Introduction

The γ-hydroxybutyric acid (GHB) is a structural analog of the γ-aminobutyric acid (GABA).

GABA GHB

It is an endogenous substance with function of an inhibitory neurotransmitter in the central nervous system in mammals. Its natural concentration in the human brain is 0.3 mmol/g. However, the compound is able to pass the blood-brain barrier, which is unique for a neurotransmitter, and, thus, its concentration in cerebrospinal fluid (CSF) can be enlarged. The main mechanism of its action consists in binding to a specific presynaptic GHB receptor which is coupled to a G protein [34]. Its further effect relies upon a decrease in adenyl cyclase activity.

A receptor complex of GHB-$GABA_B$ has also been reported and GHB was proved to be an agonist of most $GABA_B$ receptors [5]. The third observed mechanism involves an allosteric action on the calcium channels [19].

Under physiological conditions, GHB originates from GABA which is metabolized by a transaminase to succinic semialdehyde and then by a dehydrogenase to GHB. Nevertheless, most of GABA is transformed to succinic acid which enters the Krebs cycle (Fig. 1).

Neuropharmacological action

GHB can be found in both nerve and somatic cells and in body fluids such as blood and CSF. However, its function in the body outside the nervous system remains unknown.

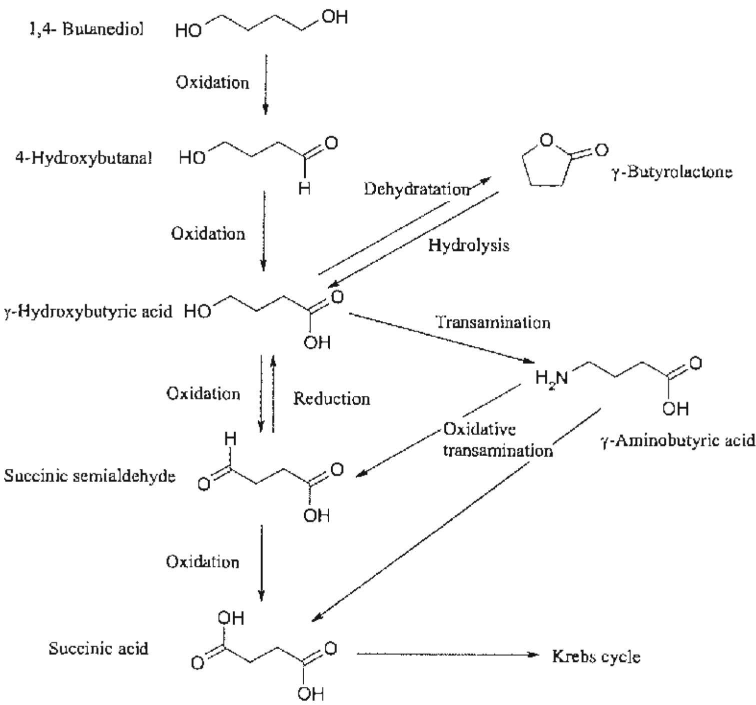

Fig. 1. Metabolism of γ-hydroxybutyric acid, γ-aminobutyric acid, 1,4-butanediol and γ-butyrolactone

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The cerebral cortex

Stimulation of the GHB-$GABA_B$ receptor complex in the frontal lobe of the cerebral cortex probably leads to absence seizures [2] due to inhibition of Ca^{2+} uptake (stimulated by K^+ ions). Moreover, agonism at presynaptic $GABA_B$ receptor results in lowered GABA release leading to the absence seizures mentioned above [14, 33]. Another result of the $GABA_B$ agonism is the hypnotic effect [37].

An increase in endogenous acetylcholine level has been reported after GHB administration as well as an increase in serotonin synthesis and metabolism [31].

In the 1970s it was observed that morphine elevated the GHB level and that GHB and morphine had synergic effect in producing euphoria [30]. Later, it was demonstrated that GHB increased synthesis of enkephalins, and that GHB-induced EEG change was reversed by naloxone [29]. Moreover, GHB was proved not to stimulate the μ, δ and κ opioid receptors [25].

In the temporal lobe, in the hippocampus, which is responsible for memory and learning, agonistic action at the $GABA_B$ receptors leads to guanyl cyclase activation, increase in cGMP level, and consequently to hyperpolarization of hippocampal neurons. Such action is responsible for amnesia due to GHB ingestion.

The thalamus

A decrease in the excitatory postsynaptic potentials in the thalamus has been reported. Such action is responsible for the anesthetic effect of GHBNa used as sodium oxybate (Somsanit) [9]. It is suspected that the GHB-$GABA_B$ receptor complex also can be formed in the ventrobasal nucleus in the thalamus and is responsible for absence seizures [1].

The hypothalamus

Administration of GHB results in activation of tyrosine hydroxylase and, as a result, the larger amount of dopamine is synthesized. Nevertheless, small doses of the drug decrease and its large doses increase secretion of dopamine [5].

Secretion of dopamine in the striatum is decreased independently of the GHB dose.

The limbic system

The limbic system is responsible for the mood. Frequent stimulation of the reward system, especially the extended amygdala, which includes the shell and the nucleus accumbens, with the same substance leads to addiction [27]. Such mechanism also concerns GHB ingestion [16].

The cerebellum

There are so few GHB receptors in the cerebellum that the effect of their stimulation is merely noticeable. What is observed in this structure is that GHB decreases the synthesis of nitric oxide, resulting in sudden reversible increase in blood pressure in the brain.

The spinal cord

Intraspinal GHB administration leads to hyperpolarization of neurons in the spinal cord [26].

The metabolic and endocrinologic effects

Inhibitory effect of GHB on the GABA-ketoglutaric acid transaminase leads to a decrease in glucose catabolism and greater tolerance of hypoxia [35]. Such an action can be considered as an advantage of GHB use in resuscitation [17].

Another effect of the drug consists in increasing the growth hormone level, which was the reason why GHB became popular among body-builders [31].

Toxicology

Only 1% of GHB is excreted with urine in the unchanged form [13]. Therefore, in order to measure its concentration in blood or urine, it is essential to use advanced chromatographic methods. One of them, using gas chromatography/mass spectrometry (GC/MS), allows to measure as small drug concentrations as 0.1 mg/l in plasma and 0.2 mg/l in urine, however, it requires conversion of GHB to γ-butyrolactone (GBL) (acidification of samples) [10]. Another GC/MS analysis, which does not require the mentioned conversion, can be used for samples within the concentration range between 0.5–2.0 mg/l [8, 18]. If a patient has ingested GBL which was partially converted to GHB in blood, it may be the easiest to use high performance liquid chromatography (HPLC) [13]. Another analytical procedure has recently been developed by Kimura et al. [15] due to the need for higher sensitivity of tests. In addition, a new ultra-rapid procedure seems promising, because a valid result of the test is accessible within 1 h [36].

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GHB is rapidly metabolized to succinic semialdehyde and then to succinic acid which enters the Krebs cycle (Fig. 1) and the final metabolic products are CO_2 and H_2O. Therefore, 4–6 h after the GHB ingestion it may be impossible to measure its concentration in urine [20, 28].

Acute intoxication can be entirely cured within 6 h in cases when it does not impair respiratory activity [22].

Some dose- and blood concentration-effect relationships are summarized in Tables 1 and 2.

Chemical modifications of GHB

Trans-4-hydroxycrotonic acid (T-HCA) (I)

T-HCA was proved to be an endogenous substance in the CNS [3]. It is able to bind to the GHB receptor and it was the first compound that showed properties required for a substance to be capable of reacting with the GHB receptor. Firstly, the active form of the compound must be non-lactonic. Secondly, there is small tolerance of the distance between the carboxyl and the hydroxyl groups.

1,4-Butanediol (II)

1,4-Butanediol is much more lipophilic than GHB. Therefore, it passes through the blood-brain barrier much faster and clinical effects are observed sooner than after GHB ingestion. However, in the CNS it is transformed to GHB as it is sbown in Figure 2. It should be noticed that administration of any alcohol dehydrogenase inhibitor, such as ethanol or 4-methylpyrazole, prevents the sedative effect from development after GHB ingestion. Disulfiram, which is an inhibitor of aldehyde dehydrogenase, partially abolishes the sedative effect. Therefore, it seems that 1,4-butanediol (1,4-BD) acts after being metabolized to GHB and/or to GABA [4, 38].

alcohol dehydrogenase → aldehyde dehydrogenase →

Fig. 2. 1,4-Butanediol metabolism to γ-hydroxybutyric acid

trans-4-hydroxycrotonic acid (I)

1,4-butanediol (II)

4-chlorobutyric acid (III)

4-metoxybutyric acid (IV)

NCS-356

sodium 4-hydroxy-5-*p*-chlorophenylpentanoate (V)

UMB68

sodium 4-hydroxy-4-methylpentanoate (VI)

UMB58

5- hydroxypentanoic acid (VII)

Fig. 3. GHB-related compounds

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Table 1. The relationship between the dose and the effect of GHB in patients [6]

Dose (g)	Effect
below 0.7	euphoria, sociability
0.7–1.4	short amnesia
1.5–2.1	weariness and sleep
2.1–3.5	intensification of the above effects
3.5–4.9	hypnosis, hypotonia, weak analgesia

Table 2. The relationship between the GHB concentration in blood and the state of consciousness in patients [12]

GHB concentration in blood (mg/l)	State of consciousness
over 260	patients in coma and did not react to pain stimuli
156-260	patients asleep and did react to pain stimuli
52-156	patients showed spontaneous movements and occasionally opened their eyes
below 52	patients woke up

N-benzylamide of α-(benzylamine)-γ-hydroxybutyric acid (**VIII**)

N-(*o*-chlorobenzyl)-amide of α-(benzylamine)-γ-hydroxybutyric acid (**IX**)

α-(4-Phenylpiperazine)-γ-hydroxybutyric acid and
α-(4-benzylpiperazine)-γ-hydroxybutyric acid derivatives (**X**)

n = 0 or 1
m = 1 or 2 (for m = 2 R = H)
R = H, 2-Cl, 4-Cl, 4-F, 4-CH_3, 4-OCH_3, 3,4-$(OCH_3)_2$

Fig. 4. Antiepileptic derivatives of γ-hydroxybutyric acid

C_4-substituted derivatives

Both 4-chlorobutyric acid (**III**) and 4-methoxybutyric acid (**IV**) have stronger ability to cause absence seizures [21] (Fig. 3).

NCS-356, sodium 4-hydroxy-5-*p*-chlorophenylpentanoate (**V**), is a specific GHB receptor agonist used for receptor studies. However, it is rapidly metabolized in the organism [37].

The following compounds have been studied for both GHB receptor agonism and their potential metabolism [37]. Both UMB68 (**VI**) and UMB58 (**VII**) are specific GHB receptor agonists. UMB68, sodium 4-hydroxy-4-methylpentanoate, is not metabolized by oxidases due to a change in the primary alcohol group (GHB) into the tertiary one (UMB68). Therefore, it is used for GHB receptor studies. UMB58, 5-hydroxypentanoic acid, can be another compound proving the tolerance of the distance between the carboxyl and the hydroxyl group in the acid for the GHB receptor agonism.

Both compounds: N-benzyl-α-(benzylamine) -γ-hydroxybutanamide (**VIII**) and N-(*o*-chlorobenzyl)-α-(benzylamine)-γ-hydroxybutanamide (**IX**) (Fig. 4) have been synthesized within the American Antiepileptic Drug Development (ADD) program and chosen as effective and the least toxic among this group of substances. Within the range of promising compounds, there are also α-(4-phenylpiperazine)-γ-hydroxybutyric acid and α-(4-benzylpiperazine)-γ-hydroxybutyric acid derivatives (**X**) [19].

Cyclic compounds

Among cyclic compounds tested for their influence on different seizures, there are γ-thiobutyrolactone (**XI**) and GBL (**XII**) (Fig. 5). The former has the potential of causing *grand mal* seizures [21] and the latter, as GHB precursor, causes *petit mal* seizures. It is suspected that GBL is first hydrolyzed to GHB in blood by the serum esterases, then the GHB passes through the blood-brain barrier and in the CNS it reacts with the GHB receptor.

NCS-382

The pharmacological GHB receptor antagonist, NCS-382 (6,7,8,9-tetrahydro-5-[H]benzocyclo-hepten-5-ol-4-ylideneacetic acid) (**XIII**) (Fig. 5), and especially its (R)-isomer [7], is accessible in NIDA

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γ-Thiobutyrolactone (**XI**)

γ-Butyrolactone (GBL) (**XII**)

NCS-382 (6,7,8,9 tetrahydro-5-[H]benzocyclohepten-5-ol-4-ylideneacetic acid) (**XIII**)

Fig. 5. Cyclic derivatives of GHB

(National Institute on Drug Abuse) for studies on the GHBergic system. It is able to antagonize GHB action, up to a certain GHB dose, due to the other mechanisms of action reported for GHB [24]. NCS-382 is also able to antagonize seizures of different sources other than those caused by GHB, although clinical data do not suggest that the effectiveness is always satisfactory [32].

Valproic acid and ethosuximide

The GHB dehydrogenase inhibitors, valproic acid (Depakene, Valproate, Valrelease) and ethosuximide (Zarontin), which are common antiepileptic medicines, intensify all the effects observed after administration of GHB [11, 23]. The effect is observed due to inhibited GHB metabolism.

Conclusions

The recent 40 years of studies on pharmacological aspects of GHB action in mammalian brain have brought a large amount of information concerning the GHB synaptic system and the role of its agonists in the brain. However, certain problems, such as the distribution of the GHB-$GABA_B$ complex or different sensitivity of $GABA_B$ receptors, still remain unsolved. Certain substances with proved action on the GHBergic system require further studies and much more specific data are expected. Nevertheless, the development of analytical procedures to test GHB, 1,4-BD and/or GBL is impressive as it seems to meet requirements of emergency toxicology departments.

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PHARMACODYNAMICS

K. Wesnes · P. M. Simpson · B. Jansson · A. Grahnén
H-J. Weimann · H. Küppers

Moxonidine and cognitive function: interactions with moclobemide and lorazepam

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Abstract *Objective*: Moxonidine represents a new generation of centrally acting antihypertensive drugs. It binds to I_1-imidazoline receptors and exerts its antihypertensive activity through a reduction in systemic vascular resistance, while cardiac output remains unchanged or even increases slightly. Moxonidine is prescribed for the treatment of mild to moderate hypertension. Typical doses are 0.4 to 2.0 mg given as one dose in the morning or as divided doses in the morning and evening.

Methods: The effects of moxonidine 0.4 mg once daily in combination with moclobemide or lorazepam were investigated in two, double-blind, randomised, placebo-controlled, two-way crossover studies in a total of 48 healthy volunteers. Safety assessments were made in each study and included pre- and post-study measurement of blood pressure, heart rate, ECG, haematology, blood biochemistry, and urinalysis, and recording of adverse events.

Results: In the first study, moxonidine alone was found to produce small but statistically significant impairments of vigilance detection speed at 4 h and 6 h. Lowering of subjective alertness was also observed. Repeat dosing with moxonidine produced an impairment of memory scanning performance. These findings were not reproduced in the second study, in which moxonidine alone produced an improvement in immediate word recall at 4 h and 6 h.

No interactions were observed when moxonidine was co-administered with moclobemide. Moxonidine, when co-administered with lorazepam, produced interactions with three tasks requiring high levels of attention: choice, simple reaction time and digit vigilance performance; memory tasks; immediate word recall, delayed word recall accuracy; and visual tracking.

A total of 47 adverse events were reported in study 1. Moxonidine produced a slight decrease of systolic and diastolic blood pressure. In study 2, a total of 55 adverse events were reported. In both trials, the most frequently reported events were tiredness and dryness of mouth, the latter occurring only under the moxonidine treatment. There were no clinically relevant changes observed in blood pressure, pulse rate, and laboratory tests in either study, nor was there any evidence of any interaction between moxonidine and either moclobemide or lorazepam.

Conclusion: Moxonidine was found to be safe and well tolerated in healthy volunteers. However, the impairments on attentional tasks were greater when moxonidine was co-administered with lorazepam 1 mg. These effects should be considered when moxonidine is co-dosed with lorazepam, although they were smaller than would have been produced by a single dose of lorazepam 2 mg.

Key words Moxonidine, Cognitive function; moclobemide, lorazepam, I_1-imidazoline-receptor, drug interaction, memory, attention, computerised cognitive assessments

Introduction

Moxonidine (4-chloro-N-(4,5-dihydro-1H-imidazol-2-yl)-6-methoxy-2-methyl-5-pyrimidine) or (4-chloro-5-(2-imidazolidin-2-ylimino)-6-methyl-pyrimidine) represents a new generation of centrally acting antihypertensive drugs. It exerts its antihypertensive activity through a reduction in systemic vascular resistance, while cardiac output remains unchanged or even increases slightly [4]. Moxonidine binds to I_1-imidazoline receptors and thus exercises its regulatory effects on the arterial blood pressure and heart rate [6, 10].

K. Wesnes (✉) · P.M. Simpson
Cognitive Drug Research Limited, Priory Court, Beech Hill, Reading RG7 2BJ, UK

B. Jansson · A. Grahnén
PMC Contract Research AB, Islandsgatan 2, S-753 18 Uppsala, Sweden

H-J. Weimann · H. Küppers
Solvay Pharmaceuticals, Hans-Böckler-Allee 20, D-30173 Hannover, Germany

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Pharmacokinetic studies have characterised moxonidine with low protein binding, a short plasma half-life, a long duration of blood pressure reduction, a low rate of metabolism, an absolute bioavailability of about 90%, excretion via the kidneys, no accumulation, no interaction with glibenclamide, digoxin or hydrochlorothiazide, no interaction with food, and similar kinetic profiles in the young and elderly [16].

In clinical trials, of 2500 subjects and volunteers administered moxonidine, 370 received the drug for 1 year or longer. Doses have ranged between 0.2 and 0.4 mg once per day, althougb doses up to 2 mg have been administered and have been well tolerated. Typical adverse effects are dry mouth and fatigue which are dose dependently mild and transient [8, 11]. Moxonidine does not undergo first pass hepatic metabolism, is loosely bound to plasma proteins and is excreted largely unchanged in urine [14, 13, 16]. These are indications that moxonidine can be administered to most patients suffering from co-existing diseases, taking concomitant medication, or both.

In clinical practice, patients on long-term anti-hypertensive therapy may occasionally require treatment with either a benzodiazepine or a monoamine oxidase inhibitor. The purpose of the studies was to simulate such situations and to investigate the possible consequences for cognitive function. Lorazepam and moclobemide were selected since data on their cognitive effects had already been obtained using the computerised test system to be employed in the study [5, 9, 17, 18].

Lorazepam is a widely used benzodiazepine used in the treatment of anxiety and epilepsy, and as a premedicant. It is known to impair a wide range of cognitive functions including those associated with attention, memory and subjective ratings of alertness [5, 9].

Moclobemide is a benzamide used in the treatment of depression and melancholic syndromes. As well as showing no interaction with alcohol [17, 18], previous work with moclobemide in the elderly has shown few cognitive effects of the drug compared with trazodone.

This paper reports the results of two separate studies of the effects of moxonidine on cognitive performance. Study 1 investigated the effects of moxonidine and moclobemide, when administered alone, and the potential interaction of moxonidine when co-administered with moclobemide. Study 2 investigated the effects of moxonidine and lorazepam alone, as well as the potential interaction of moxonidine with lorazepam. In each study, both safety assessments (blood pressure, pulse rate, ECG, and laboratory tests) and the recording of adverse events were undertaken.

Therefore, the primary objective of these studies was to determine the pharmacodynamic effects of moxonidine 0.4 mg once daily when taken with moclobemide 300 mg once daily or lorazepam 1 mg once daily using computerised tests of cognitive function in healthy volunteers. The secondary objective of the studies was to investigate the safety profile of moxonidine alone and when co-administered with moclobemide and lorazepam.

Methods

Study population

Volunteers were male and female, Caucasian, aged between 18 and 45 years and within 10% of their ideal weight. In study 1 (moclobemide), 24 (13 male, 11 female) volunteers, mean age 23 (SD 3) years, mean weight 68 (SD 9) kg were included in the study. In study 2 (lorazepam), 24 (12 male, 12 female) volunteers, mean age 23 (SD 3) years, mean weight 67 (SD 9) kg were included in the study. Screening (pre- and post-study) included a medical history, physical examination, 12-lead ECG, routine laboratory tests (haematology, blood chemistry, urinalysis, and for drugs of abuse, HIV and hepatitis B), and, where appropriate, a pregnancy test. Volunteers were excluded if they had taken any drug or medication 7 days prior to the start of the study, or any investigational medication 2 months prior to the start of the study. The study was approved by an ethics committee, and signed written informed consent was obtained from all volunteers before their participation.

Study design and study medication

Both studies were of the same design – randomised, double-blind, two-way cross-over, and placebo-controlled – in which all 24 volunteers completed two treatment periods of 6 days separated by a minimum 7-day wash-out period. The volunteers were assigned randomly to receive oral doses of each of the treatment combinations. In study 1, volunteers received two 0.2-mg moxonidine tablets in the morning for 6 days, or 2 placebo moxonidine tablets. On day 6, volunteers also received two 150-mg moclobemide tablets in the morning. In study 2, the dosing regime was the same as that for study 1 with the exception that on day 6, volunteers also received one 1-mg lorazepam tablet in the morning in place of two moclobemide tablets.

The administration of the moclobemide or lorazepam was open. On days 1, 5 and 6, dosing was undertaken at the Research Centre. On days 2, 3 and 4, dosing was undertaken at home, with the times of dosing recorded on a diary card by the volunteers.

Alcohol and tobacco were prohibited during the study, and caffeine intake was restricted.

Assessments

The assessments and assessment procedures were the same for both studies and were as follows: subjects underwent four training sessions during period 1, two being conducted on the day prior to dosing and two being conducted on day 1 of the trial. Assessments were made before, and 1, 2.5, 4, 6 and 8 h post dosing. The assessments were performed in the following sequence: cognitive performance tests then adverse event recordings.

Cognitive performance tests

A selection of tests from the Cognitive Drug Research Computerised Assessment System was used [5, 9, 17, 18, 15]. This system was run by a suite of programs installed on IBM-PC compatible machines, which control all aspects of testing, selecting appropriate parallel forms and recording all responses with millisecond accuracy. Task information was presented via colour monitors and responses to the tests were made using one of two response buttons, "YES" or "NO", on a single response module. The following tasks were atempted:

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1) Immediate word recall. The volunteer had to remember a list of 15 words presented on the monitor at the rate of one word every 2 s. Immediately following the presentation, the volunteer was given 1 min to write down as many of the 15 words as they could remember. The outcome measure was accuracy (%).
2) Simple reaction time. The word "YES" was presented on the monitor at random intervals (1–3.5 s). The volunteer was instructed to press the "YES" response button on the response module as quickly as possible following recognition of the stimulus. There were 50 trials. The outcome measure was speed of response.
3) Digit vigilance task. A computer-generated random digit was constantly displayed on the right hand side of the monitor. A series of digits was displayed in the centre of the monitor at the rate of 150 per minute, and the volunteer was required to press the "YES" response button when the two digits displayed matched. There were 45 targets in this test. The outcome measures were accuracy (per cent detections) and average speed.
4) Choice reaction time. "YES" or "NO" was presented randomly on the monitor and the corresponding response button had to be pressed as quickly as possible. There were 50 trials. The outcome measure was speed of response.
5) Visual tracking. The volunteer had 1 min to track a randomly moving target presented on the monitor using a joystick. The outcome measure was the average distance off-target per second.
6) Memory scanning. A series of five digits were presented on the monitor for the volunteer to hold in memory. This was followed by a series of 30 probe digits. The volunteer had to decide if the probe digit was in the original series and press the "YES" or "NO" response button as appropriate. Three trials were presented. The outcome measures were scanning speed and sensitivity [3].
7) Delayed word recall. The volunteer was given 1 min to write down as many words as possible from the list originally presented at the start of the session. The outcome measure was accuracy.
8) Delayed word recognition. The words presented at the start of the session (target words) were randomly re-presented one at a time, together with 15 distractor words. The volunteer had to respond to target and distractor words by pressing the "YES" or "NO" response button to indicate whether the word had appeared in the original series. The outcome measures were speed and recognition sensitivity [3].
9) Bond-Lader Visual Analogue Scales [1]. A questionnaire of 16 visual analogue scales from which three factors are derived was carried out to assess change in subjective alertness, calmness and contentment.

Safety

Routine laboratory tests, urinalysis, 12-lead ECG and vital signs (semi-supine blood pressure and pulse, measured using an Omron Automatic Blood Pressure Monitor HEM-705CP) were carried out before and after the study. In addition, vital signs were measured on days 5 and 6, 30 min before dosing and 30 min after each cognitive testing session. Reports and observations of adverse events were recorded in full at any time during the study period.

Statistics

Sample size

Sample size calculations based on the assumption that moxonidine would add 50% to the magnitude of effects of moclobemide or lorazepam indicated that a sample size of 24 volunteers would be sufficient to give a power of 80% at a 5% level of statistical significance.

Withdrawals

For non-drug-related reasons, one volunteer withdrew from study 1 following the first study period. His data were omitted from the statistical analyses of the cognitive performance tests.

Concomitant medication

Two female subjects took analgesics, under medical supervision, for menstrual pain.

Cognitive performance tests

For both study 1 and study 2, a repeated measures ANOVA on difference from baseline scores was undertaken with main factors for subject and drug condition (four levels: moxonidine alone, moxonidine with moclobemide or lorazepam, placebo moxonidine alone, and placebo moxonidine with moclobemide or lorazepam). The repeated aspect of the ANOVA was the five post-dosing assessments on each study day. At each of these assessments, estimates were made of the acute effects of moxonidine (moxonidine day 5 – placebo moxonidine day 5), the effects of moclobemide or lorazepam (placebo moxonidine day 6 – placebo moxonidine day 5). In addition, estimates were made to determine whether moxonidine interacted with moclobemide or lorazepam to produce a greater effect than would have been predicted from their individual effects [(moxonidine day 6 – moxonidine day 5) – (placebo moxonidine day 6 – placebo moxonidine day 5)]. The effects of repeated dosing of moxonidine were assessed by conducting an ANOVA on the baseline day 5 data.

Results

Cognitive performance tests

Effects of moxonidine – study 1

The speed of detections in the vigilance task was slowed relative to placebo at 4 h ($P = 0.012$) and 6 h ($P = 0.015$). No further statistically significant effects were observed. There was some evidence that moxonidine impaired the accuracy of immediate word recall at both 1 h ($P = 0.051$) and 4 h ($P = 0.061$). There was a reduction in subjective alertness at 2.5 h and 4 h ($P = 0.007$ and 0.006 respectively), and lowered contentment at 4 h ($P = 0.022$).

Effects of moxonidine – study 2

There were no statistically significant effects observed for digit vigilance speed in this study. However, there was a significant improvement in the percentage of words recalled accurately under moxonidine, which was detected at 4 h and 6 h ($P = 0.022$ and 0.026 respectively).

Effects of moxonidine – repeat dosing

Memory scanning speed was significantly impaired in study 1 by 32 (SE 9) ms following repeat dosing with moxonidine ($P = 0.0005$). No further statistically significant effects were found in this study, or in study 2.

Effects of moclobemide

Moclobemide slowed the speed of simple reaction time and digit vigilance speed at 4 h post-dose ($P = 0.008$

and $P = 0.042$, respectively). Volunteers also reported reduced alertness at 2.5 h ($P = 0.003$). Moclobemide did not show the drop in delayed word recall which was seen in the placebo condition at 1 h ($P = 0.031$).

Effects of lorazepam

Choice reaction time was slowed at 1 h ($P = 0.025$), 2.5 h ($P = 0.009$), and 4 h ($P = 0.022$). Digit vigilance accuracy was impaired at 1 h ($P = 0.028$) and 4 h post-dosing ($P = 0.029$). Memory scanning performance was consistently impaired at each of the five post-dosing assessments (1 h: $P = 0.021$; 2.5 h: $P = 0.008$; 4 h: $P = 0.015$; 6 h: $P = 0.010$; 8 h: $P = 0.015$). Delayed word recall accuracy was impaired at 2.5 h ($P = 0.009$) and 4 h ($P = 0.002$). Word recognition speed was impaired at 1 h ($P = 0.023$), 2.5 h ($P = 0.023$) and 4 h ($P = 0.01$).

Subjective alertness was reduced at 2.5 h ($P = 0.002$) and 4 h ($P = 0.008$). No other changes in subjective ratings were found.

Effects of co-administration of moxonidine and moclobemide

None of the cognitive tests showed a statistically significant interaction between moxonidine and moclobemide.

Effects of co-administration of moxonidine and lorazepam

A statistically significant interaction was observed for choice reaction time (Fig. 1). The observed impairment was greater following co-administration of both drugs at 4 h ($P = 0.016$). A similar profile of activity was also seen for the speed of detections in the digit vigilance task (Fig. 2), this being impaired at 2.5 h ($P = 0.046$). The percentage detections in this task also showed an interaction effect, this being greatest at 2.5 h ($P = 0.042$). Simple reaction time did not show a similar profile in terms of statistically significant effects to these two tasks, but the overall trend was similar (Fig. 3). A significant interaction was observed for the tracking task at 4 h ($P = 0.043$) (Fig. 4), the tracking error being consistently greater with co-dosing.

Immediate word recall accuracy showed significant interactions at 4 h and 6 h ($P = 0.009$ and $P = 0.002$, respectively Fig. 5), although inspection of the figure will reveal that these were not the same type of interactions as seen for the choice reaction, vigilance and tracking tasks. For example, when moxonidine was co-dosed with lorazepam, the interaction at 4 h shows an impairment of accuracy compared with that observed with moxonidine alone. Delayed recall showed a similar pattern at 6 h ($P = 0.045$) (Fig. 6).

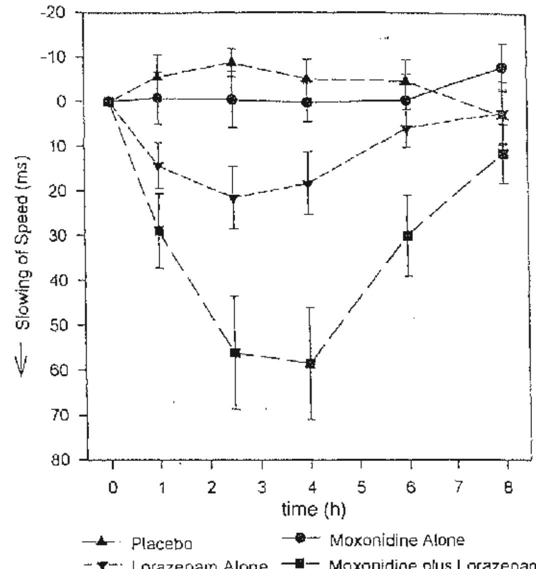


Fig. 1 The group means (SEM) of the four dosing conditions over time in study 2 for speed on the choice reaction time task

Safety assessments

There were no clinically significant changes observed in either study between pre- and post-dosing for the routine laboratory tests, urinalysis and 12-lead ECG. However, changes in blood pressure and pulse rate were observed in study 1 where there was a slight fall of approximately 8 mm Hg in both diastolic and systolic

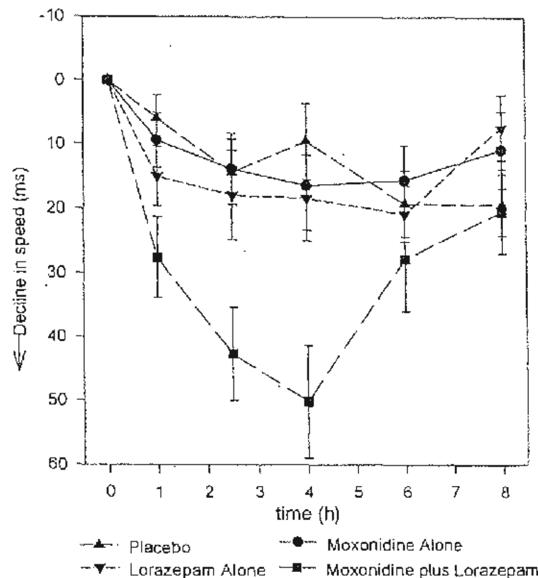


Fig. 2 The group means (SEM) of the four dosing conditions over time in study 2 for speed of detections on the digit vigilance task

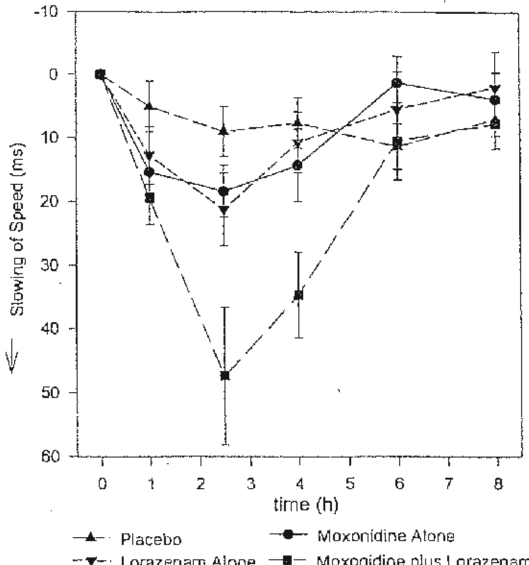
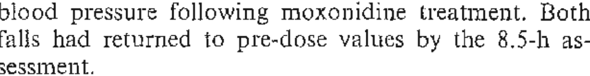


Fig. 3 The group means (SEM) of the four dosing conditions over time in study 2 for speed on the simple reaction time task



blood pressure following moxonidine treatment. Both falls had returned to pre-dose values by the 8.5-h assessment.

There was no evidence of an interaction when moxonidine was co-administered with either moclobemide or lorazepam.

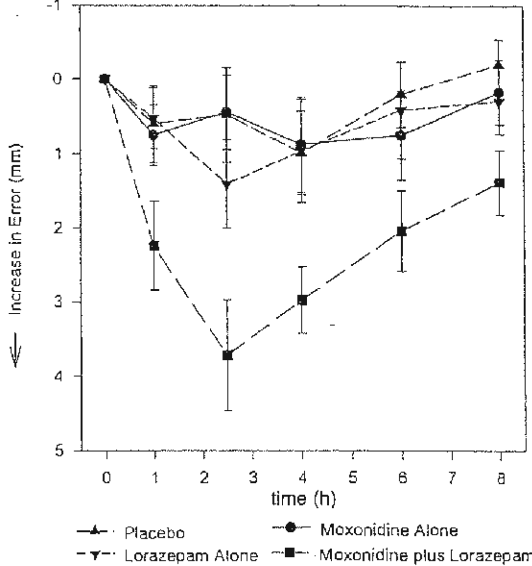


Fig. 4 The group means (SEM) of the four dosing conditions over time in study 2 for the average distance off-target on the visual tracking task

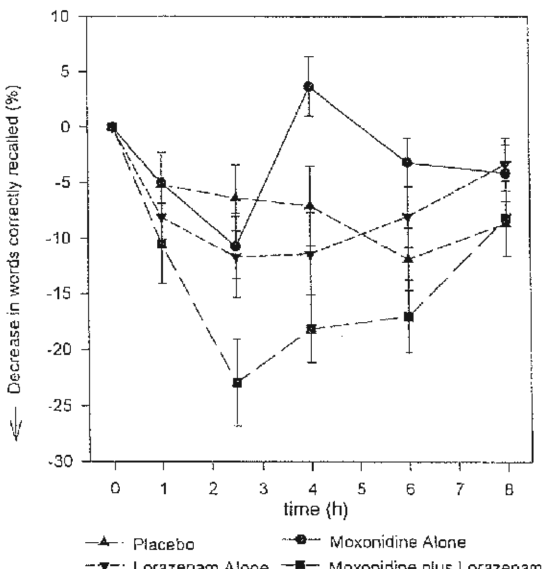


Fig. 5 The group means (SEM) of the four dosing conditions over time in study 2 for the words recalled correctly in the immediate recall task

Adverse events

A total of 47 adverse events were reported in study 1. Eighty per cent of events were mild in nature. The most frequently reported events were tiredness and dryness of mouth, the latter occurring only under the moxonidine treatment. A similar profile was observed in study 2

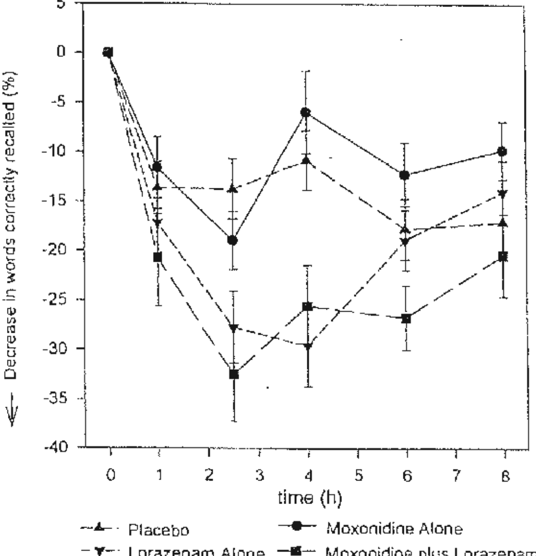


Fig. 6 The group means (SEM) of the four dosing conditions over time in study 2 for the words recalled correctly in the delayed recall task

Table 1 Summary statistics means (SEM) for the statistically significant cognitive performance tests. Statistics are based on changes from baseline

Cognitive task	Study 1			Study 2		
	Moxonidine vs. placebo	Moclobemide vs. placebo	Interaction: moxonidine/ moclobemide	Moxonidine vs. placebo	Lorazepam vs. placebo	Interaction: moxonidine/ lorazepam
Choice reaction time (ms)					1.0 h: 20 (9) 2.5 h: 30 (11) 4.0 h: 23 (10)	4.0 h: 35 (14)
Simple reaction time (ms)		4.0 h: 17 (6)				
Digit vigilance speed (ms)	4.0 h: 22 (8) 6.0 h: 20 (8)	4.0 h: 17 (8)				2.5 h: 25 (12)
Digit vigilance accuracy (%)					1.0 h: −2 (1) 4.0 h: −3 (1)	2.5 h: −4 (2)
Immediate word recall accuracy (%)	1.0 h: −8 (4) (trend) 4.0 h: −9 (5) (trend)			4.0 h: 11 (5)[a] 6.0 h: 9 (4)[a]		4.0 h: −18 (6) 6.0 h: −18 (5)
Delayed word recall accuracy (%)		1.0 h: 11 (5)			2.5 h: −14 (5) 4.0 h: −19 (6)	6.0 h: −13 (7)
Word recognition speed (ms)					1.0 h: 49 (21) 2.5 h: 60 (26) 4.0 h: 56 (21)	
Word recognition sensitivity index						
Memory scanning speed (ms)					1.0 h: 37 (16) 2.5 h: 48 (18) 4.0 h: 54 (22) 6.0 h: 43 (16) 8.0 h: 37 (14)	
Memory scanning index						
Visual tracking (mean error)						4.0 h: 2 (1)
Subjective alertness (mm)	2.5 h: −9 (3) 4.0 h: −11 (4)	2.5 h: −10 (3)			2.5 h: −12 (3) 4.0 h: −9 (3)	
Subjective contentment (mm)	4.0 h: −5 (2)					
Subjective calmness (mm)						

[a] Represents an improvement relative to placebo

Table 2 Summary of adverse events for study 1 and study 2

Event	Study 1			Study 2					Total	
	Moxonidine alone	Moxonidine + moclobemide	Placebo alone	Placebo + moclobe-mide	Moxonidine alone	Moxonidine + lorazepam	Placebo alone	Placebo + lor-azepam	Moxonidine alone	Placebo alone
Tiredness	9	3	1	1	9	4	1	2	18	2
Dizziness	3	1	0	1	0	3	1	3	3	1
Dryness of mouth	6	0	0	0	6	0	0	0	12	0
Other	7	3	6	6	5	9	2	9	12	8
Total events	25	7	7	8	20	16	4	14	45	11
Number of subjects[a]	12	7	6	5	13	15	2	12	25	8

[a] Number of subjects reporting an adverse event. Total number of subjects in each study was 24

where a total of 55 adverse events were reported. Eighty-seven per cent of events were mild in nature. Again, the most frequently reported events were tiredness and dryness of mouth, the latter occurring only under the moxonidine treatment.

These events are consistent with the known pharmacology of moxonidine, and the results of extensive clinical trials with the drug [10, 11, 12].

Discussion

The primary purpose of these studies was to determine whether moclobemide or lorazepam, when co-administered with moxonidine, produce cognitive effects which cannot be predicted from a knowledge of the effects of the individual drugs alone. When co-dosed with moxonidine, moclobemide showed no evidence of such an interaction in either cognitive performance or subjective state. However, when lorazepam was co-dosed with moxonidine, statistically significant interactions were obtained for choice reaction time, speed and accuracy of detections on the digit vigilance task, visual tracking, and both immediate and delayed word recall accuracy. Figures 1 to 4 indicate that the impairment produced by the two drugs was generally greater than would have been predicted from either drug alone. However, the interactions for word recall do not generally fit this pattern, and are most likely statistical anomalies produced by improvements in moxonidine over placebo.

The nature of the impairments with lorazepam and moxonidine co-dosing indicate that the volunteers experienced extra difficulty in maintaining attention to the experimental tasks. These disruptions were evidenced by a decrease in the number of targets detected in the vigilance task and an increase in the time taken to detect the targets. Further, the speed of choice reaction time was increased, indicating a disruption both to the speed at which the volunteers made decisions about the information presented to them and also in the selection and execution of the appropriate response. The increased error on the tracking task could either reflect an impairment of the ability to maintain attention to the task, or an inability to co-ordinate the movement of the joystick necessary to track the target. The everyday implications of these various effects would be a reduced capacity to conduct tasks which required high levels of concentration and rapid decision making. In terms of the magnitude of these additional impairments experienced when moxonidine is co-dosed with lorazepam, a recent volunteer study has looked at the effects of lorazepam 2 mg on many of the same tasks [9]. On choice reaction time, the impairments at 2 and 4 h were in excess of 200 and 400 ms, respectively, which is far in excess of the 60- to 70-ms impairment with co-dosing in the present trial. For the digit vigilance task, the peak impairment produced by lorazepam 2 mg to detections and speed was 16% and 68 ms respectively, again greater than the 5% drop in detections and the 40-ms slowing of speed seen with lorazepam and moxonidine co-dosing in the present trial. Finally, the peak impairment at 4 h in the previous trial with simple reaction time was 120 ms, which again is far in excess of the impairments with co-dosing seen at 2 h (38 ms) and 4 h (27 ms) in the present study. These data indicate, therefore, that although there is a clear and significant potentiation of the ability of lorazepam 1 mg to disrupt attention when co-dosed with moxonidine, the increased impairments are still less than would be produced by a doubling of this dose of lorazepam. This is not to state that such potentiation will not lead to everyday attentional problems, but it does provide a reference point for the amount of extra disruption experienced.

This study has confirmed previous work showing that 1 mg lorazepam impairs cognitive efficiency, the impairment peaking at 2.5 h and 4 h post-dosing. Comparing the pattern of results of this study with a previous trial using the CDR system [5], in which 1 mg lorazepam was administered to volunteers, a general similarity in the profile of impairments was evident: impairments to choice reaction time, vigilance accuracy, memory scanning speed, delayed word recall accuracy and word recognition speed were observed in both studies. Hanks et al. also observed impairments of simple reaction time and digit vigilance speed, while the present study identified an impairment of subjective alertness which did not reach statistical significance in the study by Hanks et al.

The effects on cognitive performance of moclobemide are directly consistent with previous findings. In a trial with elderly volunteers, the drug was found to improve verbal memory but disrupt performance on the digit vigilance task [17, 18], precisely what was observed in the present trial. In that trial no interaction of moclobemide with alcohol was detected, and in the present study no interaction with moxonidine was detected.

Considering the cognitive actions of moxonidine, there was little overall evidence of any effects from the two studies. Following dosing with moxonidine on day 5 (study 2), there was evidence of improvement in the accuracy of immediate word recall, although, as was described in the results section, there were trends in the opposite direction in study 1. In the first study, acute impairments were detected following dosing with moxonidine on digit vigilance speed, although this effect was not replicated in study 2. There was some evidence that repeated dosing of moxonidine impaired memory scanning performance, but again this effect was not detected in both studies. Further, Schmidt et al. [12], who studied the effects of 4-week treatment with moxonidine on the driving and psychometric test performance of hypertensive patients, reported no impairment of driving behaviour.

From the results of this study, it appears that moxonidine, in the doses administered in this study, does not produce impairment of cognitive function.

There were only two changes to the safety variables that were assessed during the study. There was a fall in diastolic and systolic blood pressure which returned to pre-dose values by the last assessment of the day. This isolated change did not require medical intervention. Recorded adverse events were consistent with the known pharmacology of moxonidine, moclobemide and lorazepam. The majority of events were mild in nature and none required further medical intervention. There was no evidence of any interaction between moxonidine and moclobemide or lorazepam with regard to the safety variables.

Taking the results of both studies together, when moxonidine is administered alone or in combination with moclobemide, it produces few significant and consistent effects on cognitive efficiency. In addition, moxonidine was found to be safe and well tolerated in healthy volunteers. However, when moxonidine is coadministered with 1 mg lorazepam, impairments in attentional efficiency are evident, which are greater than when either drug is administered alone. Nonetheless, although these effects are important and may well disrupt everyday tasks requiring attention, they are less than would be produced by a doubling of the dose of lorazepam.

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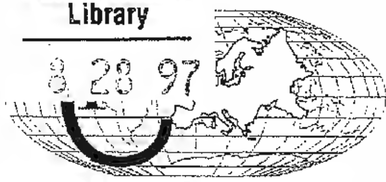

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ORIGINAL INVESTIGATION

K.A. Wesnes · T. Ward · A. McGinty · O. Petrini

The memory enhancing effects of a Ginkgo biloba/Panax ginseng combination in healthy middle-aged volunteers

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Abstract The effects of capsules containing 60 mg of a standardised extract of Ginkgo biloba (GK501) and 100 mg of a standardised extract of Panax ginseng (G115) on various aspects of cognitive function were assessed in healthy middle-aged volunteers. A double blind, placebo controlled, 14 week, parallel group, repeated assessment, multi-centre trial of two dosing regimens, 160 mg b.i.d. and 320 mg o.d. was conducted. Two hundred and fifty-six healthy middle-aged volunteers successfully completed the study. On various study days (weeks 0, 4, 8, 12 and 14) the volunteers performed a selection of tests of attention and memory from the Cognitive Drug Research computerised cognitive assessment system prior to morning dosing and again, at 1, 3 and 6 h later. The volunteers also completed questionnaires about mood states, quality of life and sleep quality. The Ginkgo/ginseng combination was found significantly to improve an Index of Memory Quality, supporting a previous finding with the compound. This effect represented an average improvement of 7.5% and reflected improvements to a number of different aspects of memory, including working and long-term memory. This enhancement to memory was seen throughout the 12-week dosing period and also after a 2-week washout. This represents the first substantial demonstration of improvements to the memory of healthy middle-aged volunteers produced by a phytopharmaceutical.

Keywords Cognitive function · Ginkgo biloba · Panax ginseng · Computerised cognitive assessments · Attention · Working memory · Secondary memory

K.A. Wesnes (✉) · T. Ward · A. McGinty
Cognitive Drug Research Ltd, 24 Portman Road, Reading RG30 1EA, UK
e-mail: keithw@cdr.org.uk
Tel.: +44-118-956-3000, Fax: +44-118-956-3001

O. Petrini
Pharmaton SA, Lugano, Switzerland

Introduction

Extracts of the leaves from *Ginkgo biloba* L. contain ginkgo-flavone glycosides and terpenoids (Foster 1991) and these are known to have vasoregulating and viscosity decreasing properties (Kleijnen and Knipschild 1992a, 1992b). Ginkgo is widely prescribed in Europe, particularly in France and Germany, for the treatment of intermittent claudication and cerebral insufficiency (Kleijnen and Knipschild 1992a, 1992b). The latter is a vague term that covers a range of problems such as confusion, absentmindedness, lack of energy, tiredness, forgetfulness, lack of concentration and depression. It is generally ascribed to the effects of poor cerebral blood flow concomitant to ageing (Kleijnen and Knipschild 1992a, 1992b). Following a review of 40 studies, Kleijnen and Knipschild (1992a, 1992b) concluded that Ginkgo was beneficial in this condition. Given these past indications for improving mental function it is not surprising that researchers should turn their attention towards degenerative conditions. There is accumulating evidence that Ginkgo may be effective to some degree in improving cognitive functioning in patients with Alzheimer's disease (Kanowski et al. 1996; Maurer et al. 1997; Le Bars et al. 1997). This application of Ginkgo is further supported by EEG work (Itil et al. 1998) and a recent review (Oken et al. 1998).

The roots of *Panax ginseng* C.A. Meyer contain several triterpene glycosides named ginsenosides (or panaxosides), which are believed to contribute to the adaptogenic and physical performance enhancing properties of the ginseng extracts (Baranov 1982). They are used in traditional Chinese medicine to treat a large number of diseases, ranging from anaemia, diabetes mellitus, insomnia and neurasthenia to gastritis, abnormal blood pressure, dyspepsia, overstrain and fatigue (Baranov 1986). Published trials showing beneficial effects of ginseng include those of D'Angelo et al. (1986), demonstrating improvements on motor performance, and Rosenfeld et al. (1989), showing benefits on neuropsychological measures. Beneficial effects on memory

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have also been reported in animals (Petkov et al. 1993, 1994).

Previous research has shown beneficial effects of Ginkgo on cognitive function in elderly people with memory impairment (Rai et al. 1991) and idiopathic memory impairment in the elderly (Wesnes et al. 1987). Specific effects of ginkgo on short-term memory have also been reported in healthy, young volunteers. As noted above, there have also been indications of benefits for cognitive functioning from ginseng in both humans (Rosenfeld et al. 1989) and animals. Given these findings, enhanced effects on cognition could be predicted from the combination of Gingko and ginseng. Given the suggestion that Ginkgo may be effective for ageing related disorders (Taillandier et al. 1986), and the observation that early use may delay the onset of deterioration (Warburton 1986), it would be interesting to observe the potential effects of the compound in healthy, middle-aged volunteers. This is a population who may frequently express concern to medical practitioners about declining mental faculties, and who may ask for advice about potential remedies, particularly given the publicity around recent high profile studies reporting positive results for products such as Ginkgo, e.g. Le Bars et al. (1997). Practitioners, however, may be unwilling to respond to such enquiries given the current lack of empirical findings with these compounds in this population. Ginkgo biloba and Panax ginseng have been combined in a product containing 60 mg Ginkgo biloba and 100 mg Panax ginseng per capsule (Ginkoba M/E; Pharmaton Natural Health Products, Lugano, Switzerland). This combined product was recently the subject of a double-blind study in 64 volunteers who satisfied criteria for neurasthenia (Wesnes et al. 1997), a condition associated with fatigue and tiredness. Significant benefits of the Ginkgo/ginseng combination on memory performance were seen 1 h after morning dosing with all three doses during 2 or more of the 3 assessment days over the 90-day study period. However, occasional impairments were seen with the higher doses after a second daily dose administered at lunchtime. This impairment was unexpected as such effects have not previously been seen with either compound, even in large literature reviews (e.g. Kleijnen and Knipschild 1992a, 1992b). Given the absence of information about the effects of either Ginkgo or ginseng in normal middle-aged volunteers, the present study was designed to test a substantial sample using the same combination of Ginkgo and ginseng. The object was to identify whether the combination has the beneficial effects on memory function seen in the previous study in middle-aged healthy individuals, and also to identify whether any evidence of a biphasic effect could be seen. The CDR computerised cognitive assessment system was used previously to study the Ginkgo/ginseng combination (Wesnes et al. 1997). It has also been shown to be sensitive to improved function with Ginkgo (Wesnes et al. 1987) and other compounds (e.g. Wesnes et al. 1989; de Wilde et al. 1995; van Harten et al. 1996; Moss et al. 1998) as well as impairments with various substances (e.g. Bailie et al. 1989; O'Neill et al. 1995; Ebert et al. 1998).

Materials and methods

Study population

Two hundred and seventy-nine volunteers (age range 38–66 years) were randomised from seven health centres, two in Sweden and five in the United Kingdom. To exclude any volunteers with any evidence of clinically relevant diseases, each was screened before drug administration. Screening included a medical history, physical examination, and routine laboratory tests. Volunteers were excluded if they had signs or history of depression, evidence of dementia, clinically relevant abnormalities in medical history or examination, history of alcohol or drug abuse, smoked more than ten cigarettes per day, had a history of food or drug allergies relevant to the study compound, had a clinically relevant deviation from normal of any finding during pre-study medical screening, were unable to perform the cognitive tests, were taking a cognition enhancing substance, or were taking medication which may have been stopped at some time during the active dosing phase. The study was conducted according the principles of the Declaration of Helsinki and good clinical practice, an ethics committee approved the protocol, and all volunteers gave written informed consent prior to participating. Twenty-three volunteers were withdrawn after randomisation; four for medical reasons not connected to the study medications; six for personal reasons; eight because of protocol violations and five suffered mild adverse events. Of the 256 volunteers who satisfactorily completed the study, 93 were male, 163 female. Mean age was 56.07 years (SD 6.87; range 38.1–66.6 years). Mean weight was 77 kg (SD 15.01; range 47–124 kg). Mean height was 167 cm (SD 9.11; range 149–196 cm).

Study design, daily procedures and study medication

The study was a placebo controlled, double-blind, parallel group, repeated measures trial of two dosing regimens of the Ginkgo/ginseng combination; 160 mg b.i.d and 320 mg o.d. The 160 mg b.i.d. contains the recommended daily doses of the two extracts, and was used in the previous study (Wesnes et al. 1997). The purpose of the single dose of 320 mg administered in the morning was to identify whether this would be a better method of administering the study compound. Each of the active conditions had an identical placebo condition.

During the screening period each volunteer performed the CDR tests on four occasions for training purposes. There were 6 study days. On each day the volunteers attended the study centres and were tested on the CDR computerised assessment system. At 8.30 a.m., the volunteers in the b.i.d. groups took a single capsule, and those in the o.d. groups took both capsules. At 1.30 p.m., the volunteers in the b.i.d. groups took their second capsule. They were tested 4 times throughout the day, at approximately 7.30 a.m., 9.30 a.m., 11.30 a.m. and 2.30 p.m. These times corresponded to 1 h before and 1, 3 and 6 h after the morning dosing time. Volunteers also completed the Bond-Lader visual analogue scales at these times. The volunteers completed the Sleep questionnaire on arrival and the other questionnaires mid-morning. The first and second study visits were 2 weeks apart and during this time all volunteers were dosed with placebo medication. After the second study visit, volunteers received their study medications. Four, 8 and 12 weeks later the volunteers returned for further study days. After the week 12 visit, dosing was discontinued, and the volunteers returned 2 weeks later for their final study day. On the days between the study visits the volunteers were instructed to take their capsules as closely as possible to the times on the study days.

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Cognitive tests

The Cognitive Drug Research (CDR) computerised cognitive assessment system. A selection of tasks from the system was administered, parallel forms of the tests being presented at each testing session. All tasks are computer-controlled, the information being presented on VGA colour monitors, and the responses recorded via response modules containing two buttons, one marked "NO" and the other "YES". The entire selection of tests took around 25 min to perform and the tests were administered in the following order:

Word presentation

A list of 15 words was presented on the monitor at the rate of 1 every 2 s for the volunteer to remember.

Immediate word recall

The volunteer was given 1 min to recall as many of the words as possible. The measures from the task were the percentage of words correctly recalled, the number of words recalled in error and the number of intrusions from previous lists.

Picture presentation

A series of 20 pictures was presented on the monitor at the rate of onw every 3 s for the volunteer to remember.

Simple reaction time

The volunteer was instructed to press the YES response button as quickly as possible every time the word YES was presented on the monitor. Thirty stimuli were presented with a varying inter-stimulus interval. The outcome measure was the average reaction time in milliseconds.

Digit vigilance task

A target digit was randomly selected and constantly displayed to the right of the monitor screen. A series of digits was presented in the centre of the screen at the rate of 150 per minute and the volunteer was required to press the YES button as quickly as possible every time the digit in the series matched the target digit. There were 45 targets. The outcome measures were the percentage of targets correctly detected, the average reaction time of these detections and the number of false positive responses (false alarms).

Choice reaction time

Either the word NO or the word YES was presented on the monitor and the volunteer was instructed to press the corresponding button as quickly as possible. There were 30 trials, for each of which the stimulus word was chosen randomly with equal probability and there was a varying inter-stimulus interval. The outcome measures were the percentage of correct responses and the average reaction time of these responses in milliseconds.

Spatial working memory

A picture of a house was presented on the screen with four of its nine windows lit. The volunteer memorised the position of the lit windows. For each of the 36 subsequent presentations of the house, the volunteer decided whether or not the one window, which is lit, was also lit in the original presentation. The volunteer recorded his response by pressing the YES or NO response button as appropriate. The measures are the percentage of correct responses and the average reaction time.

Numeric working memory

A series of 5 digits was presented for the volunteer to hold in memory. This was followed by a series of 30 probe digits for each of which the volunteer decided whether or not it was in the original series and press the YES or NO response button as appropriate. The measures are the percentage of correct responses and the average reaction time.

Joystick tracking task

In this task the volunteer used a joystick to move an object on the screen in pursuit of a randomly moving target. The task lasted for 1 min. The measure is the average distance (mm) off target.

Delayed word recall

The volunteer was again given 1 min to recall as many of the words as possible. The measures from the task were the percentage of words correctly recalled, the number of words recalled in error and the number of intrusions from previous lists.

Word recognition

The original words plus 15 distractor words were presented one at a time in a randomised order. For each word the volunteer indicated whether or not he or she recognised it as being from the original list of words by pressing the YES or NO button as appropriate. The measures are the percentage of words correctly classified (either as original or new) and the average reaction time.

Picture recognition

The original pictures plus 20 distractor pictures were presented one at a time in a randomised order. For each picture the volunteer indicated whether or not he or she recognised it as being from the original series by pressing the YES or NO button as appropriate. The measures are the percentage of pictures correctly classified (either as original or new) and the average reaction time.

Questionnaires

The Bond and Lader (1974) Visual Analogue Scales: The 16 visual analogue scales (VAS) of Bond and Lader were combined as recommended by the authors to form three Factors: alertness, calmness and contentment.

Profile of Mood States (POMS) (McNair et al. 1992)

The 65 items from this adjective check list are combined as recommended by the authors into six individual factor scores: anger, confusion, depression, fatigue, tension and vigour. There is also a Total Mood Disturbance score (TMD) which is a combination of all six factors.

General Well-Being Schedule (GWBS) (Dupuy 1984)

This 22-item questionnaire investigates a wide range of issues relevant to mood, general health, and quality of life. The overall score is a summation of the responses to the 22 individual items.

The Hopkins Symptom Checklist – SCL-90-R (Hoffmann and Overall 1978)

This questionnaire assesses a range of aspects of psychiatric well-being. The 90 items from this questionnaire are summed into an overall score.

St Mary's Hospital Sleep Questionnaire (Ellis et al. 1981)

This questionnaire assesses subjective sleep quality, volunteers answering questions about the depth of sleep, number of times awoke during night, amount of sleep, amount of sleep in the day, satisfaction with sleep, clear headedness on waking and difficulty in getting to sleep.

Analysis of cognitive function data

For the cognitive function tasks described above there are a total of 17 measures. In the present study, a total of 272 volunteers reached the pre-dose on day 0, and this was sufficient to conduct a factor analyses to provide a statistical basis for combining the 17 measures into a limited number of scores. The statistical technique used was to conduct a principal component analysis combined with a Varimax rotation. The SAS system was used to conduct the analysis. The analysis detected five factors in the data that had eigenvalues greater than unity, and these were selected for rotation. The output from the analysis of the five rotated factors is presented in Table 1. Inspection of the output table will indicate that these are strong factors; firstly, the loadings are high and secondly all but three of the 17 measures load uniquely on one factor. The factors are consistent with previous factor analyses of the CDR system (Ward and Wesnes 1999) and have internal consistency. The first factor, termed "Speed of Memory Index" involved the speed scores from the two episodic recognition tasks (word and picture recognition) and the two working memory tasks (spatial and numeric). This factor would appear to reflect the speed with which the subjects are able to identify whether or not something is being held in memory. The second factor, termed the "Quality of Episodic Secondary Memory", reflects the ability of the volunteers to hold and retrieve information from secondary memory. This was a unique factor, the four measures not loading on any other factor. The ability to recall words, recognise words and recognise pictures is reflected independently of the speed with which they can be recognised, and this factor is argued to reflect the quality of episodic memory processes. The third and fourth factors involve the tasks that have an attentional component. The third factor involves the speed scores from simple reaction time, choice reaction time and the digit vigilance task, and this is interpreted to reflect the "Power of Attention", the ability to allocate attentional processing to a particular task, the ability to focus on a particular topic for relatively short periods of time to the exclusion of other tasks. The fourth factor reflects another facet of attention, the "Ability to Sustain Attention" over the longer term, possibly independently of how hard one is concentrating; thus this task involves the ability to do the choice reaction task without making mistakes, the vigilance task without making false alarms or missing targets, and the tracking task, the ability to follow the target. The two measures which load on the fifth factor, like those on the episodic memory factor, load only on that factor. These two measures concern the capability of holding information temporarily in the two sub-systems of working memory, the articulatory loop and the visual-spatial scratchpad. This factor is termed the "Working Memory Quality Factor". There is another loading on this factor, for the speed of responses from the visual-spatial task. This is a secondary loading for this measure (–0.37), its major loading (0.67) being on the "Speed of Memory Index". It does suggest a link between the speed and accuracy of performance on this task, and its loading is in the appropriate (reverse direction) to the loading for the accuracy of responding (0.73). The purpose of the descriptions and titles which have been ascribed to the five factors

Table 1 Output of principal components analysis showing the Varimax rotated factor structure of the various measures from the CDR computerised assessment system used in this study. The significant factor loadings on the five factors are shown with asterisks

	Factor				
	1	2	3	4	5
Speed of memory processes					
Picture Recognition Speed	**0.80***	–0.16	0.17	0.05	0.09
Word Recognition Speed	**0.77***	–0.30	0.02	0.09	0.14
Numeric Working Memory Speed	**0.74***	–0.10	0.31	–0.17	–0.13
Spatial Working Memory Speed	**0.67***	0.10	0.16	–0.23	**–0.37***
Quality of episodic secondary memory					
Immediate word recall accuracy	–0.12	**0.84***	0.01	0.11	0.11
Delayed word recall accuracy	–0.17	**0.83***	0.04	–0.02	0.07
Word Recognition Accuracy	–0.13	**0.69***	0.13	0.03	–0.06
Picture Recognition Accuracy	0.09	**0.50***	–0.19	0.33	0.10
Power of attention					
Simple Reaction Time	0.10	–0.3	**0.81***	10	0.2
Choice Reaction Time	**0.39***	–0.2	**0.68***	–16	–0.11
Digit Vigilance Detection Speed	0.31	0.15	**0.65***	–24	–0.8
Continuity of attention					
Digit Vigilance Detection Accuracy	–0.08	0.04	–0.06	**0.76***	0.06
Choice Reaction Time Accuracy	0.14	0.16	0.28	**0.47***	0.27
Digit Vigilance False Alarms	0.10	–0.06	**–0.45***	**–0.53***	–0.16
Tracking Error	0.09	–0.07	0.26	**–0.62***	0.04
Quality of working memory					
Numeric Working Memory Accuracy	–0.14	–0.06	0.08	0.15	**0.77***
Spatial Working Memory Accuracy	0.08	0.24	–0.15	–0.01	**0.73***

Table 2 The four combined factor scores from the cognitive tests. Note the data at week 0 are the raw pre-dosing baseline scores and those at weeks 4–14 are the change from baseline scores. For the Quality of Memory Index and the Continuity of Attention, positive change scores reflect improvements, while for the other two indices, negative scores reflect enhancements

Week	Placebo								Active							
	−1 h		1 h		3 h		6 h		−1 h		1 h		3 h		6 h	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Quality of Memory Index																
0	365.5	7.449	326.3	6.55	320.3	7.477	326.4	7.693	366.1	4.631	321.4	4.841	318.5	4.964	311.6	5.135
4	6.772	4.969	6.209	6	6.021	6.157	5.397	6.589	6.894	3.663	13.55	4.034	9.766	4.172	21.07	4.154
8	11.87	5.926	7.918	6.518	18.94	5.734	0.222	6.406	13.65	3.666	21.6	4.281	19.65	4.344	19.94	4.617
12	18.51	6.081	12.01	5.677	17.21	6.328	12.71	5.491	15.84	3.707	23.25	4.244	18.25	3.883	24.56	4.394
14	17.53	5.438	8.386	7.243	14.28	5.647	1.249	6.458	18.49	3.906	18.34	3.946	19.72	4.552	27.66	3.95
Speed of Memory Index																
0	3335	58.93	3358	58.11	3350	58.14	3323	59.87	3328	41.19	3380	45.38	3359	41.88	3333	41.93
4	−42.9	38.91	32.65	39.14	−66.2	32.98	−64	44.53	−91.8	23.7	−39.1	27.05	−62.9	23.28	−105	28.68
8	−124	35.42	−60.7	37.62	−117	40.87	−80	42.58	−92	25.86	−101	31	−147	29.6	−129	28.11
12	−216	35.8	−159	35.72	−184	40.17	−88.9	44.5	−194	24.21	−142	30.42	−194	31.02	−173	27.27
14	−274	44.78	−226	41.31	−226	37.42	−196	35.36	−334	28.02	−263	30.09	−237	30.02	−205	27.47
Continuity of Attention																
0	57.11	1.012	59.07	0.778	58.54	0.946	58.02	1.018	56.37	0.788	57.36	0.685	57.74	0.645	57.68	0.622
4	0.934	1.118	−1.23	1.063	−0.49	0.773	1.089	0.759	1.295	0.608	0.787	0.613	0.566	0.563	−0.13	0.585
8	2.33	0.701	−0.32	0.883	0.1	0.724	0.252	0.987	1.904	0.61	1.137	0.604	−0.31	0.694	0.411	0.477
12	2.685	0.827	−1.05	1.025	−0.15	0.619	1.076	0.812	1.931	0.744	0.939	0.595	1.029	0.53	0.746	0.468
14	3.338	0.917	1.209	0.713	1.653	0.684	1.358	0.739	2.251	0.741	1.149	0.646	1.061	0.468	−0.07	0.7
Power of Attention																
0	1119	11.05	1144	12.63	1145	12.24	1141	12.94	1130	7.805	1141	7.807	1146	8.226	1148	7.987
4	14.91	6.504	12.25	7.14	10.18	8.226	13.52	7.681	8.716	4.836	23.22	5.273	17.87	4.971	8.943	5.124
8	33.96	6.744	27.14	7.515	22.61	7.028	26.55	7.65	11.36	5.245	28.97	5.8	29.26	5.415	15.52	5.563
12	22.18	7.679	11.92	7.23	17.62	6.948	23.31	7.456	13.2	5.487	24.08	5.381	25.75	5.538	15.39	5.89
14	18.48	7.487	17.89	7.75	19.72	7.649	18.61	7.175	14.93	5.694	20.15	6.08	20.75	5.86	5.64	6.006

identified by the analysis has been to provide some suggestions of the functions which might be reflected by the factors. However, it should be noted that they do not imply empirical justifications of dissociations between various aspects of function.

In the previous study with this medication (Wesnes et al. 1997), a combined measure of memory quality was used as the primary outcome variable which was a direct combination of the two memory quality factors identified in the present analysis. For this reason, together with the clear support for the individual structure of the factors reflecting the quality of episodic and working memory, these factors have been combined for the present study to form a derived score termed the Quality of Memory Index. This was selected prior to the randomisation code being broken as the primary outcome variable in this study. This index combines the accuracy scores from six of the ten tasks used in the study. The three other factor scores were also analysed as secondary measures.

Statistical analysis

The study day prior to the commencement of dosing was used as the pre-study baseline (week 0). For the cognitive tasks and VAS scores at pre-dose, 1, 3 and 6 h post-dose on week 0 were used as baselines for subsequent weeks, and subtracted from the scores at equivalent time points at weeks 4, 8, 12 and 14. These difference from baseline data were subjected to a split-plot ANOVA, fitting between subject terms for (dosing) regimen (two levels: 160 mg b.i.d., 320 mg o.d.) and (dosing) condition (two levels: placebo, verum), and within subject terms for week (four levels: weeks 4, 8, 12, 14) and daily test sessions (pre-dose, 1, 3, 6 hours post-dose). Terms were also fitted for all possible interactions between these three main factors. For the other questionnaire data, which were assessed just once per study day, the scores on week 0 were used as baselines for subsequent weeks, and subtracted from the scores at weeks 4, 8, 12 and 14. These difference from baseline data were subjected to a split-plot ANOVA, fitting between subject terms for (dosing) regimen (two levels: 160 mg b.i.d., 320 mg o.d.) and (dosing) condition (two levels: placebo, verum), and within-subject terms for week (four levels: weeks 4, 8, 12, 14). Terms were also fitted for all possible interactions between these three main factors. The software package SAS was used to analyse the data, using the procedure GLM.

Results

Cognitive function data

Primary outcome variable: Quality of Memory Index

For the primary outcome variable, the Quality of Memory Index, the analysis revealed a main effect of the Ginkgo/ginseng combination [$F(1,250)=5$; $P=0.026$], which did not interact with the dosing regimen [$F(1,250)=0.31$; $P=0.58$], nor the successive weeks of testing [$F(3,750)=0.93$; $P=0.43$], though there was a trend for an interaction with the time of testing on the study days [$F(3,750)=2.22$; $P=0.084$]. There were no higher order interactions. Inspecting the data in Table 2,

Table 3 The six tasks which comprise the Quality of Memory Index. Note the data at week 0 are the raw pre-dosing baseline scores and those at weeks 4–14 are the change from baseline scores. For all tasks positive change scores reflect improvements

Week	Placebo								Active							
	−1 h		1 h		3 h		6 h		−1 h		1 h		3 h		6 h	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Spatial Working Memory (%)																
0	85.95	2.312	77.97	2.716	77.51	2.901	76.35	3.276	86.0	1.608	76	2.112	74.7	2.189	72.32	2.456
4	0.144	2.43	4.138	3.284	0.043	3.739	1.394	3.85	2.30	81.62	4.576	2.489	2.50	72.55	4.105	2.751
8	5.647	2.084	1.017	3.598	7.428	3.302	2.155	3.979	5.05	91.7	9.048	2.226	7.5	32.33	7.737	2.698
12	6.767	2.224	4.138	3.456	8.261	2.949	5.718	3.388	4.76	31.52	6.503	2.541	8.63	22.37	10.78	2.598
14	5.273	2.132	0.761	3.675	8.664	2.904	4.871	3.517	5.23	71.74	7.738	2.3	9.81	52.40	13.12	2.328
Numeric Working Memory (%)																
0	91.8	1.493	91.19	1.196	91.11	1.571	89.42	1.505	92.2	0.91	89.01	1.109	90.0	1.033	86.94	1.245
4	−0.54	1.012	0.613	1.521	−2.53	1.795	2.682	1.555	−0.7	51.08	1.111	1.081	−0.9	10.93	4.26	1.143
8	2.069	1.309	0.62	1.585	1.379	1.313	2.605	1.596	1.49	91.07	2.103	1.087	3.86	61.06	5.128	1.305
12	1.533	1.407	1.532	1.571	0.69	1.262	4.904	1.492	1.93	31.05	4.881	1.111	2.80	11.04	5.168	1.224
14	1.226	1.585	1.225	1.161	1.226	1.192	−0.31	1.494	2.99	80.99	3.055	1.156	0.7	11.12	3.55	1.368
Immediate Word Recall (%)																
0	34.94	1.58	28.62	1.407	29.88	1.497	31.07	1.632	35.52	0.969	29.01	1.019	28.22	0.911	29.9	0.909
4	1.8	1.454	1.456	1.404	0.115	1.355	1.839	1.506	0.612	1.013	1.065	1.055	1.065	1.04	0.651	1.055
8	1.724	1.612	2.223	1.605	0.23	1.591	−0.38	1.538	1.282	1.044	1.183	1.081	1.854	1.009	−1.38	1.101
12	2.414	1.591	2.53	1.656	−0.54	1.547	−1.95	1.486	1.972	1.023	2.347	1.184	2.151	1.069	0.888	1.007
14	2.605	1.661	2.874	1.62	0.154	1.424	−0.31	1.498	3.334	1.143	1.755	1.11	2.288	1.01	1.282	1.038
Delayed Word Recall (%)																
0	20.76	1.739	7.43	1.695	5.246	2.011	9.077	1.946	22.9	1.052	9.267	1.125	8.97	1.176	8.064	1.255
4	3.563	1.559	−0.34	2.162	4.214	1.779	0.881	1.786	1.79	51.09	1.361	1.243	1.77	51.32	3.708	1.32
8	1.954	1.735	3.41	2.025	5.364	1.8	−0.42	1.656	2.44	61.19	4.28	1.292	2.42	61.28	3.708	1.346
12	4.176	1.711	2.107	2.107	4.788	2.053	0.459	1.653	3.72	81.1	3.768	1.324	1.36	11.35	3.116	1.414
14	4.061	1.605	3.64	2.47	3.946	2.192	−0.57	1.986	3.39	31.22	2.663	1.212	3.43	21.28	5.049	1.208
Word Recognition (%)																
0	56.17	2.104	49.42	2.285	46.51	2.247	49.96	2.188	55.1	1.445	47.1	1.444	47.5	1.553	46.07	1.604
4	0.996	2.121	−0.23	2.392	1.303	2.225	−3.07	2.275	−0.2	41.64	3.333	1.638	2.24	91.76	4.182	1.692
8	−2.45	2.358	−1.47	2.603	0.689	2.348	−2.76	2.314	−0.5	11.67	2.024	1.706	0.98	71.59	1.46	1.622
12	2.07	2.126	−0.08	2.251	0.612	2.601	−0.84	2.33	0.15	71.61	1.548	1.571	0.31	61.68	2.919	1.762
14	0.919	1.888	−1.61	2.688	−0.23	2.285	−2.15	2.084	0.98	61.64	0.119	1.677	−0.6	71.76	2.052	1.742
Picture Recognition (%)																
0	75.92	2.085	71.67	2.228	70.06	2.126	70.52	2.149	74.1	1.373	70.98	1.471	68.9	1.473	68.28	1.548
4	0.805	1.728	0.575	1.874	2.874	1.901	1.667	1.743	3.16	61.10	2.173	1.291	3.07	71.21	4.142	1.248
8	2.931	2.008	1.744	1.946	3.851	2.056	−0.98	1.985	3.87	61.09	3.125	1.372	2.98	81.33	3.284	1.33
12	1.552	1.861	1.782	1.967	3.391	1.908	4.425	1.737	3.28	41.37	4.286	1.424	2.98	81.38	1.686	1.485
14	3.448	1.827	1.494	1.998	0.517	2.23	−0.29	2.233	2.54	41.35	3.065	1.48	4.14	21.34	2.604	1.531

it is clear that the effect seen represents an enhancement in performance, the volunteers who were taking the medication were remembering more information than those taking placebo. Although all but one of the four time points on the 4 study days showed a benefit of active over placebo, the improvements did seem to be greater at 1 and 6 h post-dosing. The data have been collapsed over the 4 study days and are presented graphically in Fig. 1. Here we can see that the basis for the statistical trend between dosing condition and time of testing on the study days was the greater benefits seen mainly at 1 and 6 h post-dosing. Six measures have been combined to form this index, and in Table 3 these measures are shown individually to allow the reader to examine the patterns. This table illustrates the underlying continuity in the data. Taking the improvement at 6 h, for five of the six tasks performance was superior with active than placebo on each of the 4 study days, and for the sixth, immediate word recall, weeks 12 and 14 were superior, while weeks 4 and 8 were not. A similar pattern can be seen at 1 h for the two recognition and two working memory tasks, and on 3 of 4 study days for delayed word recall.

Secondary variables

There were no main effects of the Ginkgo/ginseng combination for the other three derived variables; power of attention [$F(1,250)=0.79$; $P=0.38$], continuity of attention

Table 4 The Bond and Lader VAS

Week	Placebo								Active							
	−1 h		1 h		3 h		6 h		−1 h		1 h		3 h		6 h	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Self-Rated Alertness (VAS)																
0	62.92	1.801	61.22	1.839	60.61	1.951	59.74	1.981	62.32	1.161	62	1.293	62.12	1.309	60.61	1.272
4	0.36	1.258	−0.33	1.329	0.236	1.269	−0.25	1.354	0.025	0.904	0.555	1.069	−0.74	0.906	−0.09	1.015
8	0.702	1.408	1.164	1.462	0.615	1.557	0.141	1.455	1.959	1.145	0.168	1.1	−0.28	0.945	−0.72	1.068
12	2.436	1.581	1.524	1.519	1.523	1.553	−0.81	1.491	0.879	1.119	0.955	0.958	0.793	0.967	1.479	1.021
14	3.937	1.46	3.379	1.569	4.417	1.556	2.32	1.67	2.173	1.143	1.535	1.052	1.157	0.931	2.938	1.234
Self-Rated Calmness (VAS)																
0	62.94	2.098	62.74	1.849	61.88	2.046	60.72	2.078	62.55	1.239	62.15	1.373	61.25	1.295	62.38	1.372
4	2.18	1.989	2.31	1.403	2.017	1.782	1.615	1.621	0.645	1.133	0.527	1.388	2.101	1.194	2.234	1.332
8	−0.91	2.159	−0.62	1.868	1.207	1.787	3.305	1.781	3.201	1.194	2.179	1.318	2.926	1.237	−0.41	1.329
12	3.006	1.817	2.667	1.61	4.615	1.835	4.569	2.105	2.293	1.189	1.071	1.393	3.26	1.106	3.095	1.277
14	3.262	2.172	3.385	1.843	2.092	1.853	2.397	2.184	3.751	1.279	2.5	1.388	4.92	1.309	2.325	1.454
Self-Rated Contentment (VAS)																
0	70.72	1.617	69.89	1.721	70.14	1.544	70.68	1.603	68.61	1.084	68.59	1.179	69.35	1.134	70.22	1.165
4	0.342	1.391	−0.41	1.438	0.4	1.335	−0.48	1.294	0.596	0.969	1.771	0.894	0.698	1.015	0.48	0.867
8	−0.64	1.604	1.114	1.457	0.186	1.577	−1.43	1.429	2.974	1.094	1.863	1.067	1.135	0.941	0.372	0.822
12	2.035	1.556	1.933	1.55	2.228	1.739	0.811	1.502	0.582	1.126	2.231	0.998	1.985	0.936	0.875	0.894
14	1.788	1.656	1.943	1.595	2.198	1.732	1.28	1.573	3.73	1.027	3.56	0.929	1.479	0.926	3.127	1.036

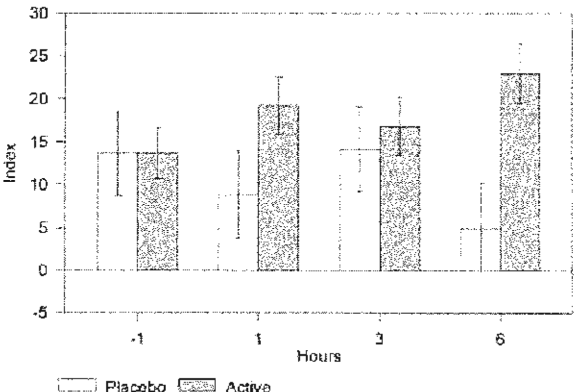

Fig. 1 The effects of the Ginkgo/ginseng combination on the Quality of Memory Index over the study days. Data are means and SE. Ascending values represent improvements over pre-dose

[F(1,250)=0.2; P=0.65], and the speed of memory processes [F(1,250)=0.05; P=0.82]. There were no significant interactions, though for the speed of memory processes there was a three-way interaction which narrowly missed significance between dosing condition, week of testing and visits over the study day [F(9,2250)= 1.85; P=0.056]. Inspecting the means in Table 2, it can be seen that the basis for this trend was improvements for active over placebo which occurred on 12 of the 16 possible occasions. This effect supports the Quality of Memory Index by showing that the improvements in accuracy are generally accompanied by improvements in speed. Importantly, at 6 h, the time when the improvement to quality was greatest, the improvements in reaction time were greater for the combination over placebo over all 4 study days.

Possible influence of age

The ages and age ranges in the four groups were comparable: 160 mg b.i.d. placebo, mean age 56.0 years, SD 7.4, range 38.7–65.7; 160 mg b.i.d. verum, mean age 55.5 years, SD 6.9, range 40.2–65.4; 320 mg o.d. placebo, mean age 56.0 years, SD 7.4, range 38.7–65.7; 320 mg o.d. verum, mean age 56.4 years, SD 6.8, range 40.9–66.6. Statistical analysis using ANOVA showed no differences between the age of the volunteers in the four groups [F(3,252)=0.35; P=0.79]. The pattern of results therefore could not have been the result of differential age ranges in the four groups.

Questionnaires

The data from the various questionnaires are summarised in Table 4 and Table 5. There were no main effects of dosing upon any derived measure from the various questionnaires, though there was a three-way interaction between dosing condition, week of testing and visits over the study day for the factor score Calmness [F(9,2241)=1.97; P=0.038] from the Bond-Lader VAS. Inspecting Table 4, it is not clear what is the basis for this interaction.

Table 5 The summary changes in the four questionnaires administered in this study, the Profile of Mood States (*POMS*), the Hopkins SCL-90-R and the General Well Being Scale (*GWBS*)

Week	Placebo		Active	
	Mean	SE	Mean	SE
POMS				
0	5.843	2.795	4.534	1.80
4	−0.229	1.517	−1.147	1.19
8	1.193	1.987	−2.245	1.12
12	−3.325	1.937	−4.742	1.21
14	−1.120	2.229	−4.706	1.27
GWBS				
0	87.04	1.24	88.16	0.86
4	−0.345	0.77	0.178	0.60
8	−2.149	1.02	−0.187	0.67
12	0.310	1.00	0.902	0.67
14	0.595	1.11	1.687	0.71
SCL-90-R				
0	100.8	2.20	98.15	1.44
4	−1.690	1.26	−1.110	0.96
8	−1.762	1.42	−2.409	1.02
12	−3.131	1.32	−3.652	0.94
14	−3.738	1.76	−4.128	0.98

Discussion

This study has demonstrated that a combination of Ginkgo biloba and Panax ginseng has beneficial effects on the memory of healthy middle-aged volunteers. This confirms the beneficial effects seen on the Quality of Memory Index in the previous study with this combination (Wesnes et al. 1997), and extends this finding from middle-aged volunteers with neurasthenia to the middle-aged in general. What is also very clear from this study is that there was no biphasic effect of the combination as was suggested in the previous study, in fact the effects were actually most potent at 6 h post-morning dose, the time at which some impairments were seen in the previous study. As this trial was four times as large as the previous trial, and as the benefits at 6 h in this study were evident at weeks 4, 8,12 and 14, whereas the deficit was only seen on day 90 in the previous trial and not on 2 previous study days; it must be concluded that the impairment identified in the previous trial was either an anomaly or instead something specific to volunteers who satisfy the criteria for neurasthenia.

The improvement to memory seen in this study was seen in an index which combined the accuracy scores from six tasks. As can be seen from Table 3, these improvements occurred for all of the tasks which contributed to the memory index. Two of the tasks involved the ability to hold information within working memory. Recent theory has proposed two sub-systems within working memory (Baddeley 1986) the articulatory loop and the visuo-spatial scratchpad. These two subsystems were assessed using the numeric working memory task and the spatial working memory task, respectively. The other tasks reflect primarily episodic secondary memory. The ability to recall words, both in the short and longer term was assessed, as was the ability to recognise these words when mixed up with other words. Also assessed was the ability to recognise previously presented pictures. The results are therefore that that a combination of Ginkgo biloba and Panax ginseng can improve the capacity to retain information in working memory as well as the ability to store and retrieve information from episodic secondary memory. It was particularly important that the "decision times" on the memory tasks were not lengthened. Had this occurred, it would have suggested the possibility that a change in task performance strategy had taken place. Clearly the time taken to recognise information is very important, and if the volunteers had taken longer, this may have accounted for the improvements in accuracy. However, exactly the opposite occurred, the trend being for speed to increase as accuracy improved, and this indicates further that this is a very robust effect.

A further important aspect of the effects of the combination on cognitive function is that they were largely restricted to memory, although attention was comprehensively assessed with tasks of proven sensitivity to change. This is also precisely what was seen in the previous study with the combination (Wesnes et al. 1997).

Finally, in considering the nature of the findings, it is important to assess the magnitude and duration of the effect. The improvements in the memory index at 6 h ranged from 6.4% to 8.8% over the 14 weeks of assessment, and averaged 7.5%. Considering that these were normal volunteers, this is in clinical terms a large improvement. There are actually very few instances in the literature of a compound producing enhancements to memory function in normal middle-aged volunteers, and consequently there are no "gold standards" or "yard sticks" available for comparative purposes. Thus, instead, the effect could be considered in terms of whether it is desirable; and it is probable that most middle-aged people would feel an improvement of between 5 and 10% would be worth having. In terms of the time course of the effect, it is clearly in place after 4 weeks of dosing, and most importantly it is still present 2 weeks after dosing is stopped. This latter effect is very exciting, as it is clearly not seen with the first generation of anti dementia drugs, the anticholinesterases (Rogers et al. 1998). This suggests either that it produces some form of structural change in the CNS, or that the mechanisms by which it produces its effects remain active for at least a fortnight. In future work, it would be worthwhile to establish precisely how long this effect persists.

This represents one of the first substantial demonstrations of improvements to the memory of healthy middle-aged volunteers. There are no known major health risks associated with either Ginkgo or ginseng, and they are readily available over the counter. As people enter their late middle age, concerns about dementia become more widespread, and the opportunity to take a "herbal" prep-

aration to help prevent the onset of mental decline will be attractive to many. Many patients will ask their family practitioner about the availability of such compounds including anti-dementia drugs. This study provides a rationale for one course of action from medical practitioners who are presented with such requests.

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Absence of Effect of Sertraline on Time-Based Sensitization of Cognitive Impairment With Haloperidol

Stephen A. Williams, M.B., B.S., Ph.D.,
Keith Wesnes, Ph.D., Stuart D. Oliver, M.A., M.B., B.Chir.,
and William G. Rapeport, M.B., B.Ch., M.R.C.P.(UK)

This double-blind, randomized, placebo-controlled study evaluated the effects of haloperidol alone and haloperidol plus sertraline on cognitive and psychomotor function in 24 healthy male subjects. ***Method:*** All subjects received placebo on Day 1 and haloperidol 2 mg on Days 2 and 25. From Days 9 to 25, snbjects were randomly assigned to either sertraline (12 subjects) or placebo (12 subjects); the sertraline dose was titrated from 50 to 200 mg/day from Days 9 to 16, and remained at 200 mg/day for the final 10 days of the drug administration period. Cognitive function testing was performed before dosing and over a 24-hour period after dosing on Days 1, 2, and 25. ***Results:*** Impairment of cognitive function was observed 6 to 8 hours after administration of haloperidol on Day 2 but was not evident 23 hours after dosing. When single-dose haloperidol was given again 25 days later, greater impairment with earlier onset was noted in several tests in both treatment groups, suggesting enhancement of this effect. There was no indication that sertraline exacerbated the impairment produced by haloperidol since an equivalent effect also occurred in the placebo group. Three subjects (2 on sertraline and 1 on placebo) withdrew from the study because of side effects. Ten subjects in each gronp reported side effects related to treatment. The side effect profiles of sertraline and of placebo were similar. ***Conclusion:*** Haloperidol produced a clear profile of cognitive impairment that was not worsened by concomitant sertraline administration.

(J Clin Psychiatry 1996;57[suppl 1]:7–11)

Antidepressant agents, such as serotonin selective reuptake inhibitors (SSRIs), and neuroleptic drugs, such as haloperidol, are frequently used in combination to treat patients with psychotic depression, bipolar disorder, or schizoaffective disorder, among other conditions. Both therapeutic drug classes act centrally and affect neurotransmitters. The putative mechanism for the antidepressant effect of sertraline and other SSRIs is inhibition of the serotonin uptake pump.[1,2] SSRIs also indirectly influence dopamine via serotonergic innervation of dopamine neurons.[3] Haloperidol is a major tranquilizer widely used in the management of psychoses that has strong central antidopaminergic activity.[4]

The SSRIs have the potential to cause extrapyramidal side effects, such as akathisia and tremor, owing to their indirect effect on dopamine neurons.[3] Since the extrapyramidal side effects associated with haloperidol therapy are attributed to its antidopaminergic activity,[4] the indirect effect of SSRIs on dopamine might potentiate these effects that are caused by haloperidol and other neuroleptic drugs.[3] Worsening of extrapyramidal side effects has been anecdotally reported with concurrent fluoxetine and haloperidol administration.[5,6]

This study was conducted to determine if concomitant administration of sertraline and haloperidol results in a drug interaction of potential clinical significance. The effects of single, 2-mg doses of haloperidol on cognitive function were assessed before and after steady-state sertraline administration.

SUBJECTS AND METHOD

A double-blind, randomized, parallel-group study was conducted in 24 healthy male volunteers to assess the influence of sertraline on the central nervous system effects of haloperidol.

Healthy male subjects between the ages of 18 and 45 years were eligible for the stndy. Subjects who had a history of asthma, eczema, or clinically significant drug allergy were excluded, as were those who had used prescription or over-the-counter drugs during the 2 weeks prior to the study. Subjects were excluded if they were recreational drug users, smoked more than 5 cigarettes daily, or were moderate-to-heavy consumers of alcohol.

From Pfizer Central Research, Sandwich (Drs. Williams and Rapeport), Cognitive Drug Research, Reading (Dr. Wesnes), and the Besselaar Clinical Research Unit, Leeds, West Yorkshire (Dr. Oliver), United Kingdom.

Reprint requests to: William G. Rapeport, M.B., B.Ch., M.R.C.P.(UK), Pfizer Ltd., Ramsgate Road, Sandwich, Kent CT13 9NJ, United Kingdom.

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All subjects gave informed consent before participating in the study.

Study Drug Administration

All subjects received placebo on Day 1 and haloperidol 2 mg on Days 2 and 25. From Days 9 to 25, subjects received oral sertraline or matching placebo. The dose of sertraline was increased to 200 mg/day over 7 days: patients were given 50 mg on Day 9; 100 mg/day on Days 10 to 12; 150 mg/day on Days 13 to 15; and 200 mg/day on Days 16 to 25. All study drugs were administered under supervision immediately before a standard breakfast. No drugs other than the study drugs and acetaminophen were allowed during the study.

Study Procedures

All subjects attended four training sessions related to the tests of cognitive and psychomotor function that were used in the study (see Table 1). Tests were performed before administration and 2, 4, 6, 8, and 23 hours after administration of the study drugs on Days 1, 2, and 25.

Cognitive testing was conducted using the Cognitive Drug Research computerized assessment system.[7,8] Information is presented on 14-inch resolution monitors and responses are recorded via a response module containing "YES"/"NO" buttons. The tests used have been described previously[9] and are listed together with the major outcome measures in Table 1. Apart from the d2 test,[10] which is a pencil and paper cancellation test, and word recall, in which the volunteers write down the words they can remember, all responding was recorded via the response buttons. In addition, after testing was completed, the volunteers completed sixteen 10-cm visual analogue scales concerning their subjective state.[11] Finally, the experimenter rated the alertness of each volunteer on a scale of 0 (comatose) to 100 (totally alert).

Physical examinations were performed at the screening visit, on Days 1 and 26, and at the follow-up visit on Day 32. Two measurements of supine blood pressure and pulse rate were taken at the screening visit; on Days 1, 2, 9, 16, and 25; and at the follow-up visit. A 12-lead electrocardiograph was done at the screening and follow-up visits.

Clinical laboratory tests, including hematology, clinical chemistry, and urinalysis, were performed at the screening visit; on Days 1, 9, 16, and 25; and at the follow-up visit to evaluate safety. Glucose-6-phosphate dehydrogenase, glucose, and hepatitis screening were performed at the screening visit, and urine drug screening was performed at the screening visit and on Day 25 before haloperidol dosing. Side effects were recorded at each visit, and extrapyramidal side effects were assessed after dosing and completion of tests of cognitive and psychomotor function on Days 1, 2, and 25 as rated by the Simpson-Angus Neurologic Rating Scale.[12]

Statistical Methods

The effects of haloperidol alone were determined by using SAS PROC GLM (SAS Institute. Cary, N.C.) to compare data from Day 1 (placebo) with data from Day 2 (haloperidol alone). Plots of residuals against predicted values and against normal scores indicated adequate homogeneity of variance and normality for the primary variables. Subjective volunteer alertness as rated by investigators demonstrated a severe departure from homogeneity of variance and normality. Therefore, nonparametric analyses were performed. The sphericity test was performed on effects of repeated measurements to test the symmetry of the variance-covariance matrix. If the data did not satisfy the assumption of type H covariance, the probability level associated with the Huynh-Feldt adjustment was used.

To identify whether sertraline altered the cognitive effects of haloperidol, a similar analysis was performed taking sertraline/placebo-sertraline as the between-group factor; taking Days 2 and 25, the two successive administrations of haloperidol, as the second factor; and taking the repeated testing sessions over each study day as the third factor. If sertraline altered the effects of haloperidol, this would be seen as a three-way interaction between these factors.

RESULTS

The mean age of the subjects was 26.3 years in the sertraline group and 23.3 years in the placebo group. Three subjects (2 sertraline, 1 placebo) withdrew because of side effects possibly or probably related to treatment. One subject given sertraline discontinued because of a syncopal episode, and another discontinued because of rigors, nausea, and vomiting. The subject given placebo who discontinued reported numerous side effects, including agitation, dizziness, hot flushes, nausea, sexual dysfunction, tremor, and vomiting.

Comparison of data from Day 1 with data from Day 2 indicates a clear profile of cognitive impairment and reduced alertness 6 to 8 hours after administration of haloperidol (Table 1). Haloperidol significantly impaired tests of attention and information processing such as simple reaction time, choice reaction time, number vigilance reaction time, number vigilance accuracy, rapid information processing accuracy, and d2 cancellation. Tracking ability was disrupted, and there was significant elevation of the critical flicker fusion threshold. There was no evidence of impaired vigilance or attentional efficiency the following morning.

The analyses identified a number of significant interactions between the 2 days of haloperidol dosing and the successive testing sessions (Table 1). These reflected greater profiles of cognitive impairment with the second administration of haloperidol. On Day 25, greater impair-

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Table 1. Summary of Effects of Haloperidol 2 mg Alone and Plus Sertraline 200 mg or Placebo on Cognitive Function and Psychomotor Performance*

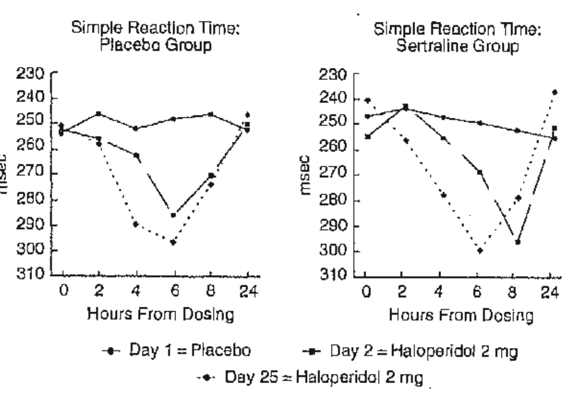
Task	Measure (Units)	Haloperidol (Day 2) vs Placebo (Day 1)	Haloperidol (Day 25) vs Haloperidol (Day 2)	Interaction of Sertraline With Change of Effects of Haloperidol From Day 2 to Day 25
Simple reaction time	Speed (msec)	p < .01	p < .05	NS
Choice reaction time	Speed (msec)	p < .05	p < .01	NS
Digit vigilance	Accuracy (%)	p < .001	NS	NS
	Speed (msec)	p < .0001	p < .05	NS
Rapid information processing	Accuracy (%)	p < .0001	NS	NS
	Speed (msec)	NS	p < .01	NS
Logical reasoning	Accuracy (%)	NS	NS	NS
	Speed (msec)	NS	NS	NS
Memory scanning	Sensitivity (SI)	NS	NS	NS
	Speed (msec)	NS	NS	NS
Tracking	Mean error (mm)	NS	p < .001	NS
Immediate recall	Accuracy (%)	NS	NS	NS
Delayed recall	Accuracy (%)	NS	p < .05	NS
Word recognition	Sensitivity (SI)	NS	p < .05	NS
	Speed (msec)	NS	NS	NS
d2 Cancellation	Efficiency	p < .05	NS	p < .05
Critical flicker fusion	Threshold (Hz)	p < .05	NS	p < .05
Visual analogue scale	Alertness (mm)	NS	p < .005	NS
	Calmness (mm)	NS	p < .05	NS
	Contentment (mm)	NS	NS	NS
Experimenter rating	Alertness of volunteers	p < .05	p < .001	NS

*Abbreviation: NS = not significant.

ment was seen on four measures (simple reaction time, choice reaction time, digit vigilance reaction time, and the alertness of the volunteers as assessed by the experimenter) with a significantly earlier onset of action (at 2 hours postdose). In addition, a wider range of measures were impaired on Day 25, including rapid information processing time, tracking, delayed word recall, word recognition sensitivity, and self-rated alertness. Importantly, the analysis (see Table 1) failed to demonstrate that sertraline dosing had any influence on the effects of haloperidol, the increased effects of haloperidol being seen equally in the patients who received sertraline as those who received placebo-sertraline. There were significant three-way interactions for two measures, d2 cancellation and critical flicker fusion. For d2 cancellation, the effect represents a faster recovery from the effects of haloperidol for the volunteers given sertraline. The effect on critical flicker fusion is unrelated to sertraline and represented a difference between the two groups of volunteers. Inspection of Figure 1 will clarify that the greater impairments with haloperidol 2 mg on Day 25 occurred independently of whether sertraline was administered to the volunteers. There was also some evidence that the sertraline group got slightly faster by Day 25 (evident in the 0-hour and 24-hour data), which while not significant for this measure, was significant for choice reaction time, number vigilance reaction time, and rapid information processing time. These effects did not interfere with the effect of haloperidol, but suggest a mild cognition enhancement produced by sertraline assuming no significant effects on the haloperidol levels.

Ten of 12 subjects in each group reported side effects of mild-to-moderate severity. The side effect profiles of sertraline and placebo were similar. The most frequently reported side effects were fatigue (8 subjects in each group), headache (3 on sertraline, 2 on placebo), nausea (3 on sertraline, 2 on placebo), dizziness (2 subjects in each group), diarrhea (2 on sertraline, 1 on placebo), increased sweating (1 on sertraline, 2 on placebo), vomiting (1 on sertraline, 2 on placebo), and somnolence (0 on sertraline, 2 on placebo). One subject in the sertraline group had evidence of extrapyramidal side effects (moderate shoulder and mild elbow stiffness) on Day 25. There were no treatment-related abnormalities in laboratory test results or other measures of safety.

Figure 1. The Effects of Haloperidol 2 mg on Day 2 and Day 25 Presented Separately for the Volunteers Who Received Placebo-Sertraline in Between and Those Who Received Sertraline



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DISCUSSION

The effects of haloperidol on cognitive function and psychomotor performance as observed in this study are consistent with those that would be expected with a drug that depresses the central nervous system and may be viewed as an extension of its pharmacologic activity. An interesting but totally unexpected finding was that the single, 2-mg dose of haloperidol produced greater impairment in cognitive function and psychomotor performance on Day 25 than on Day 2 in both placebo and sertraline groups. Inspection of Figure 1 will indicate that the effects of haloperidol on Day 2 had worn off completely by 24 hours for both groups of volunteers. The earlier onset of impairment on Day 25 is thus not a traditional carryover effect. In previous work with scopolamine, no change in the pattern of impairments was seen with three administrations of 0.6 mg scopolamine a week or more apart.[8] This effect is thus not typical of repeated acute administrations of cognitively impairing compounds. This observation of potentiation of impairment with two single doses of a drug several weeks apart has previously been demonstrated in animal experiments,[13] but has not, to our knowledge, been documented in man. This phenomenon has been termed "time-dependent sensitization," and in the case of rats exposed to haloperidol, it has been found that reexposure to the same compound up to 8 weeks later produces marked enhancement of the response. The mechanisms underlying this phenomenon are of great potential interest but are poorly understood.

Sertraline 200 mg/day—a dose four times higher than the usual, effective therapeutic dose of 50 mg/day[14]—did not potentiate the cognitive and psychomotor function impairment observed after haloperidol administration alone. Previous studies in healthy volunteers,[15,16] subjects between 50 and 67 years of age,[17] and elderly volunteers (mean age = 67 years)[18] demonstrated that sertraline alone does not impair psychomotor performance.

Although not observed in the present study, potentiation by SSRIs, such as fluoxetine, of the extrapyramidal side effects caused by neuroleptics such as haloperidol has been reported.[5,6] This effect may be the result of a pharmacodynamic drug-drug interaction related to the effects of SSRIs and haloperidol on dopamine.[3] Others have suggested that it may be caused by a pharmacokinetic drug-drug interaction that results in increased plasma neuroleptic concentrations.[6] However, there are insufficient data to establish the mechanism for the interaction.

Pharmacokinetic drug-drug interactions between SSRIs and haloperidol may occur because both are metabolized by the cytochrome P450 enzyme system and both inhibit cytochrome P450 2D6 activity.[18–26] Paroxetine and fluoxetine in clinically effective doses have been shown to have a greater potential for inhibiting cytochrome P450 2D6-mediated metabolism of drugs, such as desipramine, than does sertraline.[18,23–26] The findings of the present study are consistent with those of Lee et al.,[27] who reported that coadministration of sertraline and haloperidol was associated with a modest decrease (28%) in plasma haloperidol levels and a decrease in reduced haloperidol levels that are unlikely to be of clinical significance.

This study fonnd no pharmacodynamic interaction between sertraline and haloperidol with respect to cognitive function testing. However, the findings must be interpreted within the limitations of the study design. The dose of haloperidol evaluated was limited to relatively low, single-dose administration because the study was being carried out in normal volunteers. Plasma drug concentrations of sertraline and haloperidol were not measured. By inference, the absence of potentiation of the pharmacodynamic effects of haloperidol suggests that sertraline coadministration does not cause significant inhibition of haloperidol clearance.

In conclusion, administration of a single, low dose of haloperidol impaired cognitive function and alertness in normal volunteers. Administration of high doses of sertraline did not potentiate the impairment of cognitive function observed with haloperidol alone.

Drug names: acetaminophen (Tylenol and others), desipramine (Norpramin and others), fluoxetine (Prozac), haloperidol (Haldol and others), paroxetine (Paxil), scopolamine (Isopto-Hyoscine and others), sertraline (Zoloft).

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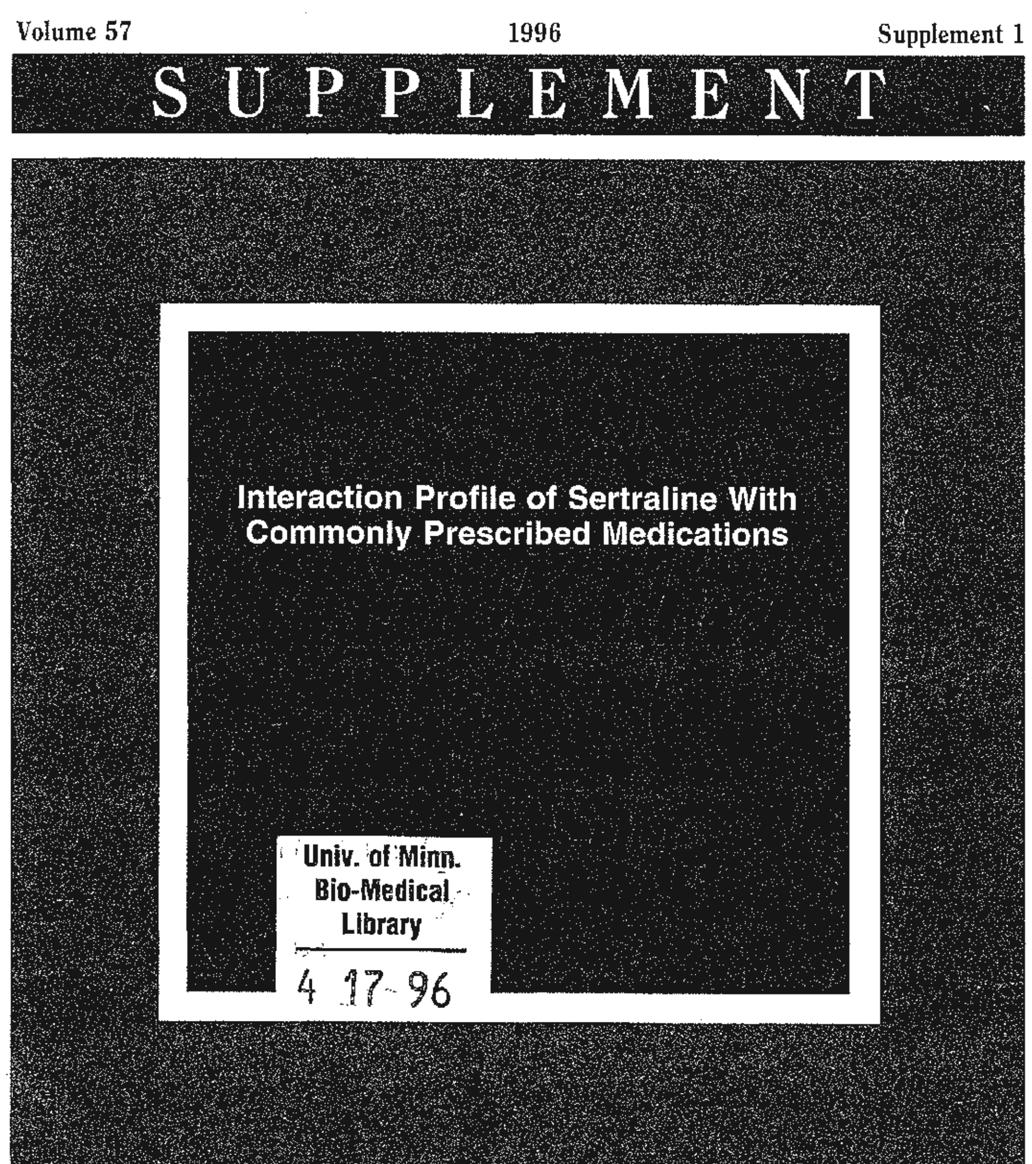

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γ-Hydroxybutyric acid (GHB) and γ-aminobutyric acid$_B$ receptor ($GABA_BR$) binding sites are distinctive from one another: molecular evidence

Ying Wu [a,e], Saima Ali [a], Gholamreza Ahmadian [a,1], Chun Che Liu [a], Yu Tian Wang [b], K. Michael Gibson [c], Andrew R. Calver [d], Joseph Francis [a,e], Menelas N. Pangalos [d,2], O. Carter Snead III [a,e,f,*]

[a] *Brain and Behavior Program, Hospital for Sick Children, University of Toronto, Toronto, ON, Canada M5G 1X8*
[b] *Brain Research Centre, University of British Columbia, Vancouver, BC, Canada*
[c] *Department of Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR, USA*
[d] *GlaxoSmithKline, Neurology, and GI CEDD, Harlow, UK*
[e] *Division of Neurology, Hospital for Sick Children, University of Toronto, Toronto, ON, Canada*
[f] *Department of Pediatrics, Faculty of Medicine, University of Toronto, Toronto, ON, Canada*

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Abstract

γ-Hydroxybutyric Acid (GHB) is thought to be a weak partial agonist at the γ-aminobutyric acid$_B$ Receptor ($GABA_BR$), but the precise relationship of the GHB receptor (GHBR) to the $GABA_BR$ remains unclear. In order to test the hypothesis that the GHBR is not identical to the $GABA_BR$, we conducted two groups of experiments. First, $GABA_BR$ subtype 1 (R1) and/or subtype 2 (R2) were over expressed in HEK 293 cells and membrane binding studies on the transfected cells done using [^{3}H]GHB and [^{3}H] (2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[a][7]annulen-6-ylidene) ethanoic acid ([^{3}H]NCS-382). The latter is a specific antagonist at the GHB binding site. Second, [^{3}H]GHB and [^{3}H]NCS-382 autoradiographic binding studies were done on the brains of mice in which the gene for $GABA_BR1a$ was deleted. Such mice do not have a functioning $GABA_BR$. There was no detectable specific [^{3}H]GHB or [^{3}H]NCS-382 binding in HEK 293 cells transfected with $GABA_BR1$, R2, or R1/R2. Binding to [^{3}H]CGP54626A, a high affinity $GABA_BR$ antagonist, was absent in $GABA_BR1a^{-/-}$ mice. There was no difference in [^{3}H]NCS-382 binding observed in the brains of $GABA_BR1a^{-/-}$, $GABA_BR1a^{+/-}$ or $GABA_BR1a^{+/+}$ mice. Specific [^{3}H]GHB binding was observed in the brain of $GABA_BR1a^{-/-}$ mice but was significantly lower than in wild type mice. These data support the hypothesis that the GHB binding site is separate and distinct from the $GABA_BR$.

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Keywords: Epilepsy; GABA receptor; γ-Hydroxybutyric Acid; Ligand binding; Gene transfection

1. Introduction

γ-Hydroxybutyric Acid (GHB) is a short chain fatty acid that occurs naturally in mammalian brain (Doherty et al., 1978). The primary precursor for GHB in the brain is γ-aminobutyric acid (GABA) (Snead et al., 1989). GHB has many properties which suggest that this compound may play a role in the brain as a neurotransmitter or neuromodulator. These characteristics include a discrete, subcellular distribution for GHB and its synthesizing enzyme and the presence of specific, high affinity [^{3}H]GHB binding sites in the brain, the anatomical distribution of which correlates with GHB turnover. As well, GHB is released by neuronal depolarization in a Ca^{+2}-dependent fashion

* Corresponding author. Tel.: +1-416-813-7851; fax: +1-416-813-7839.

E-mail address: csnead@sickkids.ca (O. Carter Snead).

[1] Present address: National Research Center for Genetic Engineering and Biotechnology, Tehran, Iran.

[2] Present address: Wyeth Research, Neuroscience Discovery, Princeton, NJ, USA.

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and a Na^+-dependent GHB uptake system has been demonstrated in brain (Maitre, 1997), as well as a distinct ontogeny for the GHB receptor (GHBR) (Snead, 1994).

GHB has the ability to induce a number of pharmacological and behavioral effects (Snead, 2002), has been shown to induce perturbation of a number of neurotransmitters (Maitre, 1997), and has found clinical use in the treatment of alcoholism and narcolepsy, and as an anesthetic (Wong et al., 2003). Further, GHB has emerged recently as a major recreational drug of abuse (Bernasconi et al., 1999; Wong et al., 2004). The mechanism(s) by which GHB induces these ubiquitous pharmacological effects is not clear. GHB has been postulated to be a weak $GABA_B$ receptor ($GABA_BR$) agonist (Bernasconi et al., 1999; Lingenhoehl et al., 1999), but there also is evidence that GHB may activate a specific, presynaptic GHBR in the brain which is separate and distinct from the $GABA_BR$ (Snead, 2000). Although a specific GHBR antagonist, NCS-382, traditionally has been used to characterize the GHBR (Maitre et al., 1990; Castelli et al., 2002), there is recent evidence (Andriamampandry et al., 2003) in support of NCS-382-insensitive GHBR subtypes in the brain.

A number of studies have been published that have assessed the affinity of GHB for the $GABA_BR$ and the ability of GHB to activate the $GABA_BR$. Virtually all of this work has examined the effect of GHB on the pharmacological, electrophysiological, biochemical, and/or binding characteristics of the $GABA_BR$ (Erhardt et al., 1998; Bernasconi et al., 1999; Lingenhoehl et al., 1999; Castelli et al., 2002; Gervasi et al., 2003). However, there are few data concerning the specific affinity of $GABA_BR$ agonists and antagonists for the GHBR as measured by [^{3}H]GHB or [^{3}H]NCS-382 binding techniques (Snead, 1996). The experiments described below were designed to test the hypothesis that the GHBR is separate and distinct from the $GABA_BR$. To this end, we investigated the binding affinity of [^{3}H]GHB and [^{3}H]NCS-382 for recombinant $GABA_BR$ as well as the binding properties of these isotopes in mice lacking the gene for $GABA_BR1$.

2. Methods

2.1. Materials

[^{3}H]CGP54626A with specific activity of 40 Ci/mmol was obtained from Tocris Cookson Ltd. (UK). [^{3}H]GHB with specific activity of 60 Ci/mmol and [^{3}H]NCS-382 with specific activity of 20 Ci/mM were obtained from American Radiolabelled Chemicals Inc. (USA). The plasmid constructs of $GABA_BR1b$ and $GABA_BR2$ clones tagged with hemagglutanin (HA) were obtained from GlaxoSmithKline, UK. The mouse monoclonal antibody against hemagglutanin (anti-HA) was purchased from Covance Inc. (USA). The guinea-pig anti-$GABA_BR1$, anti-$GABA_BR2$ polyclonal antibodies and the secondary antibodies were purchased from Chemicon International, Inc. (USA).

2.2. Cell culture and transfection

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. The cells were transiently transfected with human $GABA_BR1b$ or $GABA_BR2$ alone or together with $GABA_B$ R1/R2 using the effectene transfection reagent (Qiagen). The transfection efficiency was monitored by the expression of enhanced green fluorescent protein (EGFP) (Clontech). At least 30% of cells were transfected in all of binding assay experiments performed.

2.3. Immunoblot analysis

HEK 293 cells were harvested by centrifuge and suspended in samples buffer (62.5 mM tris-base, 2% SDS, 10% glycerol; pH 6.8). Protein quantification was determined by BCA assay (Pierce). Samples were heated for 10 min at 37 °C before loading. Equal amount of protein was loaded in each lane (15 μg/lane), separated with 4–20% gradient SDS-PAGE gel and transferred to nitrocellulose membranes. The blots were probed with guinea-pig anti-$GABA_BR1$ (1/1000) or $GABA_BR2$ polyclonal antibodies (1/1000) or mouse monoclonal anti-HA (0.5 μg/ml), followed by appropriate horseradish peroxidase-conjugated secondary antibodies. Protein–antibody complex was visualized with enhanced chemiluminescence reagents. The molecular weight was estimated with BIO-RAD Prestained SDS-PAGE Standards.

2.4. Immunofluorescence

HEK 293 cells were shocked with 2.5% DMSO 12 h after transfection. After 3 h, the cells were plated onto 35 mm dishes containing poly-L-lysine coated cover slips. Thirty-six hours after transfection, cells were fixed in 2% paraformaldehyde for 10 min, washed in 0.1 M phosphate buffer solution (PBS) and blocked for 1 h with 10% normal goat serum. To label surface proteins, the cells were incubated overnight in the primary antibody at 4 °C, washed in PBS, and incubated 30 min at room temperature with anti-mouse specific Cy3. The same cells were then permeabilized with 0.1% Triton X-100 for 10 min after rinsing with PBS, incubated in primary antibody for 3 h at 4 °C, incubated in anti-mouse specific FITC for 30 min at room temperature, and examined under a confocal microscope.

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2.5. Membrane preparation and ligand binding assays

Forty-eight hours after transfection, HEK 293 cells were collected and homogenized in buffer containing 10 mM Tris–HCl (pH 7.4) and 0.1 mM EDTA. Pellets of membrane protein were prepared by centrifuging at 1000×g for 5 min, collecting the supernatant, and then centrifuging at 15, 000×g for 20 min. Protein quantification was determined by BCA assay (Pierce).

The binding assay of [^{3}H]CGP54626A, a labeled high affinity $GABA_B$ receptor antagonist, was done by the method described by Bischoff et al. (1999). Membrane protein (50–100 μg) was resuspended with buffer containing 50 mM Tris–HCl (pH 7.4), 2.5 mM $CaCl_2$, and 0.5 nM [^{3}H]CGP54626A and agitated at room temperature for 30 min. Specific binding was determined in the presence of cold GABA in a concentration of 0.1 μM–1 mM. In the GHB competition experiments, a concentration range of 100 μM–100 mM was used.

[^{3}H]GHB binding was done by the method of Ratomponirina et al. (1998). Membrane protein (50 μg) from HEK 293 cells was resuspended with 0.1 M potassium phosphate buffer (pH 6.0) and incubated at 4 °C for 60 min with [^{3}H]GHB (50 or 100 nM). Nonspecific binding was determined in the presence of cold GHB in a concentration of 5 mM. Following incubation in triplicate, 1 ml aliquots were filtered on Whatman GF/B filters with a Brandel cell harvester and rapidly washed 3 times with 5 ml buffer. The ligand bound was counted by liquid scintillation.

The binding of [^{3}H]NCS-382, a GHBR antagonist (Maitre et al., 1990), was done by the method described by Mehta et al. (2001). Aliquots of 0.1–0.2 mg protein were incubated with [^{3}H]NCS-382 (20 nM) in Tris buffer (50 mM, pH 7.4) in 1 ml total volume. Nonspecific binding was determined in the presence of 1 mM NCS-382. Following incubation in triplicate, 1 ml aliquots were filtered on Whatman GF/B filters with a Brandel cell harvester and rapidly washed 3 times with 5 ml buffer. The ligand bound was counted by liquid scintillation.

2.6. $GABA_BR^{-/-}$ mice

$GABA_BR^{-/-}$ mice were created as described by Prosser et al. (2001). Briefly, homologous recombination in embryonic stem cells was used to generate a mouse lacking the $GABA_BR1$ subunit. Specifically, $GABA_BR1$ exons 2 to 6 were replaced with a β-galactosidase (geo) fusion gene, thus removing the translation initiation codons of both the $GABA_BR1a$ and $GABA_BR1b$ splice variants. RT-PCR analysis has revealed complete loss of $GABA_BR1$ mRNA in $GABA_BR^{-/-}$ mice and western blot analysis of brain tissue from $GABA_BR^{-/-}$ mice using antibodies specific for either $GABA_BR1a$, $GABA_BR1b$, or pan$GABA_BR1$ showed a complete lack of $GABA_BR1$ subunit expression. $GABA_BR$ wild type, heterozygous, and knockout mice were sacrificed by decapitation at the age postnatal day 18, their brains rapidly removed, rapidly frozen, and stored at −80 °C until use. All experiments were conducted according to the requirements of the United Kingdom Animals (Scientific Procedures) Act (1986) and strictly conformed to the ethical standards of GlaxoSmithKline Pharmaceuticals.

2.7. [^{3}H]CGP54626A autoradiography

[^{3}H]CGP54626A autoradiography was performed upon $GABA_BR1a^{+/+}$, $GABA_BR1a^{+/-}$, and $GABA_BR1a^{-/-}$ mice as described (Bischoff et al., 1999). The mice were sacrificed by decapitation and brains removed and immediately immersed in isopentane at −35 °C. Coronal sections were cut at 20 μm at −20 °C and thaw-mounted onto gelatin-coated slides that were dried and stored at −80 °C until used. Before [^{3}H]CGP54626A binding, brain sections were kept at room temperature for 1 h and then preincubated for 15 min in Krebs–Henseleit buffer. Total binding was performed in Krebs–Henseleit buffer containing 2 nM [^{3}H]CGP54626A for 2 h at room temperature. Ten micromolar of (−)baclofen was used to account for nonspecific binding.

2.8. [^{3}H]GHB autoradiography

[^{3}H]GHB autoradiography was performed upon $GABA_BR1a^{+/+}$, $GABA_BR1a^{+/-}$, and $GABA_BR1a^{-/-}$ mice as described (Banerjee et al., 1993). Brain sections were thawed at room temperature for 1 h and preincubated in 100 mM phosphate buffer (pH 6.0) for 30 min at 4 °C and air-dried. Triplicate tissue sections were incubated in the same buffer containing 30 nM [^{3}H]GHB for 30 min at 4 °C. In saturation experiments, equilibrium constants were generated by using 10 different concentrations of [^{3}H]GHB ranging from 2 to 500 nM. Nonspecific binding was determined in the presence of 5 mM unlabeled Na^+-free GHB since the GHB transport system in brain is strongly Na^+-dependent (Benavides et al., 1982). The nonspecific binding represented less than 10% of the total binding at concentrations near the apparent dissociation constant (K_D) for GHB. After three successive buffer washes (10 s each at 4 °C), sections were dipped in ice-cold deionized water and then air-dried.

2.9. [^{3}H]NCS-382 autoradiography

[^{3}H]NCS-382 autoradiography was performed upon $GABA_BR1a^{+/+}$, $GABA_BR1a^{+/-}$, and $GABA_BR1a^{-/-}$ mice as described (Gould et al., 2003). Brain sections

were thawed at room temperature for 1 h and incubated in 50 mM Tris–HCl buffer, pH 7.4 at 4 °C containing 1 μM [^{3}H]NCS-382 for 20 min. Nonspecific binding was determined in the presence of 500 µM unlabeled Na$^+$-free GHB. The nonspecific binding represented less than 10% of the total binding at concentrations near the K_D for [^{3}H]NCS-382 which is reported to be 0.5–1 μM (Mehta et al., 2001). Following incubation, sections on slides were washed for 3 min in 50 mM Tris–HCl buffer, pH 7.4 at 4 °C, and dipped for 1 s into ice-cold deionized distilled water. The sections were air-dried.

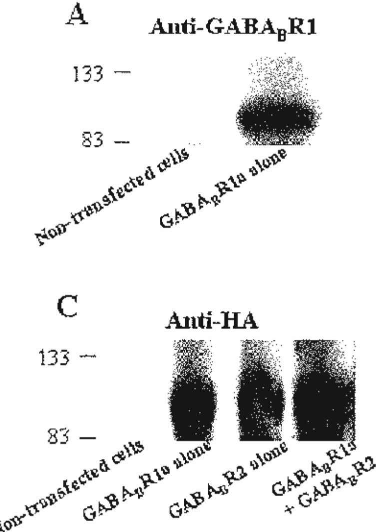
2.10. *Quantitative analysis*

The autoradiographic data were analyzed as described (Snead, 2000). Dried tissue sections were opposed to hyperfilm-bmax film (Amersham, Arlington, IL) with [^{3}H] microscale standards (Amersham) for 2–3 weeks at room temperature. The films were developed in D-19 (Kodak), fixed, and dried. Quantitative analysis of the resulting autoradiograms was performed densitometrically using a microcomputer based densitometer system (MCID; Imaging Research, Ontario, Canada). Briefly, a standard curve between the optical density (o.d.) of [^{3}H] standards and tissue radioactivity equivalents (pmol/mg of tissue) was constructed using a non-linear regression analysis. The average o.d. values of the selected brain regions were in the linear portion of this standard curve. The pmol/mg value in each brain region was calculated by interpolation using the image analyzer (Banerjee et al., 1998). Five to eight readings were determined and averaged for each anatomic area analyzed. Student's *t*-test (unpaired and two-tailed) was used to analyze the significance in all of the experiments.

3. Results

3.1. *Cell surface expression of GABA$_B$R1 was observed when GABA$_B$R1 was co-transfected with GABA$_B$R2*

The endogenous expression of GABA$_B$ receptors was undetectable in non-transfected HEK 293 cells (Fig. 1). When HEK 293 cells were transfected with plasmid cDNA of HA-GABA$_B$R1b and/or HA-GABA$_B$R2, over expression of GABA$_B$R1 and/or GABA$_B$R2 was observed by immunoblotting analysis with the antibody against GABA$_B$R1 (Fig. 1A), GABA$_B$R2 (Fig. 1B), and anti-HA (Fig. 1C). The subcellular localization of GABA$_B$ receptors in the transfected HEK 293 cells was ascertained by immunofluorescence. Fig. 2 demonstrates that GABA$_B$R1b tagged with a HA epitope at the N-terminus was absent from the cell surface of transfected HEK 293 cells under non-permeabilized condition (Fig. 2A). However, cell surface expression of HA-GABA$_B$R1b was observed when HA-GABA$_B$R1b was co-transfected with GABA$_B$R2



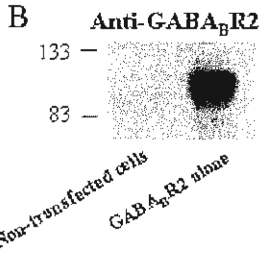


Fig. 1. Immunoblotting analysis with the whole lysate of non-transfected HEK 293 cells and HEK 293 cells transfected with HA-GABA$_B$R1b (A, C), or HA-GABA$_B$R2 (B, C), or co-transfected HA-GABA$_B$R1b with HA-GABA$_B$R2 (C). Expression of GABA$_B$R1b in transfected HEK 293 cells was detected by using guinea-pig polyclonal anti-GABA$_B$R1 (A) or mouse monoclonal anti-HA (C). Expression of GABA$_B$R2 in transfected HEK 293 cells was observed by using guinea-pig polyclonal anti-GABA$_B$R2 (B) or mouse monoclonal anti-HA (C). Relevant molecular weight markers are indicated on the left-hand side.

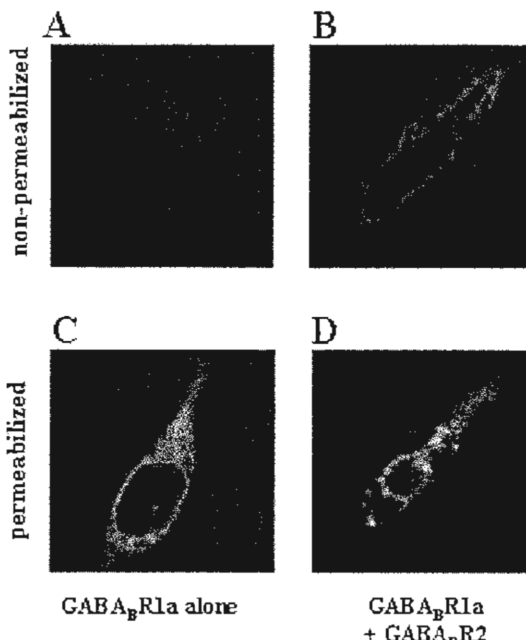


Fig. 2. Subcellular localization of GABA$_B$R1b expressed in HEK 293 cells. Surface expression was detected using HA antibody and anti-mouse specific Cy3 antibody in the non-permeabilized cells (upper panel). Intra-cellular receptors were detected in the same cells after permeabilization using HA antibody and anti-mouse specific FITC antibody (lower panel). GABA$_B$R1b alone (A, C). GABA$_B$R1b together with GABA$_B$R2 (B, D). Co-transfection with GABA$_B$R1b and GABA$_B$R2 resulted in the surface expression of GABA$_B$R1b (B).

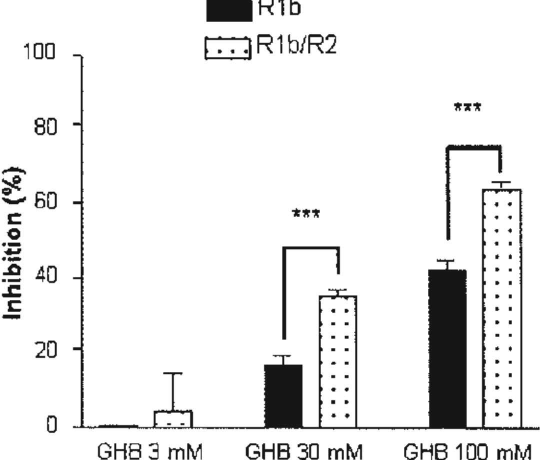


Fig. 3. The inhibition of [^{3}H]CGP54626A binding at recombinant GABA$_B$ receptors in HEK 293 cells by cold GHB. The cells were transfected with human GABA$_B$R1b alone (R1b) or together with GABA$_B$R2 (R1b/R2). The binding assay was performed using 50 μg membrane proteins from the transfected cells, 0.5 nM [^{3}H]CGP54626A and cold GHB in a concentration from 3 to 100 mM. Data were expressed as mean value ± S.E.M. from three independent experiments in triplicate. Statistical comparison using Student's t-test found a significant difference of GHB inhibition in the group (R1b/R2) vs. the group (R1b) (*** $P < 0.001$).

(Fig. 2B). Staining under permeable conditions confirmed that HA-GABA$_B$R1b was expressed intracellularly in both instances (Fig. 2C, D).

3.2. The heterodimerization between GABA$_B$R1 and GABA$_B$R2 significantly increased [^{3}H]CGP54626A binding specificity

Ligand-binding assays were used to measure the ability of GABA to compete with the antagonist [^{3}H]CGP54626A for binding sites on the recombinant GABA$_B$ receptors. HEK 293 cells transfected with GABA$_B$R1b showed specific, high affinity [^{3}H]CGP54626A binding while those transfected with GABA$_B$R2 showed no specific [^{3}H]CGP54626A binding (data not shown). In the HEK 293 cells co-transfected with GABA$_B$R1b and GABA$_B$R2, the IC_{50} value for GABA was 12.6 μM; however, the IC_{50} value for GABA was 31.6 μM in the HEK 293 cells expressing GABA$_B$R1b alone. These results are in agreement with previous reports (Kaupmann et al., 1998; Lingenhoehl et al., 1999), and demonstrate the validity of our assay system.

3.3. Partial displacement of [^{3}H]CGP54626A binding in GABA$_B$ receptor recombinant HEK cells required an extremely high concentration of GHB

The addition of 30 or 100 mM of GHB to HEK cells transfected with GABA$_B$R1b inhibited [^{3}H]CGP54626A binding by 16.3 ± 2.9% and 42.0 ± 3.9%, respectively (Fig. 3). Furthermore, co-transfection of GABA$_B$R1b with GABA$_B$R2 significantly increased the displacement rate of [^{3}H]CGP54626A binding by cold GHB in concentrations of both 30 and 100 mM in comparison to that of GABA$_B$R1b alone ($P < 0.001$) (Fig. 3). However, at concentrations of GHB of 3 mM (Fig. 3) or lower (data not shown), the inhibition of [^{3}H]CGP54626A binding by GHB disappeared, even with co-expression of GABA$_B$R1b with GABA$_B$R2.

3.4. Neither GHB nor NCS-382 had any affinity for recombinant GABA$_B$ receptors

There was no demonstrable specific [^{3}H]GHB binding using 50 and 100 nM (data not shown) of [^{3}H]GHB in membranes prepared from HEK 293 cells that had been transfected with GABA$_B$R1b or GABA$_B$R2 alone, co-transfected with GABA$_B$R1b and GABA$_B$R2, or wild type HEK 293 cells. Similarly, there was no detectable specific binding of [^{3}H]NCS-382 to membranes

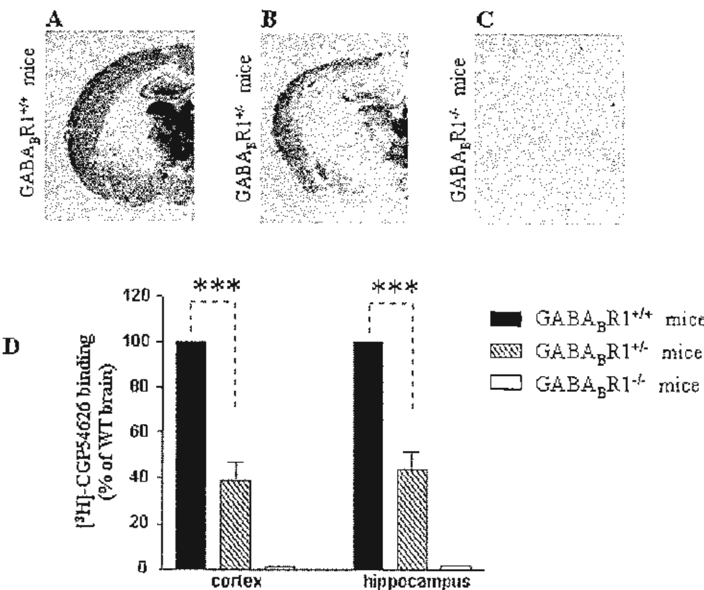


Fig. 4. [^{3}H]CGP54626A autoradiography. Coronal sections of mouse brains showed similar distribution of [^{3}H]CGP54626A binding in the brain of GABA$_B$R1$^{+/+}$ mice (A) and GABA$_B$R1$^{+/-}$ mice (B), but in the latter, the density of the binding was visibly reduced. The binding of [^{3}H]CGP54626A in the brain of GABA$_B$R1$^{-/-}$ mice was under detectable (C). Quantitative analysis (D) showed that the binding of [^{3}H]CGP54626A in the cortex and hippocampus of GABA$_B$R1$^{+/-}$ mice was equal to 40–50% of that of GABA$_B$R1$^{+/+}$ mice. Statistical analysis revealed the significant difference of the binding between GABA$_B$R1$^{+/-}$ and GABA$_B$R1$^{+/+}$ mice (*** $P < 0.001$). Data were expressed as mean value ± S.E.M. from four groups of mice.

prepared from GABA$_B$R recombinant HEK cells using 20 or 100 nM (data not shown) [^{3}H]NCS-382.

3.5. Specific [^{3}H]GHB and [^{3}H]NCS-382 binding are present in the brains of GABA$_B$R1$^{-/-}$ mice

There was no detectable specific [^{3}H]CGP54626A binding in the brains of the GABA$_B$R1$^{-/-}$ mice (Fig. 4). [^{3}H]CGP54626A binding was present in the brains of the GABA$_B$R1$^{+/-}$ mice, but was significantly reduced from that observed in the wild type mice (Fig. 4). [^{3}H]GHB binding was present in the GABA$_B$R1$^{-/-}$, GABA$_B$R1$^{+/-}$, and GABA$_B$R1$^{+/+}$ mice, but was significantly reduced by ~50% in the knockouts and ~32% in the heterozygote animals when compared to the wild type (Fig. 5). The reduction in binding observed in the heterozygote and knockout animals was due to a decrease in B_{max} (Fig. 6). The B_{max} (fmol/mg) for the wild type, heterozygote, and knockout mice was 228.2 ± 45.8, 135.5 ± 80.1, and 114.0 ± 35.3. [^{3}H]NCS-382 binding was the same in the knockouts, heterozygote, and wild type mice. There was no significant difference in the K_D (nM) of [^{3}H]GHB binding between wild type (112.2 ± 33.5), heterozygotes (136.1 ± 20.5), and knockout mice (167.31 ± 31.3) (Fig. 6). [^{3}H]NCS-382 binding was the same in the knockouts, heterozygotes, and wild type mice (Fig. 7). The regional and spatial distribution of [^{3}H]GHB and [^{3}H]NCS-382 binding was the same in the wild type and mutant animals.

4. Discussion

In these experiments, we have shown that there is little GHB binding to the GABA$_B$R of physiological relevance as measured by both [^{3}H]GHB and [^{3}H]NCS-382 binding in HEK cells in which GABA$_B$R1b, R2, or R1b/R2 are over expressed, although high millimolar concentrations of GHB do displace [^{3}H]CGP54626A binding in R1b/R2 transfected cells. The EC_{50} of GHB to activate GABA$_B$R1/R2 receptors co-expressed with Kir3 channels in *Xenopus* oocytes is reported to be approximately 5 mM (Lingenhoehl et al., 1999); however, in the current experiments, there was no significant inhibition of [^{3}H]CGP54626A binding in GABA$_B$R recombinant HEK cells by 3 mM GHB. In addition, we have demonstrated that both [^{3}H]GHB and [^{3}H]NCS-382 binding are present in the brains of animals in which there is no GABA$_B$R. However, although [^{3}H]NCS-382 binding was unchanged in brains from wild type, heterozygote, and knockout animals, [^{3}H]GHB binding was significantly reduced in the

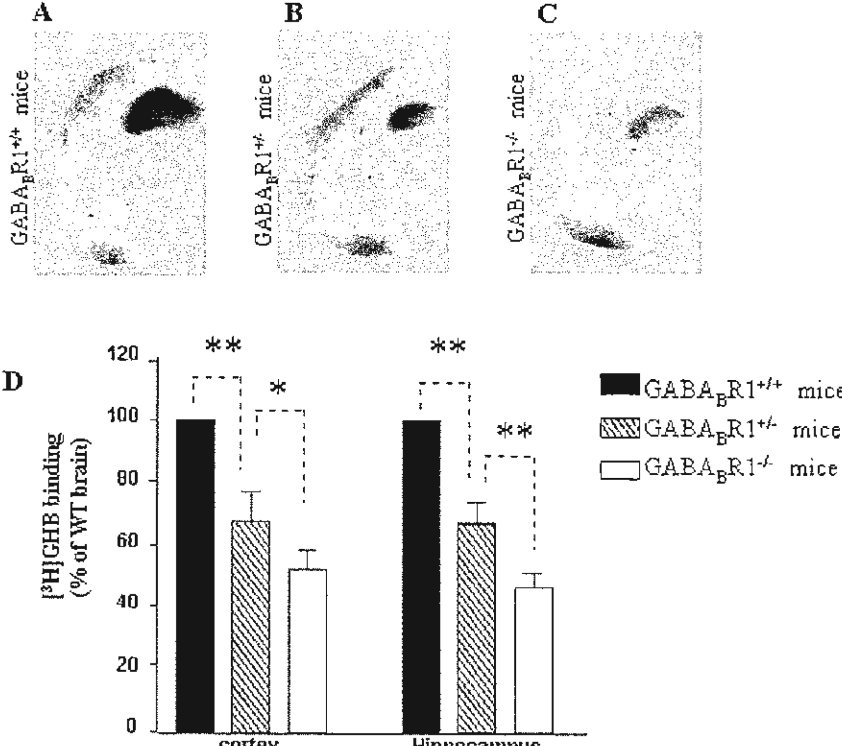


Fig. 5. [^{3}H]GHB autoradiography. Coronal sections of mouse brains showed similar distribution pattern of [^{3}H]GHB binding in the brain of GABA$_B$R1$^{+/+}$ mice (A), GABA$_B$R1$^{+/-}$ mice (B) and GABA$_B$R1$^{-/-}$ mice (C), the density of the binding was visibly reduced in the GABA$_B$R1$^{+/-}$ and GABA$_B$R1$^{-/-}$ mice. Quantitative analysis (D) showed that the binding of [^{3}H]GHB in the cortex and hippocampus of GABA$_B$R1$^{+/-}$ mice was equal to 40–50% of that of GABA$_B$R1$^{+/+}$ mice. Statistical analysis revealed the significant difference of the binding among GABA$_B$R1$^{-/-}$, GABA$_B$R1$^{+/-}$, and GABA$_B$R1$^{+/+}$ mice (* $P < 0.05$, ** $P < 0.01$). Data were expressed as mean value ± S.E.M. from four groups of mice.

heterozygote and knockout brains. The latter data suggest that in the [^{3}H]GHB binding assay, some of the

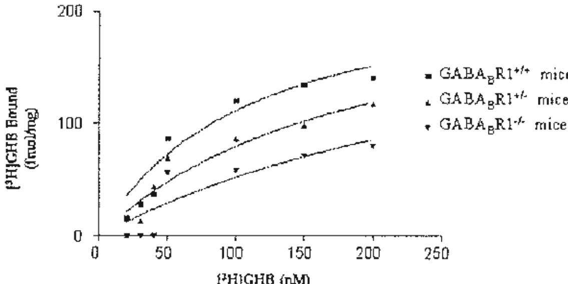


Fig. 6. Scatchard analysis of [^{3}H]GHB binding. [^{3}H]GHB binding was significantly decreased ($P < 0.05$, $N = 4$) in B_{max} in cortex of GABA$_B$R1$^{-/-}$ mice when compared to GABA$_B$R1$^{+/+}$ mice controls: B_{max} (fmol/mg) for the GABA$_B$R1$^{+/+}$ mice, GABA$_B$R1$^{+/-}$ mice, and GABA$_B$R1$^{-/-}$ mice was 228.2 ± 45.8, 135.5 ± 80.1, and 114.0 ± 35.3. There was no significant difference of [^{3}H]GHB binding in K_D (nM) of [^{3}H]GHB binding among the wild type (112.2 ± 33.5), heterozygote (136.1 ± 20.5), and knockout mice (167.5 ± 31.3).

[^{3}H]GHB may bind with low affinity to the GABA$_B$R and some with high affinity to the GHBR (Snead, 1996). This is in contradistinction to [^{3}H]NCS-382, a GHBR antagonist that has no affinity for GABA$_B$R, but which is specific only for GHBR (Snead, 1996).

GHB appears to have dual mechanisms of action in the brain (Wong et al., 2004). The biochemical data suggest that the intrinsic neurobiological activity of GHB may be mediated through the GHBR which is separate and distinct from the GABA$_B$R. However, many of the pharmacological and clinical effects of exogenously administered GHB appear to be mediated via the GABA$_B$R, where GHB may act both directly as a partial GABA$_B$R agonist and indirectly on the GABA$_B$R via GHB-derived GABA (Hechler et al., 1997).

A GHBR is suggested by specific, high affinity [^{3}H]GHB binding sites that occur in the brain with the highest density in hippocampus, followed by the cortex, and then thalamus (Hechler et al., 1987). The binding kinetics of this site comport with the physiological concentrations of GHB in brain (Doherty et al., 1978)

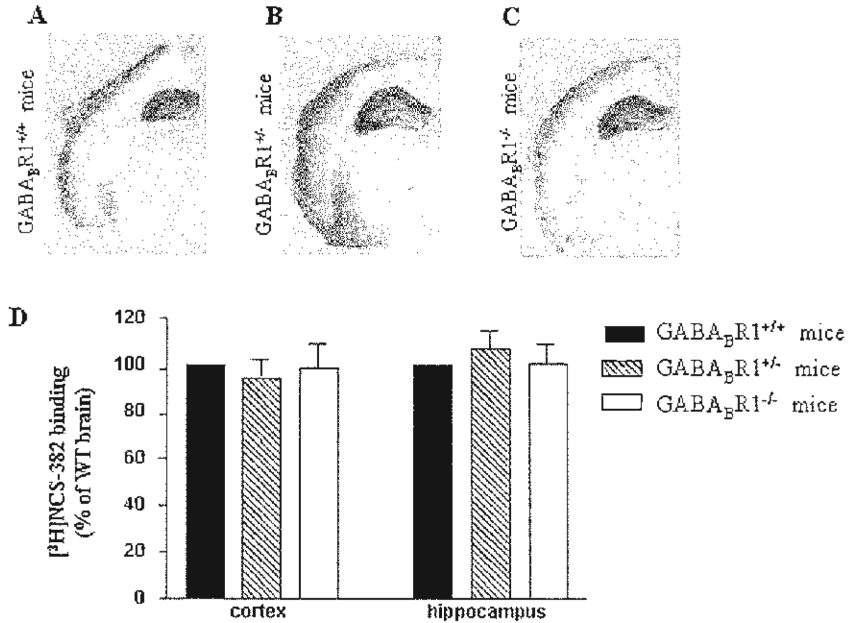


Fig. 7. [^{3}H]NCS-382 autoradiography. Coronal sections of mouse brains showed similar distribution pattern and density of [^{3}H]NCS-382 binding in the brain of GABA$_B$R1$^{+/+}$ mice (A), GABA$_B$R1$^{+/-}$ mice (B), and GABA$_B$R1$^{-/-}$ mice (C). Statistical analysis did not show significant difference of the [^{3}H]NCS-382 binding among GABA$_B$R1$^{+/+}$, GABA$_B$R1$^{+/-}$, and GABA$_B$R1$^{-/-}$ mice. Data were expressed as mean value± S.E.M. from four groups of mice.

because there is a high affinity GHB binding site with a K_D of 30–580 nM and a low affinity site with a K_D of around 1.5–16 μM (Maitre, 1997). In the current experiments, we examined only the high affinity site since the concentration range of [^{3}H]GHB for the saturation experiments was 2–500 nM. The anatomical distribution of [^{3}H]GHB binding correlates with GHB turnover (Vayer et al., 1988) and displays a distinct ontogeny with GHB binding being a postnatal event that appears in the third postnatal week of life (Snead, 1994).

There are several lines of evidence to support the hypothesis that the GHBR is not the same as the GABA$_B$R. GABA$_B$R agonists do not displace [^{3}H]GHB binding (Snead, 1996) and the ability of GHB to displace [^{3}H]baclofen binding is quite weak (Bernasconi et al., 1999). GHB and its antagonist, NCS-382 do not compete for [^{3}H]GABA binding in autoradiographic binding assays on rat brain sections (Snead, 1996). Further, the ontogeny (Snead, 1994, 2000) and regional distribution (Snead, 1994) of the GHBR and the GABA$_B$R are decidedly different. The current data showing that neither [^{3}H]GHB nor the GHBR antagonist [^{3}H]NCS-382 have any affinity for GABA$_B$R1, GABA$_B$R2, or GABA$_B$R1/R2 in recombinant HEK cells and that [^{3}H]GHB and [^{3}H]NCS-382 binding are present in the brains of mutant animals who have no GABA$_B$R are in support of this hypothesis. The GHBR has the binding kinetics to support the hypothesis that it is activated by endogenous GHB. The neurobiological function of the GHBR may be to modulate glutamate and GABA release (Hu et al., 2000).

Although the GHBR appears to be distinct from the GABA$_B$R, there is evidence that GHB is a weak or partial agonist of the GABA$_B$R with a weak affinity for the GABA$_B$R (Bernasconi et al., 1999). From a physiological perspective, GHB can activate the GABA$_B$R1/R2 co-expressed with Kir3 channels in *Xenopus* oocytes, but only with an EC_{50} of approximately 5 mM (Lingenhoehl et al., 1999), 1000 times the physiological concentration of GHB in brain. Similarly, millimolar concentrations of GHB are required to mimic the postsynaptic effects of baclofen on the GABA$_B$R and this electrophysiological effect of GHB is blocked by a specific GABA$_B$R antagonist, but not the GHBR antagonist, NCS-382 (Erhardt et al., 1998; Jensen and Mody, 2001). Indeed, it has been proposed that there is no electrophysiological evidence from in vitro investigations to support the idea that there is a neuronal GHBR-mediated electrophysiological response (Crunelli and Leresche, 2002).

Since the completion of these experiments functional aspects of the GABA$_B$R and GHB have been examined in the GABA$_B$R1$^{-/-}$ mouse created by Schuler et al. (2001) and Quéva et al. (2003). Both of those mutant

animals are phenotypically different from the $GABA_BR1^{-/-}$ mouse created by Prosser et al. (2001) that was used in the current experiments. Administration of baclofen to the mutant reported by Quéva et al. (2003) caused a large decrease in body temperature in wild type control mice and $GABA_BR^{+/-}$ mice, but the hypothermic effects of baclofen were abolished in $GABA_BR^{-/-}$. GHB administration also is usually associated with a robust hypothermia (Snead, 1990); however, in the $GABA_BR^{-/-}$ animals created by Quéva et al. (2003), GHB administration failed to induce hypothermia suggesting that the ability of GHB to reduce core body temperature is $GABA_BR$-mediated.

Recently, Kaupmann et al. (2003), in more extensive studies in the $GABA_BR^{-/-}$ mouse created by Schuler et al. (2001) have confirmed the absence of GHB-induced hypothermia in this mutant animal as well, have shown that additional pharmacological effects of GHB are absent, and demonstrated that there was a similar spatial distribution of [^{3}H]GHB and [^{3}H]GHB binding sites in the brains of the Schuler $GABA_BR^{-/-}$ mutant mouse, a finding duplicated in the present experiments using the $GABA_BR1^{-/-}$ mouse created by Prosser et al. (2001).

The principal difference between the findings of Kaupmann et al. (2003) and the current data are the binding data in the mice. While the NCS-382 data are identical, the current data showing specific, but reduced binding in the $GABA_BR1^{-/-}$ and $GABA_BR1^{+/-}$ mice compared to wild type animals differs from the findings of Kaupmann et al. (2003) who did not observe a reduction in [^{3}H]GHB binding in $GABA_BR1^{-/-}$, a puzzling finding given their in vivo data and the recombinant data in the current experiments.

The reason for the differences in [^{3}H]GHB binding observed by Kaupmann et al. (2003) and those we observed is not clear; however, the mutant mice used (Schuler et al., 2001) by Kaupmann and colleagues are phenotypically different from the mice (Prosser et al., 2001) used in the current study. The mutant animals used in the present experiments were derived from a C57B16/j background, develop seizures around postnatal day 21 and usually die from the recurrent seizures within several days of their onset. In contrast, mice lacking the $GABA_BR1$ subunit reported by Schuler et al. (2001) were derived from a Balb/c background and also exhibited spontaneous seizures, but were viable. There is about a 95% loss of specific binding to the $GABA_BR$ antagonist, [^{3}H]CGP54626A in the $GABA_BR1^{+/-}$ mice used in the current experiments (Fig. 4) vs. about 18% loss of binding to the $GABA_BR$ antagonist [^{125}I]CGP64123 in the animals used by Kaupmann et al. (2003).

One possible explanation for both the phenotypic differences and as well as the difference in GHB binding may have to do with $GABA_BR2$ (Gassmann et al., 2004) which is conserved to 30% of wild type in the $GABA_BR1^{-/-}$ mice used in the present experiments, but is undetectable in the mutant animals used by Kaupmann et al. (2003). In any event, both the $GABA_BR1^{-/-}$ mice created by Schuler et al. (2001) and those made by Prosser et al. (2001) demonstrated the unequivocal presence of GHB binding in the unequivocal absence of binding to the $GABA_BR$ providing proof that the GHB binding site is separate and distinct from the $GABA_BR$.

Another hypothesis for the discrepancy between [^{3}H]GHB binding and [^{3}H]NCS-382 binding in the $GABA_BR1$ mutant mice is that there are two pharmacological classes of GHB binding sites, one sensitive to, and one insensitive to, NCS-382. By this reasoning, the NCS-382 insensitive GHB binding site is linked to the $GABA_BR$ and therefore decreased in the $GABA_BR1^{-/-}$. Thus, the [^{3}H]GHB binding, which recognizes both NSC-sensitive and insensitive binding sites, would be decreased in the $GABA_BR1^{-/-}$. Indeed, Andriamampandry et al. (2003) have cloned a putative GHBR that is activated by GHB and is G protein coupled. However, this newly cloned receptor displays no affinity for NCS-382. There also are pharmacological data to support the hypothesis of NCS-insensitive GHBR that are $GABA_BR$-like (Koek et al., 2004).

The current data confirm that GHB is an extremely weak agonist at the $GABA_BR$ and suggest that in the [^{3}H]GHB binding assay, some of the [^{3}H]GHB binds with low affinity to an NCS-382 insensitive site and/or, the $GABA_BR$ and some with high affinity to the GHBR. These data may have direct relevance to the clinical problem of GHB abuse. Since levels of GHB are inordinately high during GHB intoxication, it would seem logical that blockade of the $GABA_BR$ may be an effective acute treatment for GHB overdose. However, studies of the mice lacking the gene for succinic semialdehyde dehydrogenase (SSADH) suggest that GHB intoxication may involve the GHBR (Gupta et al., 2003). This mutant animal ($SSADH^{-/-}$) is characterized by inordinately elevated concentrations of brain GHB and GABA that make it a potential model of chronic GHB abuse. During a critical period from postnatal days 16–22, $SSADH^{-/-}$ mice exhibit ataxia and develop generalized tonic clonic seizures that lead to rapid death. Therapeutic intervention with a $GABA_BR$ antagonist increased survival to 36% of animals, but treatment with NCS-382 was more efficacious, resulting in a survival rate of 61% (Gupta et al., 2002) suggesting that both the $GABA_BR$ and GHBR are involved in the pathogenesis of neurological morbidity in this animal.

In conclusion, these data provide further molecular evidence of the existence of a GHBR which is separate and distinct from the $GABA_BR$. The observation that

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specific binding of [^{3}H]GHB and [^{3}H]NCS-382 binding is undetectable in HEK cells in which $GABA_BR1b$, R2, or R1b/R2 have been over expressed demonstrates directly that there is little or no GHB binding of physiological relevance to the $GABA_BR$. The hypothesis of a $GABA_BR$-independent GHBR is strengthened considerably by finding that both [^{3}H]NCS-382 and [^{3}H]GHB are preserved in the brains of mutant animals in which the $GABA_BR$ is absent.

Acknowledgements

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"awake" and "drowsy"; (ii) an increase in time spent in stage 3; and (iii) a reduction in the latency to the first REM period and first stage 3. There was an increase in the number of rapid eye movement periods on the second night, which was just short of statistical significance.

The changes manifested by the depressed patients were in the same direction, though they were less marked than in the controls.

The findings support the concept of an increased state of vigilance or arousal on the first laboratory night. They also indicate a frequent tendency to miss the first REM period on the first study night.

RÉSUMÉ

ADAPTATION AU LABORATOIRE D'ÉTUDE DU SOMMEIL CHEZ DES SUJETS NORMAUX ET DES MALADES DÉPRIMÉS ("EFFET DE LA PREMIÈRE NUIT")

Les auteurs analysent les variations de l'EEG du sommeil entre la première et la deuxième nuit passées dans le laboratoire d'étude du sommeil chez 21 malades déprimés, comparés à 15 sujets de contrôle.

La deuxième nuit, plusieurs variations significatives s'observent chez les sujets de contrôle: (i) diminution du temps d'éveil et de somnolence; (ii) augmentation de la durée de stade 3; et (iii) réduction de la latence de la première période de PMO et du premier stade 3. L'augmentation du nombre de périodes de mouvements oculaires rapides au cours de la seconde nuit n'a qu'une signification statistique réduite.

Les variations présentées par les malades déprimés vont dans le même sens mais sont moins marquées que pour les sujets de contrôle.

Ces données confirment la notion d'une augmentation de l'état de veille ou d'activité pendant la première nuit passée au laboratoire. Elles indiquent aussi que la première PMO ait tendance à manquer lors de la première nuit d'étude.

C. Cochrane, Ph.D., advised on statistical procedures. Drs. G. Thrasher, R. Knapp and J. Scott participated in various aspects of these studies. We wish to thank Mrs. J. Benson, Mrs. M. Graves and Mrs. S. Kelly for their technical assistance.

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EFFECT OF BUTYROLACTONE AND GAMMA-HYDROXYBUTYRATE ON THE EEG AND SLEEP CYCLE IN MAN

Y. YAMADA, M.D., J. YAMAMOTO, M.D., A. FUJIKI, M.D., Y. HISHIKAWA, M.D. AND Z. KANEKO, M.D.

Department of Neuropsychiatry, Osaka University Medical School, Fukushima-ku, Osaka (Japan)

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It has been reported that short chain fatty acids induce in animals a "sleep-like" state indistinguishable from natural sleep and that at higher doses they induce an anesthetic state (Samson *et al.* 1956; Holmquist and Ingvar 1957; Laborit *et al.* 1960). There have been reports that sodium butyrate and its related compounds, when administered i.v., induce in cats a sleep state with low voltage fast EEG and rapid eye movements (REM period) (Jouvet *et al.* 1961; Matsuzaki *et al.* 1964). However, Winters and Spooner (1965) have reported that no significant change could be found in the sleep cycle of cats following the injection of γ-hydroxybutyrate. Metcalf *et al.* (1966) have recently reported that the oral administration of sodium γ-hydroxybutyrate in man does not induce the REM period of sleep.

The present experiments were undertaken to compare

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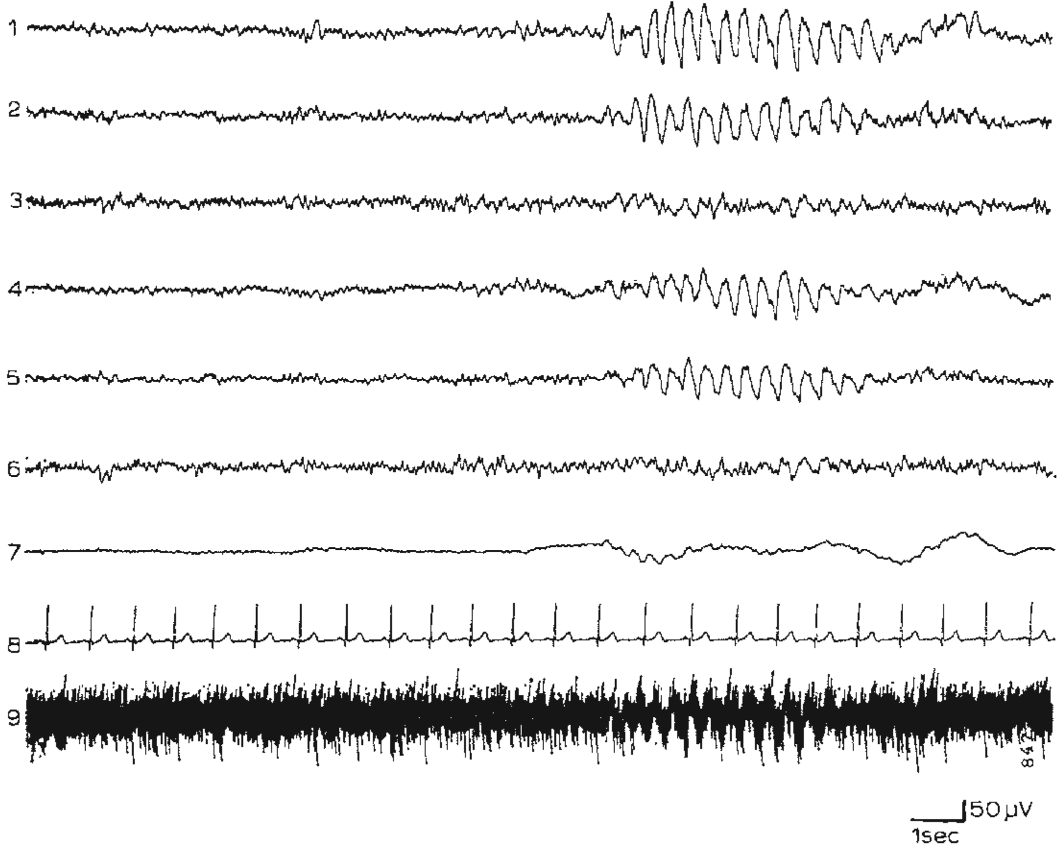

Fig. 1.
Record obtained in a normal subject (female, aged 20 years) about 9 min after the start of the i.v. injection of butyrolactone (20 mg/kg).
1–6: EEGs (1,4: left and right fronto-central; 2,5: left and right centro-parietal; 3,6: left and right parieto-occipital area); 7: electro-oculogram; 8: EKG; 9: EMG of the mental muscle recorded with the reference electrode on one of the ear lobes.

the electrographic change accompanying the sleep state induced by butyrolactone and sodium γ-hydroxybutyrate with those observed in natural sleep in man.

SUBJECTS AND METHODS

Twelve normal adults, three males and nine females, aged between 20 and 33 years, were used and a total of 50 recordings were obtained during and after the injection of either of the compounds or saline placebo. Of these recordings, 22 were carried out in five subjects in the daytime, and 28 in the remaining seven subjects (4 recordings for each: twice with the injection of butyrolactone and twice with the injection of saline) at night starting between 11 and 12 p.m. Amounts of the compounds (10, 20 or 30 mg/kg) were diluted with distilled water to a total volume of 20 ml and were injected i.v. in 10 min. The EEG, electro-oculogram, EKG and EMG were simultaneously recorded with a 12-channel electroencephalograph. Silver disc electrodes were affixed to the scalp and on the outer canthus of both eyes for recording, respectively, the EEG and eye movements. A pair of stainless steel electrodes (80 μ in diameter) insulated except for the tips were inserted into the mental muscle for recording the EMG. Sleep records were classified into the following 4 stages: the 1st (or drowsy) stage was characterized by the EEG pattern of low voltage without spindle; the 2nd (or spindle) stage by spindling against a low voltage background; the 3rd (or delta) stage by a slow, high voltage activity; and the last (or REM) stage by a low voltage EEG activity, rapid eye movements and disappearance of a tonic muscle activity (see Dement and Kleitman 1957; Hishikawa *et al.* 1965b).

RESULTS

1. *Effect on the EEG pattern and consciousness*

Within 5–10 min after the onset of the injection of

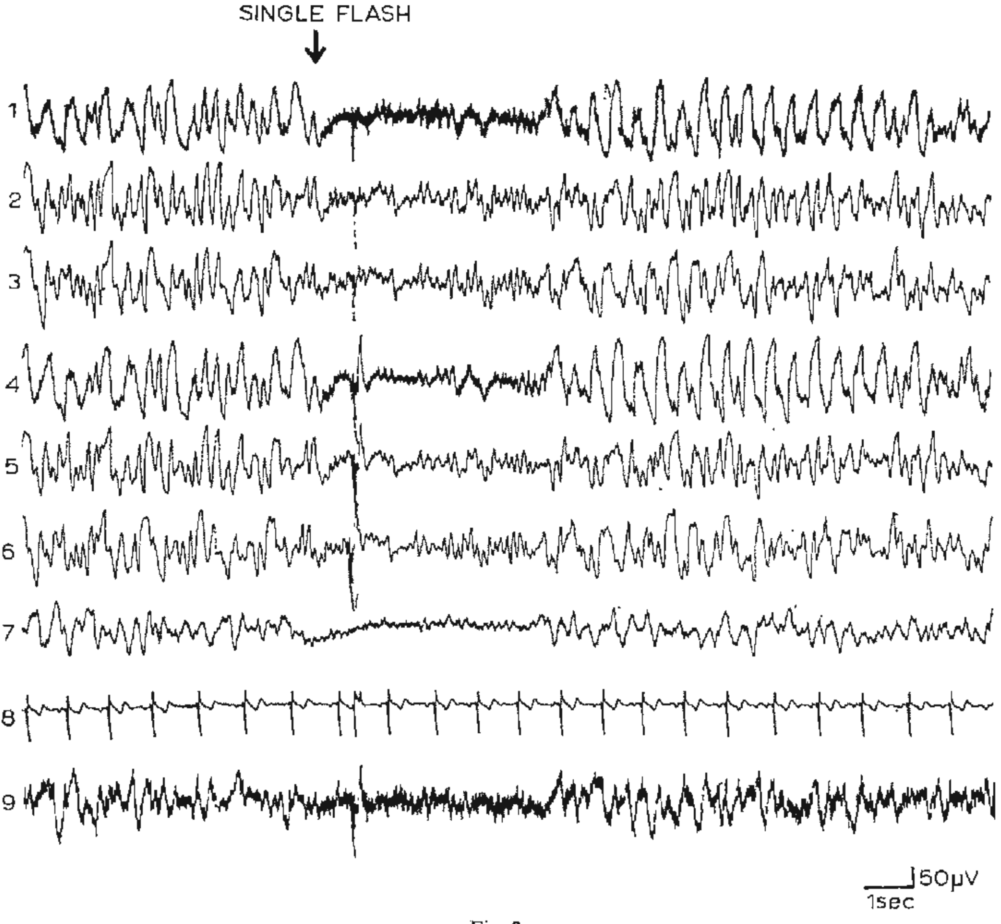

Fig. 2

Record obtained in a subject (female, aged 23 years) about 28 min after the start of the i.v. injection of butyrolactone (20 mg/kg). A flash of light (arrow) induced transient blocking of the slow waves. The subject responded to the stimulus as she had been told beforehand by pushing a key button in her hand (associated with a large and sharp artifact). Note that the tracings of the electro-oculogram (7) and EMG (9) are contaminated by the EEG activity.

1–6: EEGs recorded with the reference electrode on one of the ear lobes (1,4: left and right frontal; 2,5: left and right central; 3,6: left and right parietal area); 8: EKG.

either of the compounds, bursts of rhythmical slow waves (2–5 c/sec, 100–200 µV) would repeatedly recur in all cortical areas with the largest amplitude in the anterior derivations against a background activity of alpha rhythm (Fig. 1); the latter gradually diminished in frequency and eventually disappeared, while the bursts of slow waves became more and more frequent. About 15 min after the end of the injection the EEG pattern became one of continuous slow waves (2–2.5 c/sec, 100–200 µV) lasting for 20–30 min, after which they again appeared organized into short bursts. The subjects showing the above EEG pattern felt as if they had taken alcoholic beverage, but their consciousness was fairly clear and they could perceive and understand what was going on around them; they could count and remember the number of flash stimuli, could respond to these as instructed, etc. These behavioral responses were usually associated with a transient blocking of the high voltage slow, and appearance of low voltage fast activity intermixed with waves in the alpha and theta frequency range (Fig. 2).

In the daytime examination of three subjects, the drug effects on the EEG pattern and on the subjective feeling gradually wore off and the alpha rhythm reappeared within 50–90 min after the end of the injection. At this time, a single flash stimulus would induce an EEG response consisting of a brief (1–2 sec) burst of high voltage slow waves

such as those described above, and the subjects could respond to the stimulus. The remaining two subjects passed into sleep about 15–20 min after the end of the injection; *i.e.*, the continuous slow waves were followed by patterns characteristic of the spindle and delta stages, and both the EEG and the behavioral response to the flash stimulus completely disappeared. The bursts of rhythmical slow waves became less and less frequent as the amount of slow waves of 0.5–1 c/sec in the background EEG increased, but would reappear when the subjects were aroused. The spindle stage could be rarely identified. The above effect on the EEG pattern was similarly observed with either of the compounds at the doses of 20 and 30 mg/kg.

2. *Effect on the sleep cycle*

Effect on nocturnal sleep was studied with only butyrolactone at the dose of 20 or 30 mg/kg when the compound, at 20 mg/kg, was insufficient to induce the above described EEG effect (cases No. 2 and 7 in Table I). Since the latter did not last more than 2 h, observation was limited to the first 2 h following the injection. The average latency of each stage of sleep from the start of the injection is shown for each subject in Table I. The spindle and delta stages of sleep occurred earlier under the effect of the drug than under the control condition, but the latency of the REM period of sleep did not change significantly, whether calculated from the start of the injection or from the onset of sleep. Under the effect of the drug, the duration of the spindle stage tended to decrease, the spindle activity became less frequent and of smaller amplitude, but the delta stage preceding the initial REM period tended to last longer than under the control condition.

DISCUSSION

The results reported in this paper show that the EEG pattern induced in man by the i.v. administration of 20–30 mg/kg of butyrolactone and sodium γ-hydroxybutyrate is rather characteristic and is seldom if ever observed in the natural awake and sleeping states of normal human adult. This is in contrast to the observations of Laborit *et al.* (1960) in cats. Metcalf *et al.* (1966) have recently reported two types of paradoxical EEG–behavioral dissociation following the oral administration of sodium γ-hydroxybutyrate in man. This is generally in agreement with our observations. The fact that subjects showing the bursts of high voltage slow waves soon after the i.v. administration of the drugs could respond to the flash stimulus, suggests that this electrographic pattern is of a different nature from the slow waves that occur in natural sleep.

Under the effect of butyrolactone, the spindle and delta stages of sleep occurred earlier than under the control condition, but the latency of the REM period of sleep did not change significantly. There have been many reports about the effect of drugs on the sleep cycle (Rossi 1962; Oswald *et al.* 1963; Rechtschaffen and Maron 1964; Hishikawa *et al.* 1965a). In the present study, the REM period did not seem to be part of the direct effect of these compounds.

Differences in species of experimental subject and dosage of compounds used might account for the differences between ours and the results reported by others (see Introduction).

SUMMARY

The purpose of the present paper is to compare a

TABLE I

Latencies of various stages of sleep from the start of the injection of butyrolactone (in minutes)

Case	Sex	Disappearance of α-rhythm (drowsy stage)		Spindle stage		Delta stage		REM period	
		Control	Butyro-lactone	Control	Butyro-lactone	Control	Butyro-lactone	Control	Butyro-lactone
1	F	15	14	29	18	40	39	117	126
2	M	27	20	32	22	40	32	92	50
3	F	13	12	21	16	26	20	132	130
4	F	8	12	22	16	37	23	97	133
5	F	19	8	23	18	40	21	92	62
6	F	14	6	24	—	54	16	84	83
7	M	13	10	19	13	29	19	91	88
Mean		15.5	11.7	24.3	17.1	40.8	24.3	100	96
S.D.*		6.0	4.5	4.6	3.0	9.6	8.2	54.6	34.0
P**		>0.05		<0.01		<0.05		>0.5	

* Standard deviation.

** P values are based upon two-tailed t tests for matched samples on differences between means of the data under the effect of butyrolactone and the control condition.

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sleep state induced by butyrolactone and sodium γ-hydroxybutyrate with natural sleep in man with the aids of the EEG, electro-oculogram and EMG of the mental muscle. The following results were obtained:

(1) The i.v. administration of 20–30 mg/kg of butyrolactone and sodium γ-hydroxybutyrate induced in man a peculiar state without marked change in consciousness and with high voltage slow waves in the EEG.

(2) When butyrolactone was administered at night, just prior to bed-time, the spindle and delta stages of sleep occurred earlier, the duration of the spindle stage became shorter and the delta stage tended to last longer than in the control night. The latency to the onset of the initial REM period calculated either from the start of the injection or from the onset of sleep did not differ significantly.

RÉSUMÉ

EFFETS DU BUTYROLACTONE ET DU GAMMA-HYDROXYBUTYRATE SUR L'EEG ET LE CYCLE DU SOMMEIL CHEZ L'HOMME

Le but de la présente étude est de comparer chez l'homme un stade de sommeil induit par butyrolactone et sodium γ-hydroxybutyrate au sommeil naturel à l'aide de l'EEG, de l'oculogramme et de l'électromyogramme du muscle du menton. Les résultats obtenus sont les suivants:

(1) L'administration intra-veineuse de 20–30 mg/kg de butyrolactone et de sodium γ-hydroxybutyrate induit chez l'homme un stade particulier sans changement marqué de la conscience et avec ondes lentes de haut-voltage à l'EEG.

(2) Quand le butyrolactone est administré le soir, juste avant l'heure du coucher, les stades du sommeil caractérisés par des spindles et des ondes delta apparaissent plus tôt, la durée du stade avec spindles se raccourcit et le stade avec ondes delta tend à se prolonger par rapport aux nuits de contrôle. La latence d'apparition de la première période de mouvements oculaires rapides calculée aussi bien par rapport au début de l'injection que par rapport au début du sommeil ne change pas de façon significative.

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ANNEX I

SUMMARY OF PRODUCT CHARACTERISTICS

1. NAME OF THE MEDICINAL PRODUCT

Xyrem 500 mg/ml oral solution

2. QUALITATIVE AND QUANTITATIVE COMPOSITION

Each ml of solution contains 500 mg of sodium oxybate.

For a full list of excipients, see section 6.1.

3. PHARMACEUTICAL FORM

Oral solution.

The oral solution is clear to slightly opalescent.

4. CLINICAL PARTICULARS

4.1 Therapeutic indications

Treatment of narcolepsy with cataplexy in adult patients.

4.2 Posology and method of administration

Treatment should be initiated by and remain under the guidance of a physician experienced in the treatment of sleep disorders.

Due to the well known potential of abuse of sodium oxybate, physicians should evaluate patients for a history of or susceptibility to drug abuse prior to commencing treatment. During treatment, patients should be monitored for the risk of diversion, misuse and abuse of sodium oxybate (see section 4.4).

Posology The recommended starting dose is 4.5 g/day sodium oxybate divided into two equal doses of 2.25 g/dose. The dose should be titrated to effect based on efficacy and tolerability (see section 4.4) up to a maximum of 9 g/day divided into two equal doses of 4.5 g/dose by adjusting up or down in dose increments of 1.5 g/day (i.e. 0.75 g/dose). A minimum of one to two weeks is recommended between dose increments. The dose of 9 g/day should not be exceeded due to the possible occurrence of severe symptoms at doses of 18 g/day or above (see section 4.4).

Single doses of 4.5 g should not be given unless the patient has been titrated previously to that dose level.

Discontinuation of Xyrem

The discontinuation effects of sodium oxybate have not been systematically evaluated in controlled clinical trials (see section 4.4).

If the patient stops taking the medicinal product for more than 14 consecutive days, titration should be restarted from the lowest dose.

Special populations

Patients with hepatic impairment

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The starting dose should be halved in all patients with hepatic impairment, and response to dose increments monitored closely (see section 4.4).

Patients with renal impairment
All patients with impaired renal function should consider a dietary recommendation to reduce sodium intake (see section 4.4).

Elderly patients
Elderly patients should be monitored closely for impaired motor and/or cognitive function when taking sodium oxybate (see section 4.4).

Paediatric population
The safety and efficacy of sodium oxybate in children and adolescents aged 0-18 years has not been established. No data is available. Therefore the use of sodium oxybate in children and adolescents in not recommended.

Method of administration
Xyrem should be taken orally upon getting into bed and again between 2.5 to 4 hours later. It is recommended that both doses of Xyrem should be made up at the same time upon retiring to bed. Xyrem is provided for use with a graduated measuring syringe and two 90 ml dosing cups with child resistant caps. Each measured dose of Xyrem must be dispensed into the dosing cup and diluted with 60 ml of water prior to ingestion. Because food significantly reduces the bioavailability of sodium oxybate, patients should eat at least several (2-3) hours before taking the first dose of Xyrem at bedtime. Patients should always observe the same timing of dosing in relation to meals.

4.3 Contraindications

Hypersensitivity to the active substance or to any of the excipients.

Patients with major depression

Patients with succinic semialdehyde dehydrogenase deficiency.

Patients being treated with opioids or barbiturates.

4.4 Special warnings and precautions for use

Xyrem has the potential to induce respiratory depression

Respiratory depression
Sodium oxybate also has the potential to induce respiratory depression. Apnoea and respiratory depression have been observed in a fasting healthy subject after a single intake of 4.5 g (twice the recommended starting dose). Patients should be questioned regarding signs of Central Nervous System (CNS) or respiratory depression. Special caution should be observed in patients with an underlying respiratory disorder. Because of the higher risk of sleep apnoea, patients with a BMI ≥40 kg/m2 should be monitored closely when taking sodium oxybate.

Approximately 80% of patients who received sodium oxybate during clinical trials maintained CNS stimulant use. Whether this affected respiration during the night is unknown. Before increasing the sodium oxybate dose (see section 4.2), prescribers should be aware that sleep apnoea occurs in up to 50% of patients with narcolepsy.

Abuse potential and dependence
Sodium oxybate, which is as the sodium salt of GHB, is a CNS depressant active substance with well known abuse potential. Prior to treatment physicians should evaluate patients for a history of or

susceptibility to drug abuse. Patients should be routinely monitored and in the case of suspected abuse, treatment with sodium oxybate should be discontinued.

There have been case reports of dependence after illicit use of GHB at frequent repeated doses (18 to 250 g/day) in excess of the therapeutic dose range. Whilst there is no clear evidence of emergence of dependence in patients taking sodium oxybate at therapeutic doses, this possibility cannot be excluded.

CNS depression

The combined use of alcohol or any CNS depressant medicinal product with sodium oxybate may result in potentiation of the CNS-depressant effects of sodium oxybate. Therefore, patients should be warned against the use of alcohol in conjunction with sodium oxybate.

Patients with porphyria

Sodium oxybate is considered to be unsafe in patients with porphyria because it has been shown to be porphyrogenic in animals or *in vitro* systems.

Benzodiazepines

Given the possibility of increasing the risk of respiratory depression, the concomitant use of benzodiazepines and sodium oxybate should be avoided

Neuropsychiatric events

Patients may become confused while being treated with sodium oxybate. If this occurs, they should be evaluated fully, and appropriate intervention considered on an individual basis. Other neuropsychiatric events include anxiety, psychosis, paranoia, hallucinations, and agitation. The emergence of thought disorders and/or behavioural abnormalities when patients are treated with sodium oxybate requires careful and immediate evaluation.

The emergence of depression when patients are treated with sodium oxybate requires careful and immediate evaluation. Patients with a previous history of a depressive illness and/or suicide attempt should be monitored especially carefully for the emergence of depressive symptoms while taking sodium oxybate. Major depression is contraindicated for use with Xyrem (section 4.3).

If a patient experiences urinary or faecal incontinence during sodium oxybate therapy, the prescriber should consider pursuing investigations to rule out underlying aetiologies.

Sleepwalking has been reported in patients treated in clinical trials with sodium oxybate. It is unclear if some or all of these episodes correspond to true somnambulism (a parasomnia occurring during non-REM sleep) or to any other specific medical disorder. The risk of injury or self-harm should be borne in mind in any patient with sleepwalking. Therefore, episodes of sleepwalking should be fully evaluated and appropriate interventions considered.

Sodium intake

Patients taking sodium oxybate will have an additional daily intake of sodium that ranges from 0.82 g (for a 4.5 g/day Xyrem dose) to 1.6 g (for a 9 g/day Xyrem dose). A dietary recommendation to reduce sodium intake should be carefully considered in the management of patients with heart failure, hypertension or compromised renal function. (see section 4.2).

Patients with compromised liver function

Patients with compromised liver function will have an increased elimination half-life and systemic exposure to sodium oxybate (see Section 5.2). The starting dose should therefore be halved in such patients, and response to dose increments monitored closely (see section 4.2).

Elderly

There is very limited experience with sodium oxybate in the elderly. Therefore, elderly patients should be monitored closely for impaired motor and/or cognitive function when taking sodium oxybate.

Childhood and adolescence
Safety and effectiveness in children and adolescents has not been established, therefore use in patients under 18 years of age is not recommended.

Epileptic patients
Seizures have been observed in patients treated with sodium oxybate. In patients with epilepsy, the safety and efficacy of sodium oxybate has not been established, therefore use is not recommended.

Rebound effects and withdrawal syndrome
The discontinuation effects of sodium oxybate have not been systematically evaluated in controlled clinical trials. In some patients, cataplexy may return at a higher frequency on cessation of sodium oxybate therapy, however this may be due to the normal variability of the disease. Although the clinical trial experience with sodium oxybate in narcolepsy/cataplexy patients at therapeutic doses does not show clear evidence of a withdrawal syndrome, in rare cases, events such as insomnia, headache, anxiety, dizziness, sleep disorder, somnolence, hallucination, and psychotic disorders were observed after GHB discontinuation.

4.5 Interaction with other medicinal products and other forms of interaction

The combined use of alcohol with sodium oxybate may result in potentiation of the central nervous system-depressant effects of sodium oxybate. Patients should be warned against the use of any alcoholic beverages in conjunction with sodium oxybate.

Sodium oxybate should not be used in combination with sedative hypnotics or other CNS depressants.

Sedative hypnotics
Drug interaction studies in healthy adults with sodium oxybate (single dose of 2.25 g) and lorazepam (an anxiolytic [benzodiazepine]; single dose of 2 mg) and zolpidem tartrate (a hypnotic [non-benzodiazepine]; single dose of 5 mg) demonstrated no pharmacokinetic interactions. Increased sleepiness was observed after concomitant administration of sodium oxybate (2.25 g) and lorazepam (2 mg). The pharmacodynamic interaction with zolpidem has not been assessed. When higher doses up to 9 g/d of sodium oxybate are combined with higher doses of hypnotics (within the recommended dose range) pharmacodynamic interactions associated with symptoms of CNS depression and/or respiratory depression cannot be excluded (see section 4.3).

Tramadol
A drug interaction study in healthy adults with sodium oxybate (single dose of 2.25 g) and tramadol (a central acting opioid; single dose of 100 mg) demonstrated no pharmacokinetic/pharmacodynamic interaction. When higher doses up to 9 g/d of sodium oxybate are combined with higher doses of opioids (within the recommended dose range) pharmacodynamic interactions associated with symptoms of CNS depression and/or respiratory depression cannot be excluded (see sections 4.3).

Antidepressants
Drug interaction studies in healthy adults demonstrated no pharmacokinetic interactions between sodium oxybate (single dose of 2.25 g) and the antidepressants protriptyline hydrochloride (single dose of 10 mg) and duloxetine (60 mg at steady state). No additional effect on sleepiness was observed when comparing single doses of sodium oxybate alone (2.25 g) and sodium oxybate (2.25 g) in combination with duloxetine (60 mg at steady state). Antidepressants have been used in the treatment of cataplexy. A possible additive effect of antidepressants and sodium oxybate cannot be excluded. The rate of adverse events has increased when sodium oxybate is co-administered with tricyclic antidepressants.

Modafinil
A drug interaction study in healthy adults demonstrated no pharmacokinetic interactions between sodium oxybate (single dose of 4.5 g) and modafinil (a stimulant; single dose of 200 mg). Sodium oxybate has been administered concomitantly with CNS stimulant agents in approximately 80% of

patients in clinical studies in narcolepsy. Whether this affected respiration during the night is unknown.

The co-administration of omeprazole (a medicinal product that alters gastric pH) has no clinically significant effect on the pharmacokinetics of sodium oxybate. The dose of sodium oxybate therefore does not require adjustment when given concomitantly with proton pump inhibitors.

Studies *in vitro* with pooled human liver microsomes indicate that sodium oxybate does not significantly inhibit the activities of the human isoenzymes (see section 5.2).

Since sodium oxybate is metabolised by GHB dehydrogenase there is a potential risk of an interaction with medicinal products that stimulate or inhibit this enzyme (e.g. valproate, phenytoin or ethosuximide). No interaction studies have been conducted in human subjects.

4.6 Fertility, pregnancy and lactation

Pregnancy
Animal studies have shown no evidence of teratogenicity but embryolethality was seen in both rat and rabbit studies (see section 5.3).

Data from a limited number of pregnant women exposed in the first trimester indicate a possible increased risk of spontaneous abortions. To date no other relevant epidemiological data are available. Limited data from pregnant patients during second and third trimester indicate no malformative nor foeto/neonatal toxicity of sodium oxybate.

Sodium oxybate is not recommended during pregnancy.

Breastfeeding
It is not known whether sodium oxybate and/or its metabolites are excreted into breast milk. Breastfeeding is not recommended during treatment with sodium oxybate.

Fertility
There is no clinical data available on the effect of sodium oxybate on fertility. No effect on fertility parameters in the rat were observed (see section 5.3).

4.7 Effects on ability to drive and use machines

Sodium oxybate has a major influence on the ability to drive and use machines.

For at least 6 hours after taking sodium oxybate, patients must not undertake activities requiring complete mental alertness or motor co-ordination, such as operating machinery or driving.
When patients first start taking sodium oxybate, until they know whether this medicinal product will still have some carryover effect on them the next day, they should use extreme care while driving a car, operating heavy machines, or performing any other task that could be dangerous or require full mental alertness.

4.8 Undesirable effects

The most commonly reported adverse reactions are dizziness, nausea, and headache, all occurring in 10% to 20% of patients.

Frequency estimate: very common (≥ 1/10); common (≥ 1/100 to < 1/10); uncommon (≥ 1/1000 to < 1/100); rare (≥ 1/10,000 to < 1/1000); very rare (< 1/10,000); not known (cannot be estimated from the available data).

Within each frequency grouping, adverse events are presented in order of decreasing seriousness.

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Immune system disorders:
Uncommon : hypersensitivity

Metabolism and nutrition disorders:
Common: anorexia, decreased appetite

Psychiatric disorders:
Common: depression, cataplexy, anxiety, abnormal dreams, confusional state, disorientation, nightmares, sleepwalking, sleep disorder, insomnia, middle insomnia, nervousness
Uncommon: suicide attempt, psychosis, paranoia, hallucination, abnormal thinking, agitation, initial insomnia
Not known (cannot be estimated from the available data): suicidal ideation

Nervous system disorders:
Very common: dizziness, headache
Common: sleep paralysis, somnolence, tremor, balance disorder, disturbance in attention, hypoaesthesia, paraesthesia, sedation, dysgeusia
Uncommon: myoclonus, amnesia, restless legs syndrome
Not known (cannot be estimated from the available data): convulsion

Ear and labyrinth disorders:
Common: vertigo

Eye disorders:
Common: blurred vision

Cardiac disorders:
Common: palpitations

Vascular disorders:
Common: hypertension

Respiratory, thoracic and mediastinal disorders:
Common: dyspnoea, snoring, nasal congestion
Not known (cannot be estimated from the available data): respiratory depression, sleep apnoea

Gastrointestinal disorders:
Very common: nausea (the frequency of nausea is higher in women than men)
Common: vomiting, diarrhoea, abdominal pain upper,
Uncommon: faecal incontinence

Skin and subcutaneous tissue disorders:
Common: hyperhidrosis, rash
Not known (cannot be estimated from the available data): urticaria

Musculoskeletal, connective tissue and bone disorders:
Common: arthralgia, muscle, spasms, back pain

Renal and urinary disorders:
Common: enuresis nocturna, urinary incontinence

General disorders and administration site conditions:
Common: asthenia, fatigue, feeling drunk, oedema peripheral

Infections and infestations:
Common: nasopharyngitis, sinusitis

Investigations:
Common: blood pressure increased, weight decreased

Injury, poisoning and procedural complications
Common: fall

Description of selected adverse reactions
In some patients, cataplexy may return at a higher frequency on cessation of sodium oxybate therapy, however this may be due to the normal variability of the disease. Although the clinical trial experience with sodium oxybate in narcolepsy/cataplexy patients at therapeutic doses does not show clear evidence of a withdrawal syndrome, in rare cases, adverse reactions such as insomnia, headache, anxiety, dizziness, sleep disorder, somnolence, hallucination, and psychotic disorders were observed after GHB discontinuation.

4.9 Overdose

Information about signs and symptoms associated with overdose with sodium oxybate is limited. Most data derives from the illicit use of GHB. Sodium oxybate is the sodium salt of GHB. Events associated with withdrawal syndrome have been observed outside the therapeutic range.

Patients have exhibited varying degrees of depressed consciousness that may fluctuate rapidly between a confusional, agitated combative state with ataxia and coma. Emesis (even with impaired consciousness), diaphoresis, headache, and impaired psychomotor skills may be observed. Blurred vision has been reported. An increasing depth of coma has been observed at higher doses. Myoclonus and tonic-clonic seizures have been reported. There are reports of compromise in the rate and depth of respiration and of life-threatening respiratory depression, necessitating intubation and ventilation. Cheyne-Stokes respiration and apnoea have been observed. Bradycardia and hypothermia may accompany unconsciousness, as well as muscular hypotonia, but tendon reflexes remain intact. Bradycardia has been responsive to atropine intravenous administration.
Gastric lavage may be considered if co-ingestants are suspected. Because emesis may occur in the presence of impaired consciousness, appropriate posture (left lateral recumbent position) and protection of the airway by intubation may be warranted. Although gag reflex may be absent in deeply comatose patients, even unconscious patients may become combative to intubation, and rapid sequence induction (without the use of sedative) should be considered.

No reversal of the central depressant effects of sodium oxybate can be expected from flumazenil administration. There is insufficient evidence to recommend the use of naloxone in the treatment of overdose with GHB. The use of haemodialysis and other forms of extracorporeal medicinal product removal have not been studied in sodium oxybate overdose. However, due to the rapid metabolism of sodium oxybate, these measures are not warranted.

5. PHARMACOLOGICAL PROPERTIES

5.1 Pharmacodynamic properties

Pharmacotherapeutic group: Other nervous system medicinal products, ATC code: N07XX04.

Sodium oxybate is a central nervous system depressant which reduces excessive daytime sleepiness and cataplexy in patients with narcolepsy and modifies sleep architecture reducing fragmented nighttime sleep. The precise mechanism by which sodium oxybate produces an effect is unknown, however sodium oxybate is thought to act by promoting slow (delta) wave sleep and consolidating night-time sleep. Sodium oxybate administered before nocturnal sleep increases Stages 3 and 4 sleep and increases sleep latency, whilst reducing the frequency of sleep onset REM periods (SOREMPs). Other mechanisms, which have yet to be elucidated, may also be involved. In the clinical trial database, greater than 80 % of patients maintained concomitant stimulant use.

The effectiveness of sodium oxybate for the treatment of narcolepsy symptoms was established in four multicentre, randomised, double-blind, placebo-controlled, parallel-group trials (Trial 1, 2, 3 and 4) in patients with narcolepsy with cataplexy except for trial 2 where cataplexy was not required for enrolment Concomitant stimulant use was permitted in all trials (except for the active-treatment phase of Trial 2); antidepressants were withdrawn prior to active treatment in all trials with the exception of Trial 2. In each trial, the daily dose was divided into two equal doses. The first dose each night was taken at bedtime and the second dose was taken 2.5 to 4 hours later.

Table 1 Summary of clinical trials performed using sodium oxybate for the treatment of narcolepsy

Trial	Primary Efficacy	*N*=	Secondary Efficacy	Duration	Active treatment and Dose (g/d)
Trial 1	EDS (ESS); CGIc	246	MWT/Sleep Architecture/ Cataplexy/Naps/FOSQ	8 weeks	Xyrem 4.5 - 9
Trial 2	EDS (MWT)	231	Sleep Architecture/ ESS/CGIc/Naps	8 weeks	Xyrem 6 – 9 Modafinil 200-600 mg
Trial 3	Cataplexy	136	EDS (ESS)/CGIc/Naps	4 weeks	Xyrem 3 - 9
Trial 4	Cataplexy	55	None	4 weeks	Xyrem 3 - 9

EDS – Excessive daytime sleepiness; ESS – Epworth Sleepiness Scale; MWT – Maintenance of Wakefulness Test; Naps – Number of inadvertent daytime naps; CGIc – Clinical Global Impression of Change; FOSQ – Functional Outcomes of Sleep Questionnaire

Trial 1 enrolled 246 patients with narcolepsy and incorporated a 1 week up-titration period. The primary measures of efficacy were changes in excessive daytime sleepiness as measured by the Epworth Sleepiness Scale (ESS), and the change in the overall severity of the patient's narcolepsy symptoms as assessed by the investigator using the Clinical Global Impressions of Change (CGI-c) measure.

Table 2 Summary of ESS in Trial 1

Epworth Sleepiness Scale (ESS; range 0-24)				
Dose Group [g/d (n)]	Baseline	Endpoint	Median Change from Baseline	Change from Baseline Compared to Placebo **(p-value)**
Placebo (60)	17.3	16.7	-0.5	-
4.5 (68)	17.5	15.7	-1.0	0.119
6 (63)	17.9	15.3	-2.0	**0.001**
9 (55)	17.9	13.1	-2.0	**< 0.001**

Table 3 Summary of CGI-c in Trial 1

Clinical Global Impressions of Change (CGI-c)		
Dose Group [g/d (n)]	Responders* N (%)	Change from Baseline Compared to Placebo (p-value)
Placebo (60)	13 (21.7)	-
4.5 (68)	32 (47.1)	**0.002**
6 (63)	30 (47.6)	**< 0.001**
9 (55)	30 (54.4)	**< 0.001**

* The CGI-c data were analysed by defining responders as those patients who were very much improved or much improved.

Trial 2 compared the effects of orally administered sodium oxybate, modafinil and sodium oxybate + modafinil, with placebo in the treatment of daytime sleepiness in narcolepsy. During the 8 week double-blind period, patients took modafinil at their established dose or placebo equivalent. The sodium oxybate or placebo equivalent dose was 6 g/day for the first 4 weeks and was increased to 9 g/day for the remaining 4 weeks. The primary measure of efficacy was excessive daytime sleepiness as measured by objective response in MWT.

Table 4 Summary of MWT in Trial 2

TRIAL 2				
Dose Group	Baseline	Endpoint	Mean Change from Baseline	Endpoint Compared to Placebo
Placebo (56)	9.9	6.9	-2.7	-
Sodium Oxybate (55)	11.5	11.3	0.16	**<0.001**
Modafinil (63)	10.5	9.8	-0.6	**0.004**
Sodium Oxybate + Modafinil (57)	10.4	12.7	2.3	**<0.001**

Trial 3 enrolled 136 narcoleptic patients with moderate to severe cataplexy (median of 21 cataplexy attacks per week) at baseline. The primary efficacy measure in this trial was the frequency of cataplexy attacks.

Table 5 Summary of outcomes in Trial 3

Dosage	Number of Subjects	Cataplexy Attacks		
Trial 3		**Baseline**	**Median Change from Baseline**	**Change from Baseline Compared to Placebo (p-value)**
		Median attacks/week		
Placebo	33	20.5	-4	-
3.0 g/day	33	20.0	-7	0.5235
6.0 g/day	31	23.0	-10	**0.0529**
9.0 g/day	33	23.5	-16	**0.0008**

Trial 4 enrolled 55 narcoleptic patients who had been taking open-label sodium oxybate for 7 to 44 months. Patients were randomised to continued treatment with sodium oxybate at their stable dose or to placebo. Trial 4 was designed specifically to evaluate the continued efficacy of sodium oxybate after long-term use. The primary efficacy measure in this trial was the frequency of cataplexy attacks.

Table 6 Summary of outcome in Trial 4

	Number of Subjects	**Baseline**	**Median Change from Baseline**	Change from Baseline Compared to Placebo **(p-value)**
Trial 4		Median attacks/two weeks		
Placebo	29	4.0	21.0	-
Sodium oxybate	26	1.9	0	**p <0.001**

In Trial 4, the response was numerically similar for patients treated with doses of 6 to 9 g/day, but there was no effect seen in patients treated with doses less than 6 g/day.

5.2 Pharmacokinetic properties

Sodium oxybate is rapidly and almost completely absorbed after oral administration; absorption is delayed and decreased by a high fat meal. It is eliminated mainly by metabolism with a half-life of 0.5 to 1 hour. Pharmacokinetics are nonlinear with the area under the plasma concentration curve (AUC) versus time curve increasing 3.8-fold as dose is doubled from 4.5 g to 9 g. The pharmacokinetics are not altered with repeat dosing.

Absorption: Sodium oxybate is absorbed rapidly following oral administration with an absolute bioavailability of about 88 %. The average peak plasma concentrations (1st and 2nd peak) following administration of a 9 g daily dose divided into two equivalent doses given four hours apart were 78 and 142 µg/ml, respectively. The average time to peak plasma concentration (T_{max}) ranged from 0.5 to 2 hours in eight pharmacokinetic studies. Following oral administration, the plasma levels of sodium oxybate increase more than proportionally with increasing dose. Single doses greater than 4.5 g have not been studied. Administration of sodium oxybate immediately after a high fat meal resulted in delayed absorption (average T_{max} increased from 0.75 hr to 2.0 hr) and a reduction in peak plasma level (C_{max}) by a mean of 58% and of systemic exposure (AUC) by 37%.

Distribution: Sodium oxybate is a hydrophilic compound with an apparent volume of distribution averaging 190-384 ml/kg. At sodium oxybate concentrations ranging from 3 to 300 μg/ml, less than 1% is bound to plasma proteins.

Biotransformation: Animal studies indicate that metabolism is the major elimination pathway for sodium oxybate, producing carbon dioxide and water via the tricarboxylic acid (Krebs) cycle and secondarily by β-oxidation. The primary pathway involves a cytosolic $NADP^+$-linked enzyme, GHB dehydrogenase, that catalyses the conversion of sodium oxybate to succinic semialdehyde, which is then biotransformed to succinic acid by the enzyme succinic semialdehyde dehydrogenase. Succinic acid enters the Krebs cycle where it is metabolised to carbon dioxide and water. A second mitochondrial oxidoreductase enzyme, a transhydrogenase, also catalyses the conversion to succinic semialdehyde in the presence of α-ketoglutarate. An alternate pathway of biotransformation involves β-oxidation via 3,4-dihydroxybutyrate to Acetyl CoA, which also enters the citric acid cycle to result in the formation of carbon dioxide and water. No active metabolites have been identified.

Studies *in vitro* with pooled human liver microsomes indicate that sodium oxybate does not significantly inhibit the activities of the human isoenzymes: CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A up to the concentration of 3 mM (378 μg/ml). These levels are considerably higher than levels achieved with therapeutic doses.

Elimination: The clearance of sodium oxybate is almost entirely by biotransformation to carbon dioxide, which is then eliminated by expiration. On average, less than 5% of unchanged medicinal product appears in human urine within 6 to 8 hours after dosing. Faecal excretion is negligible.

Special populations:

Elderly patients:. In a limited number of patients greater than the age of 65 years the pharmacokinetics of sodium oxybate was not different compared to patients younger than 65 years of age.

Paediatric patients: The pharmacokinetics of sodium oxybate in paediatric patients under the age of 18 years have not been studied.

Renal impairment: Because the kidney does not have a significant role in the excretion of sodium oxybate, no pharmacokinetic study in patients with renal dysfunction has been conducted; no effect of renal function on sodium oxybate pharmacokinetics would be expected.

Hepatic disease: Sodium oxybate undergoes significant presystemic (hepatic first-pass) metabolism. After a single oral dose of 25 mg/kg, AUC values were double in cirrhotic patients, with apparent oral clearance reduced from 9.1 in healthy adults to 4.5 and 4.1 ml/min/kg in Class A (without ascites) and Class C (with ascites) patients, respectively. Elimination half-life was significantly longer in Class C and Class A patients than in control subjects (mean $t_{1/2}$ of 59 and 32 versus 22 minutes). It is prudent to reduce the starting dose of sodium oxybate by one-half in patients with liver dysfunction (see Section 4.2).

Race
The effect of race on metabolism of sodium oxybate has not been evaluated.

5.3 Preclinical safety data

Repeat administration of sodium oxybate to rats (90 days and 26 weeks) and dogs (52 weeks) did not result in any significant findings in clinical chemistry and micro- and macro pathology. Treatment-related clinical signs were mainly related to sedation, reduced food consumption and secondary changes in body weight, body weight gain and organ weights. The rat and dog exposures at the NOEL were lower (~50%) than that in humans. Sodium oxybate was non-mutagenic and non-clastogenic in *in vitro* and *in vivo* assays.

Gamma Butyrolactone (GBL), a pro-drug of GHB tested at exposures similar to the expected in man (1.21-1.64 times) has been classified by NTP as non-carcinogenic in rats and equivocal carcinogen in mice, due to slight increase of pheochromocytomas which was difficult to interpret due to high mortality in the high dose group. In a rat carcinogenicity study with oxybate no compound-related tumours were identified.

GHB had no effect on mating, general fertility or sperm parameters and did not produce embryo-foetal toxicity in rats exposed to up 1000 mg/kg/day GHB (1.64 times the human exposure calculated in nonpregnant animals). Perinatal mortality was increased and mean pup weight was decreased during the lactation period in high-dose F_1 animals. The association of these developmental effects with maternal toxicity could not be established. In rabbits, slight foetotoxicity was observed.

Drug discrimination studies show that GHB produces a unique discriminative stimulus that in some respects is similar to that of alcohol, morphine and certain GABA-mimetic medicinal products. Self-administration studies in rats, mice and monkeys have produced conflicting results, whereas tolerance to GHB as well as cross-tolerance to alcohol and baclofen has been clearly demonstrated in rodents.

6. PHARMACEUTICAL PARTICULARS

6.1 List of excipients

Purified water
Malic acid for pH adjustment
Sodium hydroxide for pH adjustment

6.2 Incompatibilities

This medicinal product must not be mixed with other medicinal products.

6.3 Shelf life

5 years

After first opening: 40 days

After dilution in the dosing cups (see section 4.2), the preparation should be used within 24 hours.

6.4 Special precautions for storage

This medicinal product does not require any special storage conditions.
For storage conditions of the diluted medicinal product see section 6.3.

6.5 Nature and contents of container

Amber oval PET bottle which is delivered with a plastic/foil seal and closed with a child resistant closure composed of HDPE/polypropylene with a pulpboard inner liner.

Each carton contains one bottle of 180 ml solution, a press-in bottle adaptor consisting of an LDPE bottle-well housing, a Silastic Biomedical ETR Elastomer valve, an acrylonitrile butadiene styrene terpolymer valve retainer and LDPE tubing, a graduated measuring device (polypropylene syringe), two polypropylene dosing cups and two HDPE child resistant screw closures.

6.6 Special precautions for disposal

No special requirements

7. MARKETING AUTHORISATION HOLDER

UCB Pharma Ltd
208 Bath Road
Slough
Berkshire
SL1 3WE
UK

8. MARKETING AUTHORISATION NUMBER

EU/1/05/312/001

9. DATE OF FIRST AUTHORISATION/RENEWAL OF AUTHORISATION

Date for first Authorisation: 13/10/2005
Date of latest renewal: 18/10/2010

10. DATE OF REVISION OF THE TEXT

Detailed information on this medicinal product is available on the website of the European Medicines Agency http://www.ema.europa.eu/

ANNEX II

A. MANUFACTURING AUTHORISATION HOLDER RESPONSIBLE FOR BATCH RELEASE

B. CONDITIONS OF THE MARKETING AUTHORISATION

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A MANUFACTURING AUTHORISATION HOLDER RESPONSIBLE FOR BATCH RELEASE

Name and address of the manufacturer responsible for batch release

UCB Pharma Ltd
208 Bath Road
Slough
Berkshire SL1 3WE
United Kingdom

B CONDITIONS OF THE MARKETING AUTHORISATION

- **CONDITIONS OR RESTRICTIONS REGARDING SUPPLY AND USE IMPOSED ON THE MARKETING AUTHORISATION HOLDER**

Medicinal product subject to special and restricted medical prescription (See Annex I: Summary of Product Characteristics, section 4.2).

- **CONDITIONS OR RESTRICTIONS WITH REGARD TO THE SAFE AND EFFECTIVE USE OF THE MEDICINAL PRODUCT**

Not applicable.

- **OTHER CONDITIONS**

Pharmacovigilance system
The MAH must ensure that the system of pharmacovigilance as presented in module 1.8.1 of the Marketing Authorisation, is in place and functioning before and whilst the medicinal product is on the market.

Risk Management Plan (RMP)
The MAH commits to performing the additional pharmacovigilance activities detailed in the Pharmacovigilance Plan as agreed in the RMP presented in module 1.8.2 of the Marketing Authorisation and any subsequent updates agreed by the Committee for Medicinal Products for Human Use (CHMP).

As per the CHMP Guideline on Risk Management Systems for medicinal products for human use, any updated RMP should be submitted at the same time as the following Periodic Safety Update Report (PSUR).

In addition, an updated RMP should be submitted:

- When new information is received that may impact on the current Safety Specification, Pharmacovigilance Plan or risk minimisation activities
- Within 60 days of an important (pharmacovigilance or risk minimisation) milestone being reached
- At the request of the European Medicines Agency

PSURs
The MAH will provide the PSUR annually.

ANNEX III

LABELLING AND PACKAGE LEAFLET

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A. LABELLING

PARTICULARS TO APPEAR ON THE OUTER PACKAGING

Carton and bottle

1. NAME OF THE MEDICINAL PRODUCT

Xyrem 500 mg/ml oral solution
sodium oxybate

2. STATEMENT OF ACTIVE SUBSTANCE

Each ml of solution contains 500 mg sodium oxybate

3. LIST OF EXCIPIENTS

4. PHARMACEUTICAL FORM AND CONTENTS

Oral solution

One 180 ml bottle

5. METHOD AND ROUTE OF ADMINISTRATION

Oral use. Read the package leaflet before use.

6. SPECIAL WARNING THAT THE MEDICINAL PRODUCT MUST BE STORED OUT OF THE REACH AND SIGHT OF CHILDREN

Keep out of the reach and sight of children.

7. OTHER SPECIAL WARNING(S), IF NECESSARY

Keep the container tightly closed.

8. EXPIRY DATE

EXP {MM/YYYY}

9. SPECIAL STORAGE CONDITIONS

The medicinal product should be used within 40 days after the first opening.
After dilution in the dosing cups the preparation should be used within 24 hours

10. SPECIAL PRECAUTIONS FOR DISPOSAL OF UNUSED MEDICINAL PRODUCTS

OR WASTE MATERIALS DERIVED FROM SUCH MEDICINAL PRODUCTS, IF APPROPRIATE

No special requirements

11. NAME AND ADDRESS OF THE MARKETING AUTHORISATION HOLDER

UCB Pharma Ltd
208 Bath Road
Slough
Berkshire
SL1 3WE.
UK.

12. MARKETING AUTHORISATION NUMBER

EU/1/05/312/001

13. BATCH NUMBER

Batch

14. GENERAL CLASSIFICATION FOR SUPPLY

Medicinal product subject to medical prescription.

15. INSTRUCTIONS ON USE

16. INFORMATION IN BRAILLE

Xyrem 500 mg/ml (applies to carton only)

B. PACKAGE LEAFLET

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PACKAGE LEAFLET: INFORMATION FOR THE USER

Xyrem 500 mg/ml oral solution
Sodium oxybate

Read all of this leaflet carefully before you start using this medicine.
- Keep this leaflet. You may need to read it again.
- If you have any further questions, ask your doctor or your pharmacist.
- This medicine has been prescribed for you. Do not pass it on to others. It may harm them, even if their symptoms are the same as yours.
- If any of the side effects gets serious, or if you notice any side effects not listed in this leaflet, please tell your doctor or pharmacist.

In this leaflet:

1. What Xyrem is and what it is used for
2. Before you take Xyrem
3. How to take Xyrem
4. Possible side effects
5 How to store Xyrem
6. Further information

1. WHAT XYREM IS AND WHAT IT IS USED FOR

Xyrem works by consolidating night-time sleep, though its exact mechanism of action is unknown.

Xyrem is used to treat narcolepsy with cataplexy in adult patients.

Narcolepsy is a sleep disorder that may include attacks of sleep during normal waking hours, as well as cataplexy, sleep paralysis, hallucinations and poor sleep. Cataplexy is the onset of sudden muscle weakness or paralysis without losing consciousness, in response to a sudden emotional reaction such as anger, fear, joy, laughter or surprise.

2. BEFORE YOU TAKE XYREM

Do not take Xyrem
- if you are allergic (hypersensitive) to sodium oxybate or any of the other ingredients of Xyrem
- if you have succinic semialdehyde dehydrogenase deficiency (a rare metabolic disorder)
- if you suffer from major depression
- if you are being treated with opioid or barbiturate medicines

Take special care with Xyrem
- if you have breathing or lung problems (and especially if you are obese), because Xyrem has the potential to cause respiratory depression
- if you have or have previously had depressive illness
- if you have heart failure, hypertension (high blood pressure), liver or kidney problems as your dose may need to be adjusted
- if you are taking other central nervous system depressants or alcohol
- if you have previously had experience with drug abuse
- if you suffer from epilepsy as the use of Xyrem is not recommended in this condition
- if you have porphyria (an uncommon metabolic disorder)

If any of these apply to you, tell your doctor before you take Xyrem.

While you are taking Xyrem, if you experience bed wetting and incontinence (both urine and faeces), confusion, hallucinations, episodes of sleepwalking or abnormal thinking you should tell your doctor

straight away. Whilst these effects are uncommon, if they do occur they are usually mild-to-moderate in nature.

If you are elderly, your doctor will monitor your condition carefully to check whether Xyrem is having the desired effects.

Xyrem should not be taken by children and adolescents.

When you discontinue taking Xyrem you need to follow your doctor's instructions as it may result in side effects e.g. headache, lack of sleep, mood changes and hallucinations.

Xyrem has a well known abuse potential. Cases of dependency have occurred after the illicit use of sodium oxybate.

Your doctor will ask if you have ever abused any drugs before you start taking Xyrem and whilst you are using the medicine

Taking other medicines
Please tell your doctor or pharmacist if you are taking or have recently taken any other medicines, including medicines obtained without a prescription.

In particular Xyrem should not be taken together with sleep inducing medicines and medicines that reduce central nervous system activity (the central nervous system is the part of the body related to the brain and spinal cord).

Also care should be taken to tell your doctor and pharmacist if your are taking any of the following types of medicines:

- medicines that increase central nervous system activity and antidepressants
- medicines that may be processed in a similar way by the body (eg valproate, phenytoin or ethosuximide)

Taking Xyrem with food and drink
You must not drink alcohol while taking Xyrem, as its effects can be increased.

Xyrem is to be taken at a set time well after a meal (two - three hours) as food decreases the amount of Xyrem that is absorbed by your body.

You need to monitor the amount of salt you take as Xyrem contains sodium (which is found in table salt) which may affect you if you have had high blood pressure, heart or kidney problems in the past. If you take two 2.25 g doses of sodium oxybate each night you will take 0.82 g of sodium, or if you take two 4.5 g doses of sodium oxybate each night you will take in 1.6 g sodium. You may need to moderate your intake of salt.

Pregnancy and breast-feeding
There have been very few women who have taken Xyrem sometime during their pregnancy and a few of them had spontaneous abortions. The risk of taking Xyrem during pregnancy is unknown, and therefore the use of Xyrem in pregnant women or women trying to become pregnant is not recommended. Tell your doctor if you are pregnant or are planning to become pregnant.

It is not known whether Xyrem passes into breast milk. Patients taking Xyrem should stop breast feeding.

Ask your doctor or pharmacist for advice before taking any medicine.

Driving and using machines
Xyrem will affect you if you drive or operate tools or machines. Do not drive a car, operate heavy machinery, or perform any activity that is dangerous or that requires mental alertness for at least 6

hours after taking Xyrem. When you first start taking Xyrem, until you know whether it makes you sleepy the next day, use extreme care while driving a car, operating heavy machinery or doing anything else that could be dangerous or needs you to be fully mentally alert.

3. HOW TO TAKE XYREM

Always take Xyrem exactly as your doctor has told you to. You should check with your doctor or pharmacist if you are not sure.

The usual starting dose is 4.5 g/day, given as two equally divided doses of 2.25 g/dose. Your doctor may gradually increase your dose up to a maximum of 9 g/day given as two equally divided doses of 4.5 g/dose.

Take Xyrem orally two times each night. Take the first dose upon getting into bed and the second dose 2.5 to 4 hours later. You may need to set an alarm clock to make sure you wake up to take the second dose. Food decreases the amount of Xyrem that is absorbed by your body. Therefore, it is best to take Xyrem at set times well after a meal (two-three hours). Prepare both doses before bedtime.

If you stop taking Xyrem for more than 14 consecutive days you should consult your doctor as you should restart taking Xyrem at a reduced dose.

Instructions on how to dilute Xyrem

The following instructions explain how to prepare Xyrem. Please read the instructions carefully and follow them step by step.

To help you, the Xyrem carton contains 1 bottle of medicine, a measuring syringe and two dosing cups with child-resistant caps.

1. Remove the bottle cap by pushing down while turning the cap anticlockwise (to the left). After removing the cap, set the bottle upright on a table-top. There is a plastic covered foil seal on the top of the bottle, which must be removed before using the bottle for the first time. While holding the bottle in its upright position, insert the press-in-bottle-adaptor into the neck of the bottle. This needs only to be done the first time that the bottle is opened. The adaptor can then be left in the bottle for all subsequent uses
2. Next, insert the tip of the measuring syringe into the centre opening of the bottle and press down firmly (See Figure 1).

Figure 1

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3. While holding the bottle and syringe with one hand, draw up the prescribed dose with the other hand by pulling on the plunger. NOTE: Medicine will not flow into the syringe unless you keep the bottle in its upright position (See Figure 2).

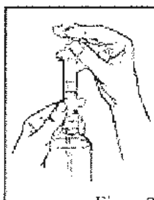
Figure 2

4. Remove the syringe from the centre opening of the bottle. Empty the medicine from the syringe into one of the dosing cups provided by pushing on the plunger (See Figure 3). Repeat this step for the second dosing cup. Then add about 60 ml of water to each dosing cup (60 ml is about 4 tablespoons).

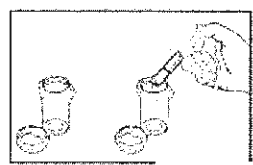
Figure 3

5. Place the caps provided on the dosing cups and turn each cap clockwise (to the right) until it clicks and locks into its child-resistant position (See Figure 4). Rinse out the syringe with water.

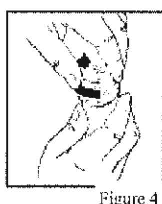
Figure 4

6. Just before going to sleep, place your second dose near your bed. You may need to set an alarm so you wake up to take your second dose no earlier than 2.5 hours and no later than 4 hours after your first dose. Remove the cap from the first dosing cup by pressing down on the child-resistant locking tab and turning the cap anticlockwise (to the left). Drink all of the first dose while sitting in bed, recap the cup, and then lie down right away.

7. When you wake up 2.5 to 4 hours later, remove the cap from the second dosing cup. While sitting in bed, drink all of the second dose right before lying down to continue sleeping. Recap the second cup.

If you have the impression that the effect of Xyrem is too strong or too weak, talk to your doctor or pharmacist.

If you take more Xyrem than you should
Symptoms of Xyrem overdose may include agitation, confusion, impaired movement, impaired breathing, blurred vision, profuse sweating, headache, vomiting, decreased consciousness leading to coma and seizures. If you take more Xyrem than you were told to take, or take it by accident, get emergency medical help right away. You should take the labelled medicine bottle with you, even if it is empty.

If you forget to take Xyrem
If you forget to take the first dose, take it as soon as you remember and then continue as before. If you miss the second dose, skip that dose and do not take Xyrem again until the next night. Do not take a double dose to make up for forgotten individual doses.

If you stop taking Xyrem
You should continue to take Xyrem for as long as instructed by your doctor. You may find that your cataplexy attacks return if your medicine is stopped and you may experience insomnia, headache, anxiety, dizziness, sleeping problems, sleepiness, hallucination and abnormal thinking.

If you have any further questions on the use of this product, ask your doctor or pharmacist.

4. POSSIBLE SIDE EFFECTS

Like all medicines, Xyrem can have side effects. These are usually mild to moderate. If you experience any of these, tell your doctor straight away.

The frequency of possible side effects listed below is defined using the following convention:
very common (affects more than 1 user in 10)
common (affects 1 to 10 users in 100)
uncommon (affects 1 to 10 users in 1,000)
rare (affects 1 to 10 users in 10,000)
very rare (affects less than 1 user in 10,000)
not known (frequency cannot be estimated from the available data)

Very common side effects (affects more than 1 user in 10) include:
Nausea, dizziness, headache

Common side effects (affects 1 to 10 users in 100) include:
Sleeping problems including insomnia, blurred vision, feeling the heart beat, vomiting, stomach pains, diarrhoea, anorexia, decreased appetite, weight loss, weakness, abnormal dreams, tiredness, feeling drunk, sleep paralysis, sleepiness, trembling, confusion/ disorientation, nightmares, sleep walking, bed wetting, sweating, depression, muscle cramps, swelling, fall, joint pain, back pain, cataplexy, balance disorder, disturbance in attention, disturbed sensitivity particularly to touch, abnormal touch sensation, sedation, abnormal taste, anxiety, difficulty in falling asleep in the middle of the night, nervousness, feeling of "spinning" (vertigo), urinary incontinence, shortness of breath, snoring, congestion of the nose, rash, inflammation of the sinuses, inflammation of nose and throat, increased blood pressure

Uncommon side effects (affects 1 to 10 users in 1,000) include:
Psychosis (a mental disorder that may involve hallucinations, incoherent speech, or disorganized and agitated behaviour), paranoia, abnormal thinking, hallucination, agitation, suicide attempt, difficulty in falling asleep, restless legs, forgetfulness, myoclonus (involuntary contractions of muscles), involuntary passage of faeces, hypersensitivity

Side effects with unknown frequency (cannot be estimated from the available data) include:
Convulsion, decreased breathing depth or rate, hives, , suicidal thoughts, short cessation of breathing during sleep.

If any of these affect you severely, tell your doctor.
If you are concerned about any side effect, or if you notice any side effects not mentioned in this leaflet, please tell your doctor or pharmacist.

5. HOW TO STORE XYREM

Keep out of the reach and sight of children.

Do not use Xyrem after the expiry date stated on the bottle (EXP). The expiry date refers to the last day of that month.

Store in the original container.
Do not store Xyrem solutions diluted with water for more than 24 hours.

Once you open a bottle of Xyrem, any contents that you have not used with 40 days of opening should be disposed of.

Medicines should not be disposed of via wastewater or household waste. Ask your pharmacist how to dispose of medicines no longer required. These measures will help to protect the environment.

6. FURTHER INFORMATION

What Xyrem contains

- The active substance is sodium oxybate. Each ml of solution contains 500 mg of sodium oxybate.
- The other ingredients are purified water, malic acid and sodium hydroxyde.

What Xyrem looks like and contents of the pack
Xyrem is supplied as an oral solution in a 180 ml amber plastic bottle, which is closed with a childresistant cap. When the bottle is delivered, there is a plastic covered foil seal which is on the top of the bottle, underneath the cap. Each pack contains one bottle, a press-in-bottle-adaptor (PIBA), a plastic measuring syringe and two dosing cups with child-resistant caps.
Xyrem is a clear to slightly opalescent solution.
The amber plastic bottle contains 180 ml of oral solution.
Marketing Authorisation Holder and Manufacturer

Marketing Authorisation Holder: UCB Pharma Ltd, 208 Bath Road, Slough, Berkshire, SL1 3WE, United Kingdom.
Manufacturer:
UCB Pharma Ltd, 208 Bath Road, Slough, Berkshire SL1 3WE, United Kingdom

For any information about this medicine, please contact the local representative of the Marketing Authorisation Holder.

You should have received a Xyrem Information Pack from your physician, which includes a booklet all about Xyrem and a video showing you how to take the medicine. If you have not received this, please contact the local representative of the Marketing Authorisation Holder, below.

België/Belgique/Belgien
UCB Pharma SA/NV
Tel/Tél: +32 / (0)2 559 92 00

България
ЮСИ БИ България ЕООД
Тел.: + 359 (0) 2 962 30 49

Česká republika
UCB s.r.o.
Tel: + 420 221 773 411

Luxembourg/Luxemburg
UCB Pharma SA/NV
Tél/Tel: +32 / (0)2 559 92 00

Magyarország
UCB Magyarország Kft.
Tel.: + 36-(1) 391 0060

Malta
Pharmasud Ltd.
Tel: +356 / 21 37 64 36

Danmark
UCB Nordic A/S
Tlf: + 45 / 32 46 24 00

Deutschland
UCB Pharma GmbH
Tel: + 49 /(0) 2173 48 4848

Eesti
UCB Pharma Oy Finland
Tel: + 358 10 234 6800 (Soome)

Ελλάδα
UCB A.E.
Τηλ: +30 / 2109974000

España
UCB Pharma, S.A.
Tel: + 34 / 91 570 34 44

France
UCB Pharma S.A.
Tél: + 33 / (0)1 47 29 44 66

Ireland
UCB (Pharma) Ireland Ltd.
Tel: + 353 / (0)1-46 37 395

Ísland
Vistor hf.
Tel: +354 535 7000

Italia
UCB Pharma S.p.A.
Tel: + 39 / 02 300 791

Κύπρος
Lifepharma (Z.A.M.) Ltd
Τηλ: + 357 22 34 74 40

Latvija
UCB Pharma Oy Finland
Tel: + 358 10 234 6800 (Somija)

Lietuva
UCB Pharma Oy Finland
Tel: + 358 10 234 6800 (Suomija)

Nederland
UCB Pharma B.V.
Tel.: +31 / (0)76-573 11 40

Norge
UCB Nordic A/S
Tel: +45 / 32 46 24 00

Österreich
UCB Pharma GmbH
Tel: + 43 (1) 291 80 00

Polska
UCB Pharma Sp. z o.o.
Tel.: + 48 22 696 99 20

Portugal
UCB Pharma (Produtos Farmacêuticos), Lda
Tel: + 351 / 21 302 5300

România
UCB Pharma Romania S.R.L.
Tel: +40 21 300 29 04

Slovenija
Medis, d.o.o.
Tel: + 386 1 589 69 00

Slovenská republika
UCB s.r.o., organizačná zložka
Tel: + 421 (0) 2 5920 2020

Suomi/Finland
UCB Pharma Oy Finland
Puh/ Tel: + 358 10 234 6800

Sverige
UCB Nordic A/S
Tel: + 46 / (0) 40 29 49 00

United Kingdom
UCB Pharma Ltd.
Tel : +44 / (0)1753 534 655

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This leaflet was last approved in

Detailed information on this medicine is available on the European Medicines Agency website: http://www.ema.europa.eu/

PRESCRIBING INFORMATION

Rx only

Xyrem®
(sodium oxybate) oral solution
CIII

WARNING: Central nervous system depressant with abuse potential.
Should not be used with alcohol or other CNS depressants.

Sodium oxybate is GHB, a known drug of abuse. Abuse has been associated with some important central nervous system (CNS) adverse events (including death). Even at recommended doses, use has been associated with confusion, depression and other neuropsychiatric events. Reports of respiratory depression occurred in clinical trials. Almost all of the patients who received sodium oxybate during clinical trials were receiving CNS stimulants.

Important CNS adverse events associated with abuse of GHB include seizure, respiratory depression and profound decreases in level of consciousness, with instances of coma and death. For events that occurred outside of clinical trials, in people taking GHB for recreational purposes, the circumstances surrounding the events are often unclear (e.g., dose of GHB taken, the nature and amount of alcohol or any concomitant drugs).

Xyrem is available through the Xyrem Success Program, using a centralized pharmacy 1-866-XYREM88® (1-866-997-3688). The Success Program provides educational materials to the prescriber and the patient explaining the risks and proper use of sodium oxybate, and the required prescription form. Once it is documented that the patient has read and/or understood the materials, the drug will be shipped to the patient. The Xyrem Success Program also recommends patient follow-up every 3 months. Physicians are expected to report all serious adverse events to the manufacturer. (See WARNINGS).

DESCRIPTION

Xyrem (sodium oxybate) is a central nervous system depressant that reduces excessive daytime sleepiness and cataplexy in patients with narcolepsy. Sodium oxybate is intended for oral administration. The chemical name for sodium oxybate is sodium 4-hydroxybutyrate. The molecular formula is $C_4H_7NaO_3$ and the molecular weight is 126.09 grams/mole The chemical structure is:

$$Na^{+}\ {}^{-}O-\overset{\displaystyle O}{\overset{\|}{C}}-CH_2-CH_2-CH_2-O-H$$

Sodium oxybate is a white to off-white, crystalline powder that is very soluble in aqueous solutions. Xyrem oral solution contains 500 mg of sodium oxybate per milliliter of USP Purified Water, neutralized to pH 7.5 with malic acid.

CLINICAL PHARMACOLOGY

Mechanism of Action

The precise mechanism by which sodium oxybate produces an effect on cataplexy is unknown.

Pharmacokinetics

Sodium oxybate is rapidly but incompletely absorbed after oral administration; absorption is delayed and decreased by a high fat meal. It is eliminated mainly by metabolism with a half-life of 0.5 to 1 hour. Pharmacokinetics are nonlinear with blood levels increasing 3.7-fold as dose is doubled from 4.5 to 9 grams (g). The pharmacokinetics are not altered with repeat dosing.

Absorption

Sodium oxybate is absorbed rapidly following oral administration with an absolute bioavailability of about 25%. The average peak plasma concentrations (1st and 2nd peak) following administration of a 9 g daily dose divided into two equivalent doses given four hours apart were 78 and 142 micrograms/milliliter (mcg/mL), respectively. The average time to peak plasma concentration (T_{max}) ranged from 0.5 to 1.25 hours in eight pharmacokinetic studies. Following oral administration, the plasma levels of sodium oxybate increase more than proportionally with increasing dose. Single doses greater than 4.5 g have not been studied. Administration of sodium oxybate immediately after a high fat meal resulted in delayed absorption (average T_{max} increased from 0.75 hr to 2.0 hr) and a reduction in peak plasma level (C_{max}) by a mean of 58% and of systemic exposure (AUC) by 37%.

Distribution

Sodium oxybate is a hydrophilic compound with an apparent volume of distribution averaging 190–384 mL/kg. At sodium oxybate concentrations ranging from 3 to 300 mcg/mL, less than 1% is bound to plasma proteins.

Metabolism

Animal studies indicate that metabolism is the major elimination pathway for sodium oxybate, producing carbon dioxide and water via the tricarboxylic acid (Krebs) cycle and secondarily by beta-oxidation. The primary pathway involves a cytosolic $NADP^{+}$-linked enzyme, GHB dehydrogenase, that catalyses the conversion of sodium oxybate to succinic semialdehyde, which is then biotransformed to succinic acid by the enzyme succinic semialdehyde dehydrogenase. Succinic acid enters the Krebs cycle where it is metabolized to carbon dioxide and water. A second mitochondrial oxidoreductase enzyme, a transhydrogenase, also catalyses the conversion to succinic semialdehyde in the presence of α-ketoglutarate. An alternate pathway of biotransformation involves β-oxidation via 3,4-dihydroxybutyrate to carbon dioxide and water. No active metabolites have been identified.

Studies *in vitro* with pooled human liver microsomes indicate that sodium oxybate does not significantly inhibit the activities of the human isoenzymes: CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A up to the concentration of 3 mM (378 mcg/mL). These levels are considerably higher than levels achieved with therapeutic doses.

Elimination

The clearance of sodium oxybate is almost entirely by biotransformation to carbon dioxide, which is then eliminated by expiration. On average, less than 5% of unchanged drug appears in human urine within 6 to 8 hours after dosing. Fecal excretion is negligible.

Special Populations

Geriatric

The pharmacokinetics of sodium oxybate in patients greater than the age of 65 years have not been studied

Pediatric

The pharmacokinetics of sodium oxybate in patients under the age of 18 years have not been studied.

Gender

In a study of 18 female and 18 male healthy adult volunteers, no gender differences were detected in the pharmacokinetics of sodium oxybate following a single oral dose of 4.5 g.

Race

There are insufficient data to evaluate any pharmacokinetic differences among races.

Renal Disease

Because the kidney does not have a significant role in the excretion of sodium oxybate, no pharmacokinetic study in patients with renal dysfunction has been conducted; no effect of renal function on sodium oxybate pharmacokinetics would be expected.

Hepatic Disease

Sodium oxybate undergoes significant presystemic (hepatic first-pass) metabolism. The kinetics of sodium oxybate in 16 cirrhotic patients, half without ascites, (Child's Class A) and half with ascites (Child's Class C) were compared to the kinetics in 8 healthy adults after a single oral dose of 25 mg/kg. AUC values were double in the cirrhotic patients, with apparent oral clearance reduced from 9.1 in healthy adults to 4.5 and 4.1 mL/min/kg in Class A and Class C patients, respectively. Elimination half-life was significantly longer in Class C and Class A patients than in control subjects (mean $t_{1/2}$ of 59 and 32 versus 22 minutes). It is prudent to reduce the starting dose of sodium oxybate by one-half in patients with liver dysfunction (see Dosage and Administration).

Drug-Drug Interaction

Drug interaction studies in healthy adults demonstrated no pharmacokinetic interactions between sodium oxybate and protriptyline hydrochloride, zolpidem tartrate, and modafinil. However, pharmacodynamic interactions with these drugs cannot be ruled out. Alteration of gastric pH with omeprazole produced no significant change in the oxybate kinetics.

CLINICAL TRIALS

Cataplexy

The effectiveness of sodium oxybate in the treatment of cataplexy was established in two randomized, double-blind, placebo-controlled trials (Trials 1 and 2) in patients with narcolepsy, 85% and 80%, respectively, of whom were also being treated with CNS stimulants. The high percentages of concomitant stimulant use make it impossible to assess the efficacy and safety of Xyrem independent of stimulant use. In each trial, the treatment period was 4 weeks and the total daily doses ranged from 3 to 9 g, with the daily dose divided into two equal doses. The first dose each night was taken at bedtime and the second dose was taken 2.5 to 4 hours later. There were no restrictions on the time between food consumption and dosing.

Trial 1 was a multi-center, double-blind, placebo-controlled, parallel-group trial that enrolled 136 narcoleptic patients with moderate to severe cataplexy (median of 21 cataplexy attacks per week) at baseline. Prior to randomization, medications with possible effects on cataplexy were withdrawn, but stimulants were continued at stable doses. Patients were randomized to receive placebo, sodium oxybate 3 g/night, sodium oxybate 6 g/night, or sodium oxybate 9 g/night.

Trial 2 was a multi-center, double-blind, placebo-controlled, parallel-group, randomized withdrawal trial that enrolled 55 narcoleptic patients who had been taking open-label sodium oxybate for 7 to 44 months. To be included, patients were required to have a history of at least 5 cataplexy attacks per week prior to any treatment for cataplexy. Patients were randomized to continued treatment with sodium oxybate at their stable dose or to placebo. Trial 2 was designed specifically to evaluate the continued efficacy of sodium oxybate after long-term use.

The primary efficacy measure in Trials 1 and 2 was the frequency of cataplexy attacks.

Table 1
Summary of Outcomes in Clinical Trials Supporting the Efficacy of Sodium Oxybate

Trial/ Dosage Group (n)	Baseline	Median Change From Baseline	Comparison to Placebo p-value
CATAPLEXY ATTACKS			
Trial 1			
		(median attacks/week)	
Placebo (33)	20.5	-4	------
6.0 g/night (31)	23.0	-10	0.0451
9.0 g/night (33)	23.5	-16	0.0016
Trial 2			
		(median attacks/two weeks)	
Placebo (29)	4.0	21.0	–
Sodium oxybate (26)	1.9	0	<0.001

In Trial 1, both the 6 g/night and 9 g/night doses gave statistically significant reductions in the frequency of cataplexy attacks. The 3 g/night dose had little effect. In Trial 2, following the discontinuation of long-term open-label sodium oxybate therapy, patients randomized to placebo experienced a significant increase in cataplexy ($p < 0.001$), providing evidence of long-term efficacy of sodium oxybate. In Trial 2, the response was numerically similar for patients treated with doses of 6 to 9 g/night, but there was no effect seen in patients treated with doses less than 6 g/night, suggesting little effect at these doses.

Excessive Daytime Sleepiness

The effectiveness of sodium oxybate in the treatment of excessive daytime sleepiness in narcolepsy was established in two randomized, double-blind, placebo-controlled trials (Trials 3 and 4) in patients with narcolepsy. Seventy-eight percent of patients in Trial 3 were also being treated with CNS stimulants.

Trial 3 was a multi-center, randomized, double-blind, placebo-controlled, parallel-arm trial that evaluated 228 patients with moderate to severe symptoms at entry into the study including a median Epworth Sleepiness Scale (see below) score of 18, and Maintenance of Wakefulness Test (see below) score of 8.25 minutes. These patients were randomized to one of 4 treatment groups: placebo; sodium oxybate 4.5 g/night; sodium oxybate 6 g/night; and sodium oxybate 9 g/night. The period of double-blind treatment in this trial was 8 weeks. Antidepressants were withdrawn prior to randomization; stimulants were continued at stable doses

The primary efficacy measures in Trial 3 were the Epworth Sleepiness Scale and the Clinical Global Impression of Change. The Epworth Sleepiness Scale is intended to evaluate the extent of sleepiness in everyday situations by asking the patient a series of questions. In these questions, patients are asked to rate their chances of dozing during each of 8 activities on a scale from 0–3 (0=never; 1=slight; 2=moderate; 3=high). Higher total scores indicate a greater tendency to sleepiness. The Clinical Global Impression of Change is a 7-point scale, centered at *No Change*, and ranging from *Very Much Worse* to *Very Much Improved*. In Trial 3, patients were rated by evaluators who based their assessments on the severity of narcolepsy at baseline.

Trial 4 was a multi-center randomized, double-blind, double-dummy placebo-controlled, parallel-arm trial that evaluated 222 patients with moderate to severe symptoms at entry into the study including a median Epworth Sleepiness Scale score of 15, and Maintenance of Wakefulness Test (see below) score of 10.25 minutes. At entry, patients had to be taking modafinil for ≥1 month and at stable doses of 200, 400, or 600 mg daily for at least 1 month prior to randomization. The patients enrolled in the study were randomized to one of 4 treatment groups: placebo; sodium oxybate; modafinil; and sodium oxybate plus modafinil. Sodium oxybate was administered in a dose of 6 g/night for 4 weeks, followed by 9 g/night for 4 weeks. Modafinil was continued at the prior dose. Patients taking antidepressants could continue these medications at stable doses.

The only primary efficacy measure in Trial 4 was the Maintenance of Wakefulness Test. The Maintenance of Wakefulness Test measures latency (in minutes) to sleep onset averaged over 4 sessions at 2 hour intervals following nocturnal polysomnography. For each test session, the subject is asked to remain awake without using extraordinary measures. Each test session is terminated after 20 minutes if no sleep occurs, or after 10 minutes, if sleep occurs. The overall score is the mean sleep latency for the 4 sessions.

In Trial 3, statistically significant improvements were seen on the Epworth Sleepiness Scale and on the Clinical Global Impression of Change at the 6 g/night and 9 g/night doses of sodium oxybate.

Table 2
Daytime Sleepiness in Trial 3

Epworth Sleepiness Scale (Range 0–24)				
Dose Group [g/night (n)]	Baseline	Endpoint	Median Change from Baseline	Change from Baseline Compared to Placebo (p-value)
Placebo (59)	17.5	17.0	-0.5	–
6 (58)	19.0	16.0	-2.0	< 0.001
9 (47)	19.0	12.0	-5.0	< 0.001

Table 3
Clinical Global Impression of Change in Day and Nighttime Symptoms (Responder Analysis) in Trial 3

Dose Group [g/night (n)]	Percent Responders (Very Much Improved or Much Improved)	Significance Compared to Placebo (p-value) Change from Baseline
Placebo (59)	22%	–
6 (58)	52%	<0.001
9 (47)	64%	<0.001

In Trial 4, a statistically significant improvement on the Maintenance of Wakefulness Test score was seen in the sodium oxybate and sodium oxybate plus modafinil groups.

Table 4
Daytime Sleepiness as Evaluated in Trial 4

Maintenance of Wakefulness Test (minutes)				
Dose Group (n)	Baseline	Endpoint	Mean Change from Baseline	Endpoint Compared to Placebo
Placebo (55)	9.7	6.9	-2.7	--
Sodium Oxybate (50)	11.3	12.0	0.6	<0.001
Sodium Oxybate plus Modafinil (54)	10.4	13.2	2.7	<0.001

This trial was not capable by design of comparing the effects of sodium oxybate to modafinil, because patients receiving modafinil were not titrated to a maximally effective dose.

INDICATIONS AND USAGE

Xyrem (sodium oxybate) oral solution is indicated for the treatment of excessive daytime sleepiness and cataplexy in patients with narcolepsy.

In Xyrem clinical trials, approximately 80% of patients maintained concomitant stimulant use (see BLACK BOX WARNINGS).

CONTRAINDICATIONS

Sodium oxybate is contraindicated in patients being treated with sedative hypnotic agents.

Sodium oxybate is contraindicated in patients with succinic semialdehyde dehydrogenase deficiency. This rare disorder is an inborn error of metabolism variably characterized by mental retardation, hypotonia, and ataxia.

WARNINGS

SEE BOXED WARNING

Due to the rapid onset of its CNS depressant effects, sodium oxybate should only be ingested at bedtime, and while in bed. For at least 6 hours after ingesting sodium oxybate, patients must not engage in hazardous occupations or activities requiring complete mental alertness or motor coordination, such as operating machinery, driving a motor vehicle, or flying an airplane. When patients first start taking Xyrem or any other sleep medicine, until they know whether the medicine will still have some carryover effect on them the next day, they should use extreme care while performing any task that could be dangerous or requires full mental alertness.

The combined use of alcohol (ethanol) with sodium oxybate may result in potentiation of the central nervous system-depressant effects of sodium oxybate and alcohol. Therefore, patients should be warned strongly against the use of any alcoholic beverages in conjunction with sodium oxybate. Sodium oxybate should not be used in combination with sedative hypnotics or other CNS depressants.

Central Nervous System Depression/Respiratory Depression

Sodium oxybate is a CNS depressant with the potential to impair respiratory drive, especially in patients with already-compromised respiratory function. In overdoses, life-threatening respiratory depression has been reported (see OVERDOSAGE). In clinical trials two subjects had profound CNS depression. A 39 year-old woman, a healthy volunteer received a single 4.5 g dose of sodium oxybate after fasting for 10 hours. An hour later, while asleep, she developed decreased respiration and was treated with an oxygen mask. An hour later, this event recurred. She also vomited and had fecal incontinence. In another case, a 64 year-old narcoleptic man was found unresponsive on the floor on Day 170 of treatment with sodium oxybate at a total daily dose of 4.5 g/night. He was taken to an emergency room where he was intubated. He improved and was able to return home later the same day. Two other patients discontinued sodium oxybate because of severe difficulty breathing and an increase in obstructive sleep apnea.

The respiratory depressant effects of Xyrem, at recommended doses, were assessed in 21 patients with narcolepsy, and no dose-related changes in oxygen saturation were demonstrated in the group as a whole. One of these patients had significant concomitant pulmonary illness, and 4 of the 21 had moderate-to-severe sleep apnea. One of the 4 patients with sleep apnea had significant worsening of the apnea/hypopnea index during treatment, but worsening did not increase at higher doses. Another patient discontinued treatment because of a perceived increase in clinical apnea events. In the randomized controlled Trials 3 and 4, a total of 40 narcolepsy patients were included with a baseline apnea/hypopnea index of 16 to 67 events per hour indicative of mild to severe sleep disordered breathing. None of the 40 patients had a clinically significant worsening of their respiratory function as measured by apnea/hypopnea index and pulse oximetry while receiving sodium oxybate at dosages of 4.5 to 9 g/night in divided dosages. Nevertheless, caution should be observed if Xyrem is prescribed to patients with compromised respiratory function. Prescribers should be aware that sleep apnea has been reported with a high incidence (even 50%) in some cohorts of narcoleptic patients.

Confusion/Neuropsychiatric Adverse Events

During clinical trials, 2.6% of patients treated with sodium oxybate experienced confusion. Fewer than 1% of patients discontinued the drug because of confusion. Confusion was reported at all recommended doses from 6 to 9 g/night. In a controlled trial where patients were randomized to fixed total daily doses of 3, 6, and 9 g/night or placebo, a dose-response relationship for confusion was demonstrated with 17% of patients at 9 g/night experiencing confusion. In all cases in that controlled trial, the confusion resolved soon after termination of treatment. In Trial 3 where sodium oxybate was titrated from an initial 4.5 g/night dose, there was a single event of confusion in one patient at the 9 g/night dose. In the majority of cases in all clinical trials, confusion resolved either soon after termination of dosing or with continued treatment. However, patients treated with Xyrem who become confused should be evaluated fully, and appropriate intervention considered on an individual basis.

Other neuropsychiatric events included psychosis, paranoia, hallucinations, and agitation. The emergence of thought disorders and/or behavior abnormalities when patients are treated with sodium oxybate requires careful and immediate evaluation.

Depression

In clinical trials, 3.2% of patients treated with sodium oxybate reported depressive symptoms. In the majority of cases, no change in sodium oxybate treatment was required. Four patients (<1%) discontinued because of depressive symptoms. In the controlled clinical trial where patients were randomized to fixed doses of 3, 6, 9 g/night or placebo, there was a single event of depression at the 3 g/night dose. In Trial 3, where patients were titrated from an initial 4.5 g/night starting dose, the incidence of depression was 1 (1.7%), 1 (1.5%), 2 (3.2%), and 2 (3.6%) for the placebo, 4.5 g, 6 g, and 9 g/night doses respectively.

In the 717 patient dataset, there were two suicides and one attempted suicide recorded in patients with a previous history of depressive psychiatric disorder. Of the two suicides, one patient used sodium oxybate in conjunction with other drugs. Sodium oxybate was not involved in the second suicide. Sodium oxybate was the only drug involved in the attempted suicide. A fourth patient without a previous history of depression attempted suicide by taking an overdose of a drug other than sodium oxybate

The emergence of depression when patients are treated with Xyrem requires careful and immediate evaluation. Patients with a previous history of a depressive illness and/or suicide attempt should be monitored especially carefully for the emergence of depressive symptoms while taking Xyrem.

Usage in the Elderly

There is very limited experience with sodium oxybate in the elderly. Therefore, elderly patients should be monitored closely for impaired motor and/or cognitive function when taking sodium oxybate.

PRECAUTIONS

Incontinence

During clinical trials, 7% of narcoleptic patients treated with sodium oxybate experienced either a single episode or sporadic nocturnal urinary incontinence and <1% experienced a single episode of nocturnal fecal incontinence. Less than 1% of patients discontinued as a result of incontinence. Incontinence has been reported at all doses tested.

In a controlled trial where patients were randomized to fixed total daily doses of 3, 6, and 9 g/night or placebo, a dose-response relationship for urinary incontinence was demonstrated with 14% of patients initiated at 9 g/night experiencing urinary incontinence. In the same trial, one patient experienced fecal incontinence when initiated at a dose of 9 g/night and discontinued treatment as a result.

If a patient experiences urinary or fecal incontinence during Xyrem therapy, the prescriber should consider pursuing investigations to rule out underlying etiologies, including worsening sleep apnea or nocturnal seizures, although there is no evidence to suggest that incontinence has been associated with seizures in patients being treated with Xyrem.

Sleepwalking

The term "sleepwalking" in this section refers to confused behavior occurring at night and, at times, associated with wandering. It is unclear if some or all of these episodes correspond to true somnambulism, which is a parasomnia occurring during non-REM sleep, or to any other specific medical disorder. Sleepwalking was reported in 4% of 717 patients treated in clinical trials with sodium oxybate. In sodium oxybate-treated patients <1% discontinued due to sleepwalking. In controlled trials of up to 4 weeks duration, the incidence of sleepwalking was 1% in both placebo and sodium oxybate-treated patients. Sleepwalking was reported by 32% of patients treated with sodium oxybate for periods up to 16 years in one independent uncontrolled trial. Fewer than 1% of the patients in that trial discontinued due to sleepwalking. Five instances of significant injury or potential injury were associated with sleepwalking during a clinical trial of sodium oxybate including a fall, clothing set on fire while attempting to smoke, attempted ingestion of nail polish remover, and overdose of oxybate. Therefore, episodes of sleepwalking should be fully evaluated and appropriate interventions considered.

Sodium Intake

Daily sodium intake in patients taking sodium oxybate is provided below and should be considered in patients with heart failure, hypertension or compromised renal function.

Table 5
Sodium Content per Total Nightly Dose

Xyrem Dose (g)	Xyrem (mL)	Sodium Content /Dose
3	6	546 mg
4.5	9	819 mg
6	12	1092 mg
7.5	15	1365 mg
9	18	1638 mg

Hepatic Insufficiency

Patients with compromised liver function will have an increased elimination half-life and systemic exposure to sodium oxybate (see Pharmacokinetics). The starting dose should therefore be decreased by one-half in such patients, and response to dose increments monitored closely (see Dosage and Administration).

Renal Insufficiency

No studies have been conducted in patients with renal failure. Because less than 5% of sodium oxybate is excreted via the kidney, no dose adjustment should be necessary in patients with renal impairment. The sodium load associated with administration of sodium oxybate should be considered in patients with renal insufficiency.

Information for Patients

The Xyrem Patient Success Program® includes detailed information about the safe and proper use of sodium oxybate, as well as information to help the patient prevent accidental use or abuse of sodium oxybate by others. Patients must read and/or understand the materials before initiating therapy. Prescribers will discuss dosing (including the procedure for preparing the dose to be administered) prior to the initiation of treatment. Patients should also be informed that they should be seen by the prescriber frequently during the course of their treatment to review dose titration, symptom response and adverse reactions. Food significantly decreases the bioavailability of sodium oxybate (see Pharmacokinetics). Whether sodium oxybate is taken in the fed or fasted state may affect both the efficacy and safety of sodium oxybate for a given patient. Patients should be made aware of this and try to take the first dose several hours after a meal. Patients should be informed that sodium oxybate is associated with urinary and, less frequently, fecal incontinence. As a safety precaution, patients should be instructed to lie down and sleep after each dose of sodium oxybate, and not to take sodium oxybate at any time other than at night, immediately before bedtime and again 2.5 to 4 hours later. Patients should be instructed that they should not take alcohol or other sedative hypnotics with sodium oxybate.

For additional information, patients should see the Medication Guide for Xyrem.

Laboratory Tests

Laboratory tests are not required to monitor patient response or adverse events resulting from sodium oxybate administration.

In an open-label trial of long term exposure to sodium oxybate, which extended as long as 16 years for some patients, 30% (26/87) of patients tested had at least one positive anti-nuclear antibody (ANA) test. Of the 26, 17 patients had multiple positive ANA tests over time. The clinical course of these patients was not always clearly recorded, but one patient was clearly diagnosed with rheumatoid arthritis at the time of the first recorded positive ANA test. No instances of systemic lupus erythematosus have been reported in patients taking sodium oxybate.

Drug Interactions

Interactions between sodium oxybate and three drugs commonly used in patients with narcolepsy (zolpidem tartrate, protriptyline HCl, and modafinil) have been evaluated in formal studies. Sodium oxybate, in combination with these drugs, produced no significant pharmacokinetic changes for either drug (see Pharmacokinetics). However, pharmacodynamic interactions cannot be ruled out. Nonetheless, sodium oxybate should not be used in combination with sedative hypnotics or other CNS depressants. Alteration of gastric pH with omeprazole produced no significant change in the oxybate kinetics.

Carcinogenicity, Mutagenicity, Impairment of Fertility

Sodium oxybate was not carcinogenic in rats administered oral doses of up to 1000 mg/kg/day (2 times the exposure in humans receiving the maximum recommended dose (MRHD) of 9 g/day, on an AUC basis) for 83 weeks in the male rats and for 104 weeks in female rats. The results of 2-year carcinogenicity studies in mouse and rat with gamma-butyrolactone, a compound that is metabolized to sodium oxybate *in vivo*, showed no clear evidence of carcinogenic activity. The plasma AUCs of sodium oxybate achieved at the high doses in these studies were 1/2 (mice and female rats) and 1/10 (male rats) the plasma AUCs at the MRHD.

Sodium oxybate was negative in the Ames microbial mutagen test, an *in vitro* chromosomal aberration assay in CHO cells, and an *in vivo* rat micronucleus assay.

Sodium oxybate did not impair fertility in rats at doses up to 1000 mg/kg (approximately equal to the maximum recommended human daily dose on a mg/m² basis).

Pregnancy

Pregnancy Category B: Reproduction studies conducted in pregnant rats at doses up to 1000 mg/kg (approximately equal to the maximum recommended human daily dose on a mg/m² basis) and in pregnant rabbits at doses up to 1200 mg/kg (approximately 3 times the maximum recommended human daily dose on a mg/m² basis) revealed no evidence of teratogenicity. In a study in which rats were given sodium oxybate from Day 6 of gestation through Day 21 post-partum, slight decreases in pup and maternal weight gains were seen at 1000 mg/kg; there were no drug effects on other developmental parameters. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

Labor and Delivery

Sodium oxybate has not been studied in labor or delivery. In obstetric anesthesia using an injectable formulation of sodium oxybate newborns had stable cardiovascular and respiratory measures but were very sleepy, causing a slight decrease in Apgar scores. There was a fall in the rate of uterine contractions 20 minutes after injection. Placental transfer is rapid, but umbilical vein levels of sodium oxybate were no more than 25% of the maternal concentration. No sodium oxybate was detected in the infant's blood 30 minutes after delivery. Elimination curves of sodium oxybate between a 2-day old infant and a 15-year old patient were similar. Subsequent effects of sodium oxybate on later growth, development and maturation in humans are unknown.

Nursing Mothers

It is not known whether sodium oxybate is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when sodium oxybate is administered to a nursing woman.

Pediatric Use

Safety and effectiveness in patients under 16 years of age have not been established.

Race and Gender Effects

There were too few non-Caucasian patients to permit evaluation of racial effects on safety or efficacy. More than 90% of the subjects in clinical trials were Caucasian.

The database was 58% female. No important differences in safety or efficacy of Xyrem were noted between men and women. The overall percentage of patients with at least one adverse event was slightly higher in women (80%) than in men (69%). The incidence of serious adverse events and discontinuations due to adverse events were similar in both men and women.

ADVERSE REACTIONS

A total of 717 narcoleptic patients were exposed to sodium oxybate in clinical trials. The most commonly observed adverse events associated with the use of sodium oxybate were:

Headache (22%), nausea (21%), dizziness (17%), nasopharyngitis (8%), somnolence (8%), vomiting (8%), and urinary incontinence (7%).

Two deaths occurred in these clinical trials, both from drug overdoses. Both of these deaths resulted from ingestion of multiple drugs, including sodium oxybate in one patient.

In these clinical trials, 10% of patients discontinued because of adverse events. The most frequent reasons for discontinuation (>1%) were nausea (2%), dizziness (2%) and vomiting (1%).

Approximately 9% of patients receiving sodium oxybate in 5 placebo-controlled clinical trials (n=443) withdrew due to an adverse event, compared to 1% receiving placebo (n=79). The reasons for discontinuation that occurred more frequently in sodium oxybate-treated patients than placebo-treated patients were: nausea (2%), dizziness (2%), vomiting (1%); as well as urinary incontinence, confusional state, dyspnea, hypesthesia, paresthesia, somnolence, tremor, vertigo, and blurred vision, all occurring in <1% of patients.

Incidence in Controlled Clinical Trials

Most Commonly Reported Adverse Events in Controlled Clinical Trials

The most commonly reported adverse events (≥5%) in placebo controlled clinical trials associated with the use of sodium oxybate and occurring more frequently than seen in placebo-treated patients were: nausea (19%), dizziness (18%), headache (18%), vomiting (8%), somnolence (6%), urinary incontinence (6%), and nasopharyngitis (6%). These incidences are based on combined data from Trial 1, Trial 2, Trial 3, and two smaller randomized, double-blind, placebo-controlled, cross-over trials (n=655).

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice. The adverse reaction information from clinical trials does, however, provide a basis for identifying the adverse events that appear to be related to drug use and for approximating incidence rates.

The data presented below come from two placebo-controlled clinical trials, Trial 1 and Trial 3.

Tables 6 and 7 list the incidence of treatment-emergent adverse events in Trials 1 and 3, respectively, for which there was an incidence of ≥5% and the incidence in at least one dosage group on sodium oxybate was greater than placebo. The number of patients in each dosage group represents the total number of patients treated at each dose. Treatment was initiated at assigned doses of 3, 6, and 9 g in Trial 1.

Table 6
Incidence (%) of Treatment-Emergent Adverse Events in Trial 1

System Organ Class	Placebo	Sodium Oxybate Dosage (g/night) at Onset		
MedDRA Preferred Term	N = 34	3 N = 34	6 N = 33	9 N = 35
Ear and labyrinth disorders				
Tinnitus	0	2 (5.9%)	0	0
Eye disorders				
Vision blurred	1 (2.9%)	2 (5.9%)	0	0
Gastrointestinal disorders				
Abdominal pain upper	0	0	1 (3.0%)	4 (11.4%)
Diarrhea	0	0	2 (6.1%)	3 (8.6%)
Dyspepsia	2 (5.9%)	1 (2.9%)	3 (9.1%)	3 (8.6%)
Nausea	2 (5.9%)	3 (8.8%)	8 (24.2%)	14 (40.0%)
Vomiting	0	0	3 (9.1%)	8 (22.9%)
General disorders and administration site conditions				
Feeling drunk	0	0	0	3 (8.6%)
Lethargy	0	2 (5.9%)	0	0
Pain	1 (2.9%)	1 (2.9%)	1 (3.0%)	2 (5.7%)
Infections and infestations				
Gastroenteritis viral	0	0	2 (6.1%)	0
Nasopharyngitis	1 (2.9%)	1 (2.9%)	2 (6.1%)	2 (5.7%)
Upper respiratory tract infection	1 (2.9%)	1 (2.9%)	2 (6.1%)	0
Injury, poisoning and procedural complications				
Post procedural pain	0	0	0	2 (5.7%)
Investigations				
Blood pressure increased	1 (2.9%)	0	2 (6.1%)	0
Musculoskeletal and connective tissue disorders				
Back Pain	2 (5.9%)	0	2 (6.1%)	2 (5.7%)
Cataplexy	0	0	0	3 (8.6%)
Muscular weakness	0	2 (5.9%)	1 (3.0%)	0
Nervous system disorders				
Disturbance in attention	0	1 (2.9%)	0	3 (8.6%)
Dizziness	2 (5.9%)	8 (23.5%)	10 (30.3%)	13 (37.1%)
Headache	8 (23.5%)	3 (8.8%)	7 (21.2%)	13 (37.1%)
Hypoaesthesia	0	2 (5.9%)	0	0
Sleep Paralysis	1 (2.9%)	1 (2.9%)	2 (6.1%)	5 (14.3%)
Somnolence	3 (8.8%)	4 (11.8%)	4 (12.1%)	5 (14.3%)
Psychiatric disorders				
Confusional state	0	2 (5.9%)	1 (3.0%)	2 (5.7%)
Depression	0	2 (5.9%)	0	0
Disorientation	1 (2.9%)	1 (2.9%)	0	3 (8.6%)
Nightmare	0	1 (2.9%)	2 (6.1%)	0
Sleep disorder	0	0	2 (6.1%)	1 (2.9%)
Sleep walking	0	0	0	2 (5.7%)
Renal and urinary disorders				
Enuresis	0	0	1 (3.0%)	6 (17.1%)
Respiratory, thoracic and mediastinal disorders				
Pharyngolaryngeal pain	2 (5.9%)	0	3 (9.1%)	1 (2.9%)
Skin and subcutaneous tissue disorders				
Hyperhidrosis	0	1 (2.9%)	1 (3.0%)	2 (5.7%)

Table 7
Incidence (%) of Treatment-Emergent Adverse Events in Trial 3 where dose titration from 4.5 to 9 grams occurred in weekly intervals

System Organ Class	Placebo	Sodium Oxybate Dosage (g/night) at Onset		
MedDRA Preferred Term	N = 60	4.5 N = 185	6 N = 114	9 N = 46
Gastrointestinal disorders				
Nausea	2 (3.3%)	14 (7.6%)	12 (10.5%)	9 (19.6%)
Vomiting	1 (1.7%)	3 (1.6%)	4 (3.5%)	4 (8.7%)
Nervous system disorders				
Disturbance in Attention	0	2 (1.1%)	0	3 (6.5%)
Dizziness	1 (1.7%)	17 (9.2%)	9 (7.9%)	4 (8.7%)
Somnolence	0	2 (1.1%)	0	5 (10.9%)
Renal and urinary disorders				
Enuresis	1 (1.7%)	6 (3.2%)	4 (3.5%)	6 (13.0%)

Dose Response Information

Discontinuations of treatment due to adverse events were most common at the highest dose of sodium oxybate. A dose-response relationship was observed for nausea, vomiting, paresthesia, disorientation, irritability, disturbance in attention, feeling drunk, sleepwalking and enuresis. The incidence of all these events was notably higher at 9 g/d. Dizziness was most common at 3 and 9 g/night.

Less Common Adverse Events

During clinical trials sodium oxybate was administered to 717 patients with narcolepsy, and 182 healthy volunteers. A total of 283 patients and 25 healthy volunteers received 9 g/night, the maximum recommended dose. A total of 334 patients received sodium oxybate for at least one year. To establish the rate of adverse events, data from all subjects receiving any dose of sodium oxybate were pooled. All adverse events reported by at least two people are included except for those already listed elsewhere in the labeling, terms too general to be informative, or events unlikely to be drug induced. Events are classified by body system and listed under the following definitions: frequent adverse events (those occurring in at least 1/100 people); infrequent events (those occurring in 1/100 to 1/1000 people). These events are not necessarily related to sodium oxybate treatment.

Blood and lymphatic system disorders

Frequent: none; **Infrequent:** leukopenia, lymphadenopathy.

Cardiac disorders

Frequent: none; **Infrequent:** tachycardia.

Ear and labyrinth disorders

Frequent: ear pain, vertigo; **Infrequent:** ear discomfort, tinnitus.

Eye disorders

Frequent: vision blurred; **Infrequent:** conjunctivitis, eye irritation, eye pain, eye redness, eye swelling, keratoconjunctivitis sicca, miosis.

Gastrointestinal disorders

Frequent: constipation, dyspepsia, toothache; **Infrequent:** abdominal distension, dysphagia, eructation, fecal incontinence, flatulence, gastroesophageal reflux disease, oral pain, retching, salivary hypersecretion, stomach discomfort.

General disorders and administration site conditions

Frequent: asthenia, chest pain, fatigue, influenza like illness, malaise, pyrexia; **Infrequent:** chest discomfort, discomfort, edema, feeling abnormal, feeling cold, feeling hot, feeling hot and cold, feeling jittery, gait abnormal, hangover, lethargy, sensation of foreign body, sluggishness.

Immune system disorders

Frequent: none; **Infrequent:** hypersensitivity, multiple allergies

Infections and infestations

Frequent: bronchitis, gastroenteritis viral, influenza, nasopharyngitis, sinusitis, upper respiratory tract infection, urinary tract infection; **Infrequent:** bladder infection, bronchial infection, cellulitis, dental caries, ear infection, fungal infection, gastroenteritis, herpes simplex, herpes zoster, laryngitis, localized infection, otitis externa, pharyngitis, pneumonia, tinea pedis, tooth abscess, tooth infection, vaginal infection, vaginal mycosis.

Injury, poisoning and procedural complications

Frequent: contusion, fall, pain trauma activated; **Infrequent:** ankle fracture, back injury, concussion, head injury, joint sprain, limb injury, muscle strain, post procedural pain, road traffic accident, skin laceration, tooth injury.

Investigations

Frequent: weight decreased, **Infrequent:** alanine aminotransferase increased, blood alkaline phosphatase increased, blood calcium decreased, blood cholesterol increased, blood glucose increased, blood uric acid increased, blood urine, electrocardiogram abnormal, heart rate increased, liver function test abnormal, protein urine, respiratory rate increased, urine analysis abnormal.

Metabolism and nutrition disorders

Frequent: anorexia; **Infrequent:** decreased appetite, hypernatremia, hypocalcemia, increased appetite.

Musculoskeletal and connective tissue disorders

Frequent: arthralgia, back pain, myalgia, neck pain; **Infrequent:** arthritis, chest wall pain, joint stiffness, joint swelling, muscle tightness, muscle twitching, muscular weakness, musculoskeletal discomfort, musculoskeletal stiffness, polyarthritis, sensation of heaviness, tendonitis.

Neoplasms benign, malignant and unspecified

Frequent: none; **Infrequent:** cyst.

Nervous system disorders

Frequent: balance disorder, headache, hypoaesthesia, memory impairment; **Infrequent:** coordination abnormal, depressed level of consciousness, dizziness postural, dysarthria, dysgeusia, dyskinesia, dysstasia, head discomfort, hyperaesthesia, mental impairment, migraine, myoclonus, paralysis, psychomotor hyperactivity, restless leg syndrome, sedation, sinus headache, sleep talking, sudden onset of sleep, syncope, tension headache

Psychiatric disorders

Frequent: abnormal dreams, confusional state, depression, insomnia, nervousness, nightmare, sleep disorder; **Infrequent:** affect lability, crying, emotional disorder, euphoric mood, fear, hallucination-auditory, hypnagogic hallucination, initial insomnia, libido increased, middle insomnia, mood altered, panic disorder, paranoia, restlessness, sleep attacks, stress symptoms.

Renal and urinary disorders

Frequent: none; **Infrequent:** chromaturia, hematuria, incontinence, micturition urgency, nocturia, pollakiuria, proteinuria, urinary incontinence.

Reproductive system and breast disorders

Frequent: none; **Infrequent:** ovarian cyst, vaginal hemorrhage.

Respiratory, thoracic and mediastinal disorders

Frequent: cough, dyspnea, nasal congestion, pharyngolaryngeal pain, sinus congestion; **Infrequent:** allergic sinusitis, apnea, asthma, dry throat, hiccups, hyperventilation, nocturnal dyspnea, oropharyngeal swelling, respiratory disorder, rhinitis, rhinitis allergic, sinus disorder, snoring, throat secretion increased, upper respiratory tract congestion.

Skin and subcutaneous tissue disorders

Frequent: pruritis; **Infrequent:** acne, alopecia, cold sweat, dermatitis contact, night sweats, rosacea, skin irritation, urticaria.

Surgical and medical procedures

Frequent: none; **Infrequent:** endodontic procedure.

Vascular disorders

Frequent: hypertension; **Infrequent:** hypotension, peripheral coldness.

DRUG ABUSE AND DEPENDENCE

Controlled Substance Class

Xyrem is classified as a Schedule III controlled substance by Federal law. The active ingredient, sodium oxybate or gamma-hydroxybutyrate (GHB), is listed in the most restrictive schedule of the Controlled Substances Act (Schedule I). Thus, non-medical uses of sodium oxybate (Xyrem or GHB) are classified under Schedule I

Abuse, Dependence, and Tolerance

Abuse

See applicable directions for use under **HANDLING AND DISPOSAL** below. Although sodium oxybate (also known as GHB) has not been systematically studied in clinical trials for its potential for abuse, illicit use and abuse have been reported. Sodium oxybate is a psychoactive drug that produces a wide range of pharmacological effects. It is a sedative-hypnotic that produces dose and concentration dependent central nervous system effects in humans. The onset of effect is rapid, enhancing its desirability as a drug of abuse or misuse.

The rapid onset of sedation, coupled with the amnestic features of sodium oxybate, particularly when combined with alcohol, has proven to be dangerous for the voluntary and involuntary (assault victim) user.

GHB is abused in social settings primarily by young adults. GHB has some commonalties with ethanol over a limited dose range and some cross tolerance with ethanol has been reported as well. Cases of severe dependence and craving for GHB have been reported. Dependence is indicated by the use of increasingly large doses, increased frequency of use, and continued use despite adverse consequences. Some of the doses reported abused in the "rave" setting have been similar to the dose range studied for therapeutic treatment of cataplexy.

Hospital emergency department reports increased 100-fold from 1992 to 1999 (source: Substance Abuse Mental Health Services Administration, Drug Abuse Warning Network [DAWN]). Sixty percent of the ED reports involved individuals 25 years and younger. Numerous deaths had been reported over that period of time, typically involving GHB in combination with alcohol and other drugs, including five in the DAWN system in which GHB was the only drug that could be identified. However, the incidence of hospital emergency department reports of events involving GHB and GHB-related analogs has decreased by about 33% since 2000, and reports to the American Association of Poison Control Centers of GHB exposures has decreased from 1916 (involving 6 deaths) in 2001 to 800 (without any deaths) in 2003

Dependence

There have been case reports of dependence after illicit use of GHB at frequent repeated doses (18 to 250 g/day), in excess of the therapeutic dose range. In these cases, the signs and symptoms of abrupt discontinuation included an abstinence syndrome consisting of insomnia, restlessness, anxiety, psychosis, lethargy, nausea, tremor, sweating, muscle cramps, and tachycardia. These symptoms generally abated in 3

to 14 days. The discontinuation effects of sodium oxybate have not been systematically evaluated in controlled clinical trials. An abstinence syndrome has not been reported in clinical investigations. Although the clinical trial experience with sodium oxybate in narcolepsy/cataplexy patients at therapeutic doses does not show clear evidence of a withdrawal syndrome, two patients reported anxiety and one reported insomnia following abrupt discontinuation at the termination of the clinical trial; in the two patients with anxiety, the frequency of cataplexy had increased markedly at the same time.

Tolerance

Tolerance to sodium oxybate has not been systematically studied in controlled clinical trials. Open-label, long-term (≥6 months) clinical trials did not demonstrate development of tolerance. There have been some case reports of symptoms of tolerance developing after illicit use at dosages far in excess of the recommended Xyrem dosage regimen. Clinical studies of sodium oxybate in the treatment of alcohol withdrawal suggest a potential cross-tolerance with alcohol. Because illicit use and abuse of GHB have been reported, physicians should carefully evaluate patients for a history of drug abuse and follow such patients closely, observing them for signs of misuse or abuse of GHB (e.g. increase in size or frequency of dosing, drug-seeking behavior). Physicians should document the diagnosis and indication for Xyrem, being alert to drug-seeking behavior and/or feigned cataplexy.

OVERDOSAGE

Human Experience

Information regarding overdose with sodium oxybate is derived largely from reports in the medical literature that describe symptoms and signs in individuals who have ingested GHB illicitly. In these circumstances the co-ingestion of other drugs and alcohol is common, and may influence the presentation and severity of clinical manifestations of overdose. In addition, overdose with GHB may be indistinguishable from overdose with other drugs, or from several other medical conditions that result in similar symptoms.

In clinical trials two cases of overdose with Xyrem were reported. In the first case, an estimated dose of 150 g, more than 15 times the maximum recommended dose, caused a patient to be unresponsive with brief periods of apnea and to be incontinent of urine and feces. This individual recovered without sequelae. In the second case, death was reported following a multiple drug overdose consisting of Xyrem and numerous other drugs.

Signs and Symptoms

Information about signs and symptoms associated with overdosage with sodium oxybate derives from reports of its illicit use. Patient presentation following overdose is influenced by the dose ingested, the time since ingestion, the co-ingestion of other drugs and alcohol, and the fed or fasted state. Patients have exhibited varying degrees of depressed consciousness that may fluctuate rapidly between a confusional, agitated combative state with ataxia and coma. Emesis (even when obtunded), diaphoresis, headache, and impaired psychomotor skills may be observed. No typical pupillary changes have been described to assist in diagnosis; pupillary reactivity to light is maintained. Blurred vision has been reported. An increasing depth of coma has been observed at higher doses. Myoclonus and tonic-clonic seizures have been reported. Respiration may be unaffected or compromised in rate and depth. Cheyne-Stokes respiration and apnea have been observed. Bradycardia and hypothermia may accompany unconsciousness, as well as muscular hypotonia, but tendon reflexes remain intact.

Recommended Treatment of Overdose

General symptomatic and supportive care should be instituted immediately, and gastric decontamination may be considered if co-ingestants are suspected. Because emesis may occur in the presence of obtundation, appropriate posture (left lateral recumbent position) and protection of the airway by intubation may be warranted. Although the gag reflex may be absent in deeply comatose patients, even unconscious patients may become combative to intubation, and rapid-sequence induction (without the use of a sedative) should be considered. Vital signs and consciousness should be closely monitored. The bradycardia reported with GHB overdose has been responsive to atropine intravenous administration. No reversal of the central depressant effects of sodium oxybate can be expected from naloxone or flumazenil administration. The use of hemodialysis and other forms of extracorporeal drug removal have not been studied in GHB overdose. However, due to the rapid metabolism of sodium oxybate, these measures are not warranted.

Poison Control Center

As with the management of all cases of drug overdosage, the possibility of multiple drug ingestion should be considered. The physician is encouraged to collect urine and blood samples for routine toxicologic screening, and to consult with a regional poison control center (1-800-222-1222) for current treatment recommendations.

DOSAGE AND ADMINISTRATION

Xyrem is required to be taken at bedtime while in bed and again 2.5 to 4 hours later. The dose of Xyrem should be titrated to effect. The recommended starting dose is 4.5 g/night divided into two equal doses of 2.25 g. The starting dosage can then be increased to a maximum of 9 g/night in increments of 1.5 g/night (0.75 g per dose). One to two weeks are recommended between dosage increases to evaluate clinical response and minimize adverse effects. The effective dose range of Xyrem is 6 to 9 g/night. The efficacy and safety of Xyrem at doses higher than 9 g/night have not been investigated, and doses greater than 9 g/night ordinarily should not be administered.

Prepare both doses of Xyrem prior to bedtime. Each dose of Xyrem must be diluted with two ounces (60 mL, 1/4 cup, or 4 tablespoons) of water in the child-resistant dosing cups provided prior to ingestion. The first dose is to be taken at bedtime and the second taken 2.5 to 4 hours later; both doses should be taken while seated in bed. Patients will probably need to set an alarm to awaken for the second dose. The second dose must be prepared prior to ingesting the first dose, and should be placed in close proximity to the patient's bed. After ingesting each dose patients should then lie down and remain in bed.

Because food significantly reduces the bioavailability of sodium oxybate, the patient should allow at least 2 hours after eating before taking the first dose of sodium oxybate. Patients should try to minimize variability in the timing of dosing in relation to meals.

Hepatic Insufficiency

Patients with compromised liver function will have increased elimination half-life and systemic exposure along with reduced clearance (see Pharmacokinetics). As a result, the starting dose should be decreased by one-half and dose increments should be titrated to effect while closely monitoring potential adverse events.

Preparation and Administration Precautions

Each bottle of Xyrem is provided with a child resistant cap. The pharmacy provides two dosing cups with child-resistant caps with each Xyrem shipment.

Care should be taken to prevent access to this medication by children and pets.

See the Medication Guide for a complete description.

HOW SUPPLIED

Xyrem (sodium oxybate) is a clear to slightly opalescent oral solution. It is supplied in kits containing one bottle of Xyrem, a press-in-bottle-adaptor, a 10 mL oral measuring device (plastic syringe), a Medication Guide and a professional insert. The pharmacy provides two 90 mL dosing cups with child-resistant caps with each Xyrem shipment. Each amber oval PET bottle contains 180 mL of Xyrem oral solution at a concentration of 500 mg/mL and is sealed with a child resistant cap.

NDC 68727-100-01. Each tamper evident single unit carton contains one 180 mL bottle (500 mg/mL) of Xyrem, one press-in-bottle-adaptor and one oral dispensing syringe.

STORAGE

Store at 25°C (77°F); excursions permitted up to 15°–30°C (59°–86° F). See USP Controlled Room Temperature.

Solutions prepared following dilution should be consumed within 24 hours to minimize bacterial growth and contamination.

HANDLING AND DISPOSAL

Xyrem is a Schedule III drug under the Controlled Substances Act. Xyrem should be handled according to state and federal regulations. It is safe to dispose of Xyrem oral solution down the sanitary sewer.

Rx only

CAUTION

Federal law prohibits the transfer of this drug to any person other than the patient for whom it was prescribed.

Distributed By:
Jazz Pharmaceuticals, Inc.
Palo Alto, CA 94304

For questions of a medical nature or to order Xyrem call the Xyrem Success Program® at 1-866-XYREM88 (1-866-997-3688).

Protected by US Patent Numbers 6780889, 6472431; Additional US Patents Pending

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BLOOD-BRAIN BARRIER TO H^3-γ-AMINOBUTYRIC ACID IN NORMAL AND AMINO OXYACETIC ACID-TREATED ANIMALS*

K. KURIYAMA and P. Y. SZE
Division of Neuropharmacology and Neurochemistry, Department of Psychiatry, State University of New York, Downstate Medical Center, Brooklyn, New York, and
Department of Biobehavioral Sciences, University of Connecticut, Storrs, Connecticut, U.S.A.

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Summary—Distribution of radioactivity was examined in various tissues following the administration of H^3-γ-aminobutyric acid, injected intraperitoneally to adult mice and intraventricularly to adult rabbits. In mice treated with amino oxyacetic acid, a potent inhibitor of γ-aminobutyrate-α-ketoglutarate transaminase, no significant amount of radioactivity was found in brain after intraperitoneal injection of labeled γ-aminobutyric acid. After intraventricular injection of labeled γ-aminobutyric acid to rabbits similarly treated, small amounts of radioactivity did appear in liver and blood, but electrophoretic characterization showed that the label was not in γ-aminobutyric acid itself but in other metabolites. In untreated mice and rabbits, in which metabolism of γ-aminobutyric acid occurred normally, the label from injected H^3-γ-aminobutyric acid was found by electrophoresis to be distributed over several major metabolites. The radioactivity that seemed to move into or from brain in these untreated animals was largely attributable to these labeled metabolites. These labeled derivatives of γ-aminobutyric acid, at present unidentified except glutamine, were discussed.

The present results demonstrate that the blood–brain barrier in adult animals is impermeable to both blood-borne γ-aminobutyric acid and endogenous cerebral γ-aminobutyric acid.

γ-AMINOBUTYRIC ACID (GABA), a possible inhibitory neurotransmitter substance, is found in large quantity in vertebrate brains (ROBERTS and KURIYAMA, 1968). It is generally considered that the blood–brain barrier in adult animals is impermeable to GABA. This inference is made mainly from the observations that the steady-state concentration of GABA in adult brains remains unchanged after parenteral administration of large amounts of GABA (ROBERTS *et al.*, 1958; TSUKADA *et al.*, 1960; VAN GELDER and ELLIOTT, 1958). These results, however, did not answer the question whether a mechanism exists which excludes the movement of GABA into brain tissue, or whether the steady-state concentration of cerebral GABA is maintained unchanged by an active process of metabolism and/or transport, regardless of the extracerebral GABA concentration. In the latter case, an extensive exchange between extracerebral and cerebral GABA would be expected to take place. If an impermeable barrier indeed exists for the entry of GABA into brain tissue, it remains to be seen if the exit of GABA from brain tissue is similarly barred under physiological conditions. Such a two-way barrier would indicate that endogenous GABA in the brain is synthesized, transported and metabolized within an entirely isolated pool and would not be readily equilibrated with the other GABA pools in the body.

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In a recent study by HESPE *et al.* (1969), it was demonstrated by whole-body autoradiographic techniques that after intraperitoneal injection of C^{14}-GABA into mice, pretreated with amino oxyacetic acid hemihydrochloride to prevent metabolic degradation of injected C^{14}-GABA, no radioactivity was distributed in the brain. Their results provided the first direct experimental evidence that extracerebral GABA fails to penetrate into the brain in mature animals. However, in whole-body autoradiographic methods, radioactivity in various tissues is visually estimated as relative to that in blood (background). The interpretation that no significant amounts of C^{14}-GABA were found in the brain was apparently made on the assumption that radioactivity in the circulating blood was negligibly low. In order to reach an unequivocal conclusion, therefore, confirming data from a more sensitive method appeared to be necessary.

The present work aimed to study both influx and efflux of GABA in adult brain in their relation to the permeability of the blood–brain barrier. The distribution of radioactivity after intraperitoneal and intraventricular injection of labeled GABA into animals, treated with amino oxyacetic acid or not, was examined quantitatively by radiochemical measurement of tissue extracts. Amino oxyacetic acid, a carbonyl trapping agent, is known to be a potent inhibitor of γ-aminobutyrate-γ-ketoglutarate transaminase (GABA-T) (WALLACH, 1961). The labeled compounds found in various tissues were further identified as GABA or other metabolites by electrophoresis.

METHODS

In the experiments designed to study the entry of GABA into the brain, male C57BL/6J mice, which weighed 20–25 g and had been deprived of food for 17 hr, were used. Amino oxyacetic acid (25 mg/kg), or physiological saline, was administered by subcutaneous injection. Ninety minutes later, 1μC (spec. act.: 2μC/μg) of 2,3-H^3-GABA (New England Nuclear, radiopurity >98%), dissolved in 0·2 ml of physiological saline containing 0·1 mg of unlabeled GABA as carrier, was injected intraperitoneally. Thirty minutes after injection of H^3-GABA, the animal was lightly anesthetized with ether and the peritoneal cavity and thorax were opened. The whole animal was perfused through puncture of the left cardiac ventricle and was drained out from the right atrium with a large amount of saline until the perfusate was clear of radioactivity. Liver and brain were then immediately removed and washed with ice-cold saline. Blood samples were taken from separate animals through the cervical blood vessels.

In the experiments designed to study the exit of GABA from the brain, male albino rabbits, which weighed 2–2·5 kg and had been deprived of food for 17 hr, were used. Amino oxyacetic acid (20 mg/kg), or saline, was administered subcutaneously. Thirty minutes after amino oxyacetic acid injection, the animal was lightly anesthetized by inhalation of ether and intravenous injection of sodium barbitone (100 mg/kg). The animal was mounted in the stereotaxic apparatus and tracheotomy was performed. After artificial respiration was started, 0·5 mg/kg of Flaxedil was injected intravenously to immobilize the animal. Ninety minutes after amino oxyacetic acid injection, 20μC of 2,3-H^3-GABA (spec. act.: 2μC/μg), dissolved in 50μl of saline, was injected stereotaxically into the left ventricle of the brain with the aid of a Hamilton syringe. The injection site was ascertained in each experiment by measurement of radioactivity in the cerebrospinal fluid and visual inspection of the dissected brain. Two blood samples (1–2 ml), at 15 and 30 min after H^3-GABA injection, were taken from the left femoral vein. Sixty minutes after H^3-GABA injection, the animal was killed by terminating the artificial respiration, and the liver, kidneys and

whole brain were immediately removed and washed with ice-cold saline. The brain was further washed after opening both sides of the lateral, third and fourth ventricles. The content of the urinary bladder was thoroughly washed out and combined with the urine collected throughout the entire period after the injection of H^3-GABA.

All tissue, blood and urine samples from the mice and rabbits were homogenized in a final concentration of 75% ethanol. The ethanolic homogenate was centrifuged, and the sediment was washed with 75% ethanol and re-centrifuged. The combined supernatants were then evaporated to dryness under a stream of warm air. The residue was taken up in equal amounts of water and chloroform. After centrifugation to separate the layers, aliquots were taken from the aqueous phase for measurement of radioactivity and for electrophoresis.

Radioactivity was measured by liquid scintillation-counting procedures in a toluene -PPO-POPOP solution containing 1 ml of Hyamine. One-dimensional electrophoresis was employed on 18×2 in Whatman No 1 paper strips in a buffer (Pyridine–acetic acid–water 10 : 40 : 950), pH 4·12, at 1000 V for 2 hr. Radioactive spots were detected with a Packard radiochromotogram scanner. Identification of the unknown spots was made by comparing with unlabeled spots from known compounds developed on the same strip and located by bromocresol or trinitrobenzene-1-sulfonic acid spray.

RESULTS

The distribution of radioactivity in the liver, brain and blood 30 min after intraperitoneal injection of H^3-GABA into mice is shown in Table 1. This time interval was chosen because

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN MOUSE TISSUES AFTER INTRAPERITONEAL INJECTION OF H^3-GABA

Tissues†	Untreated*		AOAA-treated*	
	dpm × 10^{-3}	percent of H^3-GABA injected	dpm × 10^{-3}	percent of H^3-GABA injected
Whole liver	13·6±2·1(6)	0·61	869·1±63·0(6)	39·50
Whole brain	7·5±1·6(6)	0·34	0·9± 0·2(6)	0·04
Total blood‡	10·3±1·5(3)	0·46	8·7± 1·2(3)	0·40

*H^3-GABA (1μC/100μg in 0·2 ml of physiological saline) was injected intraperitoneally into mice treated with amino oxyacetic acid (AOAA) (25 mg/kg) 99 min earlier. Untreated mice were given saline instead. All values indicate mean±S.D., with the number of animals shown in parentheses.

†Tissue samples were taken 30 min after H^3-GABA injection.

‡Total blood volume was assumed to be 78 ml/kg.

it was shown in a previous study (HESPE *et al.*, 1969) that radioactivity was gradually diminished after 1 hr. In the amino oxyacetic acid-treated animals, almost 40% of the total injected radioactivity was taken up in the liver. The label in the mouse liver was identified by paper electrophoresis predominantly as unchanged GABA (Fig. 1, peak B), indicating that blockade of GABA metabolism by amino oxyacetic acid was complete or nearly complete. Only 900 dpm (0·04% of total radioactivity injected; or one-tenth of the blood level) was found in the whole brain. Since this amount of radioactivity was not significant, compared with either the total injected H^3-GABA or the liver level of H^3-GABA, no attempt was made to identify it. This result confirms the autoradiographic observation of HESPE *et al.* (1969) that there is no penetration of intraperitoneally-injected H^3-GABA into the brain. Both findings contradict the suggestion of VAN GELDER (1965), based on gross

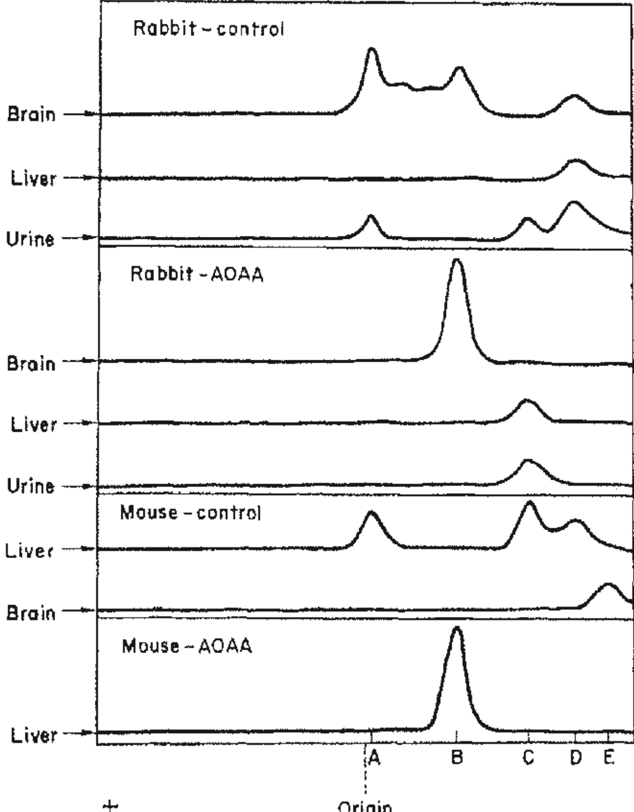

FIG. 1. Electrophoretic patterns of labeled metabolites from H^3-GABA in various tissues. Animals untreated with amino oxyacetic acid (AOAA) are indicated as control. No quantitative relation exists between individual diagrams, as the amount of tissue extract applied (in electrophoresis) and the recording sensitivity (in radioactivity scanning) were variable. Peaks A and B were identified as glutamine and GABA, respectively. Peaks C, D, and E were not identified.

pharmacological observation, that amino oxyacetic acid may alter the blood–brain barrier and thereby enable the entry of GABA into the brain. In the mice untreated with amino oxyacetic acid, less than 1% of the total injected radioactivity was found to remain in the liver, as compared with 40% taken up in the treated mice. Electrophoretic pattern showed that the label in the liver appeared in metabolic substances other than GABA, one of which was identified as glutamine (Fig. 1, peak A). The small amount of label (0·34% of total radioactivity injected) found in the brain was not in GABA but mainly in an unidentified metabolite (Fig. 1, peak E). These result indicate that rapid metabolic degradation occurred in the liver in these untreated animals, and the appearance of some label in the brain was evidently from some metabolite of GABA.

Table 2 summarizes the distribution of radioactivity in several tissues, as well as blood and urine, 1 hr after intraventricular injection of H^3-GABA into rabbits. In the rabbits treated with amino oxyacetic acid, 39% of the injected H^3-GABA was taken up into the brain and remained predominantly as unchanged H^3-GABA (Fig. 1, peak B). A result not presented here showed that more than 50% of injected H^3-GABA remained in the ventricles. The combined radioactivity found in the liver, kidneys, blood and urine accounted for approximately 2% of total injected radioactivity. However, as shown by the electrophoretic patterns of liver and urine (Fig. 1), the radioactivity appeared mainly in an

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN RABBIT TISSUES AFTER INTRAVENTRICULAR INJECTION OF H^3-GABA

Tissues†	Untreated*		AOAA-treated*	
	dpm × 10^{-3}	percent of H^3-GABA injected	dpm × 10^{-3}	percent of H^3-GABA injected
Whole brain	3906·0	8·80	17318·1	39·00
Whole liver	116·1	0·26	580·0	1·30
Whole kidneys	52·4	0·12	90·3	0·20
Total blood‡	66·0	0·15	250·8	0·56
Total urine	29·5	0·07	10·5	0·02

*H^3-GABA (20μC/10μg in 50μl of physiological saline) was injected intraventricularly into rabbits treated with amino oxyacetic acid (AOAA) (20 mg/kg) 90 min earlier. Untreated rabbits were given saline instead. All values indicate the means from 3 animals.

†Brain, liver, kidneys and blood were taken 1 hr after H^3-GABA injection. Urine was collected throughout after H^3-GABA injection.

‡Total blood volume was assumed to be 60 ml/kg.

unidentified metabolite (peak C), and not in GABA. In the animals untreated with amino oxyacetic acid, less than 9% of total injected radioactivity was found in the brain. Unlike the rapid removal of H^3-GABA from the liver of untreated mice, an appreciable amount of the label in the brain was unchanged H^3-GABA (Fig. 1, peak B), in addition to several other labeled metabolites (Fig. 1, peaks A and D). In spite of the presence of unchanged H^3-GABA in the brain of these animals, the small amounts of label appearing in the liver and urine (0·26% and 0·07%) were identified not as GABA but as some GABA metabolites (Fig. 1, peak C; peaks A, C, and D, respectively).

DISCUSSION

It is evident from these results that neither a flux of intraperitoneally injected H^3-GABA into the brain, nor a flux of intraventricularly injected H^3-GABA from the brain was demonstrated. In instances where some label did seem to move into or from the brain, movement of the label was apparently via some metabolites of GABA rather than GABA itself. The present results also indicate that amino oxyacetic acid, a potent GABA-T inhibitor which is widely used in the study of the GABA system *in vivo*, does not change the permeability of the blood–brain barrier to GABA.

The low recovery of radioactivity in the rabbits untreated with amino oxyacetic acid should be noted. The total radioactivity found in the brain, liver, kidneys, blood and urine represented less than 10% of the total injected radioactivity. Although over 50% of the intraventricularly injected H^3-GABA was found to remain unabsorbed in the ventricles, a major amount of the radioactivity taken up by brain tissue was apparently distributed in ways not accounted for here. Since the labeled end-product of complete metabolism of 2,3-H^3-GABA is labeled H_2O and since total urine was collected and examined, excretion of label after complete metabolism cannot be the explanation. Conceivably, the label was distributed either in tissues other than those examined, or over metabolic substances that are not readily extractable by 75% ethanol and water. It is also interesting to compare the metabolic fate of intraventricularly and intraperitoneally injected H^3-GABA. When transamination was not blocked by amino oxyacetic acid, the label in rabbit brain (also in mouse liver) was distributed over several metabolites, one of which was identified as glutamine. Labeled metabolites (A, C and D) present in urine from intraventricularly injected

H^3-GABA showed a similar electrophoretic pattern as those present in liver from intraperitoneally injected H-^{3}GABA, when transamination of GABA was not blocked. Among these metabolites, substance C was also present in liver and urine after intraventricular injection of H^3-GABA even when transamination was blocked by amino oxyacetic acid, but it was not found in brain itself, regardless of the activity of GABA transamination. It seems therefore that this substance C is not formed via the well-known GABA shunt; and that it can readily be formed in the liver, but it may or may not also be a brain metabolite of GABA. Substance D was readily found in untreated animals after either intraventricular or intraperitoneal injection of H^3-GABA, indicating that it may be formed from GABA in both liver and brain. Its absence in the amino oxyacetic acid-treated rabbits suggests that the formation of this substance D may require active transamination of GABA. Substance E was the only labeled metabolite found in brain after intraperitoneal injection of H^3-GABA into mice not treated with amino oxyacetic acid, but it is not known whether this substance was transported into the brain or formed *in situ*. These metabolites C, D and E are unlikely to be acidic substances (such as dicarboxylic acids or dicarboxylic amino acids), as judged from their electrophoretic migration to the cathode at pH 4·12. Preliminary characterization indicated that substances D and E are distinct in their electrophoretic mobilities from either γ-aminobutyrylcholine, γ-amino-β-hydroxybutyrate, or carnosine. Substance C appeared to have similar electrophoretic mobility to carnosine in the pyridine-acetate buffer used in this study, but further characterization is necessary. Detailed identification of these interesting metabolic derivatives of GABA is under way.

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8

GAMMA–HYDROXYBUTYRATE, SUCCINIC SEMIALDEHYDE AND SLEEP

H. LABORIT
Laboratoire d'Eutonologie, Hôpital Boucicaut, Paris 15, France

1. Introduction

The coma-inducing action of short-chain fatty acids from C_4 to C_{10} has been known for quite a few years now (White and Samson, 1956). The importance of butyric acid in cell metabolism and its eventual role in the functional activity of certain organs such as the brain led our team to study its effects in animals following intravenous injection (Jouany *et al.*, 1960a). It was thus possible to demonstrate its sleep-inducing property, and also observe that a major portion underwent β-oxidation. In order to influence its metabolic fate, we introduced an OH group on C_4 with the hope that the electronegative properties of this functional group would influence the electron distribution of the molecules and thus prevent β-oxidation. We thus synthesized sodium 4-hydroxybutyrate (GHB)* from gamma-butyrolactone (GBL). In addition, since it is known that gamma-aminobutyric acid (GABA) which is a central synaptic inhibitor, does not cross the blood–brain barrier, it was hoped that GHB would be able to do so and furnish a precursor that would stimulate GABA synthesis. The hypothesis was not confirmed inasmuch as the increase in GABA cerebral concentration following GHB injection, which is actually open to question (Mitoma and Neubauer, 1968), is not sufficiently marked to account for the central inhibiting property of GHB.

In fact, GHB was found to possess a marked hypnotic action which varied according to the species considered; marked in mice, rats and man, weaker in rabbits and dogs. Electro-encephalographic studies have shown that the sleep it produces was similar to physiological sleep, and that, in particular, the appearance of the parodoxical phase was facilitated (Jouvet *et al.*, 1961; Matsuzaki *et al.*, 1964; Matsuzaki and Takagi, 1967; Hoshi, 1970). Moreover, Bessman and Fishbein (1963) and later Roth and Giarman (1970) and Roth (1970) demonstrated that GHB is a normal brain metabolite in mammals. Its lack of action on oxidative processes (Laborit, 1964b; Ornellas and Laborit, 1966), together with a lack of action on central neurones (Crawford and Curtis, 1964), leads one to believe that it acts

* Abbreviations used in this article: GHB, gamma-hydroxybutyric acid; GABA, gamma-amino butyric acid; GBL, gamma-butyrolactone; SSA, succinic semialdehyde; STH, somatotropic hormone (growth hormone); 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophane; 5-HIAA, 5-hydroxyindolacetic acid; NE, norepinephrine; CA, catecholamines; pCPA, para-chlorphenylalanine; REM sleep, rapid eye movement sleep; NREM, slow wave sleep.

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on neuroglial metabolism and, consequently, to consider the role of the latter in sleep mechanisms (Laborit, 1964c). The hypothesis of the activation of the pentose pathway by GHB in the brain (Laborit, 1964b) has been confirmed with the use of labelled glucose on C_1 or C_6 by Taberner *et al.* (1972).

In addition, Gessa *et al.* (1968) have demonstrated that GHB causes an increase in brain dopamine during sleep with a return to a normal level upon awakening. The mechanism of action raises a certain number of interesting problems concerning the metabolism of brain amines and the alternance between catecholamine (CA) and 5-hydroxytryptamine (5-HT) storage and synthesis.

Finally, the observation that GHB induces an increase in plasma levels of hydrocortisone (Oyama *et al.*, 1968) and above all, in growth hormone (Oyama and Takiguchi, 1970) plasma levels, as seen during physiological sleep, raises also the problem of the relationships between sleep, endocrine activity and protein synthesis.

Moreover, molecules closely related to GHB such as 1,4-butanediol (Sprince *et al.*, 1966) possess hypnotic properties similar to those of GHB (Fig. 1). The same holds for imidazole-4-acetic acid (IMA) (Marcus *et al.*, 1971). It helps to rule out for GHB the possibility of acting by causing the formation of succinic semialdehyde (SSA) which can subsequently lead to the synthesis of succinic acid and enter into the tricarboxylic acid cycle. The study of the central activity of SSA shows that it differs markedly from that of GHB and does not possess any hypnotic components (Laborit *et al.*, 1963). The metabolic mechanism of this action is also of interest. Thus a general study of the hypnotic activity mechanism of GHB could lead to useful hypotheses concerning sleep mechanisms and its different phases, for it would involve the establishment of certain relationships among the enzymatic,

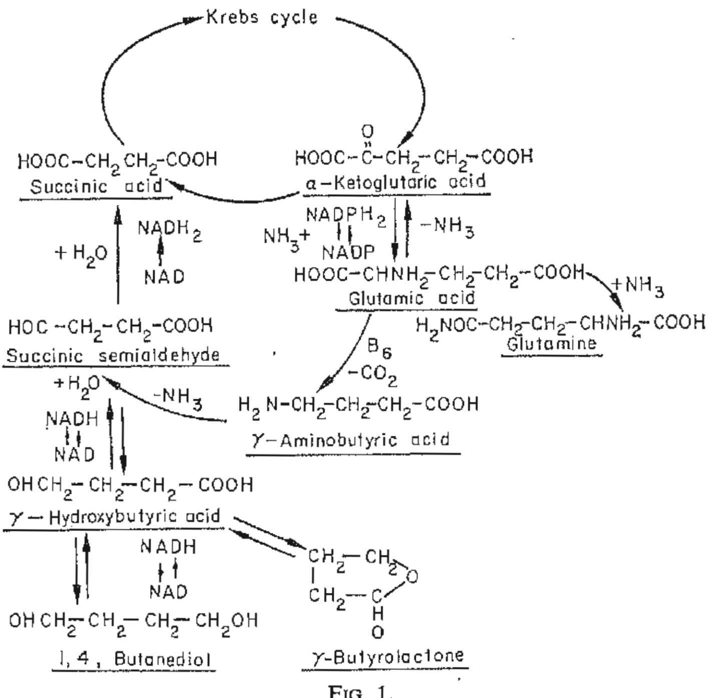

FIG. 1.

metabolic, glial and neuronal activities, the activities of the neurotransmitters, the various aspects of the EEG and of behaviour. In other words, this would establish relationships between the various organization levels of central nervous processes.

2. Biochemical Aspects

Figure 1 shows the relative position of the different factors mentioned above with respect to the tricarboxylic cycle and the GABA shunt in particular.

2.1. GHB AND GABA

It appears that GHB is capable of forming GABA in the brain (Wolleman and Devenyi, 1963; Della Pietra *et al.*, 1966). Some authors believe that this does not involve glutamic acid (Mitoma and Neubauer, 1968), while others, on the contrary, believe that it is a GABA intermediate (Margolis, 1969). There is no doubt, however, that following the injection of labelled GHB in the mouse, labelled GABA can be found in the brain, although the total quantity of GABA is not increased (de Feudis and Collier, 1970). On the other hand, GABA seems to give rise more readily to GHB formation. Roth and Giarman (1969) demonstrated that GHB has a relatively high specific activity when ^{3}H-GABA is introduced by the intracisternal route. Even *in vitro* GABA can serve as a GHB precursor (Roth, 1970).

2.2. GHB AND GBL

Roth and Giarman have shown that when 4-butyrolactone (GBL) is introduced into the blood circulation, it is changed into GHB in the presence of a blood lactonase. They have also shown that contrary to the opinion of Jouvet *et al.* (1961), the prolonged action of the lactone is not due to the fact that it represents the *active form* since when it is placed on the cortex or on cerebral ventricles it remains completely inactive, for the brain does not contain any lactonase. These authors believe that its prolonged action would result from its fixation on the muscle, which could account for its delayed action (Roth *et al.*, 1966).

2.3. GHB AND SSA

It seemed worthwhile to determine whether the hypnotic action of GHB could not be due to its conversion into SSA and to its secondary penetration into the tricarboxylic acid cycle. Since succinate is a central stimulant, such a mechanism seemed to be rather remote. Nevertheless, we have studied the pharmacological activity of SSA (Laborit *et al.*, 1963). In rats, mice and rabbits this substance has a tranquillizing effect with decrease of spontaneous activity, but does not induce sleep. Its action on the EEG is also distinct from that of GHB (see below). SSA, as well as GHB, does not decrease O_2 consumption in animals. It can be reduced by an enzyme coupled to NADH to yield GHB. On the other hand, on cerebral tissue slices it produces a higher P:O ratio than any other of the substrates used by McKhann *et al.* (1960). Finally, it can also be used as a substrate for aldehyde dehydrogenases, and reduce NAD and NADP. In the latter case it furnishes possibly an excellent substrate for oxidative processes (Jakoby, 1960), but it also competes with the aldehydes that originate from biogenic amine oxidation whose central role is unquestionable although still poorly understood.

2.4. GHB OXIDATION

Wollemann (1964) could not demonstrate that purified lactic dehydrogenase (LDH) could oxidize GHB. On the other hand, she did discover an action of alcohol dehydrogenase (ADH) on this substrate. But until quite recently it had been supposed that the brain did not contain any ADH. Raskin and Sokoloff (1972) seem to have shown, however, that alcohol dehydrogenase is present in cerebral tissue. If so, it is possible that slow sleep induced by alcohols could be associated with NAD reduction. Taborsky (1971) believes that slow sleep induced by serotonin (5-HT) is the result of the presence of a hydroxyl group in 5-hydroxytryptophol, which is its metabolite. It may be presumed that since NAD reduction depresses glycolysis, it participates in the orientation of glucose-6-phosphate toward the pentose pathway, the increased activity of which as will be pointed out later, is possibly at the origin of slow sleep (Laborit, 1964c). Such a hypothesis would explain why, according to Sprince *et al.* (1966), pyruvate could prevent or even reverse the effects of GHB, and also why McCabe *et al.* (1971) observed a strong potentiation of its hypnotic activity by alcohol.

On the other hand, Hardman and Stadtman (1963) have demonstrated that in *Clostridium butyricum* there is a β-OH-butyryl CoA dehydrogenase that can oxidate GHB and GABA to yield butyryl CoA. Should this enzyme be present in mammalian brain, butyryl CoA could well be the precursor of lipogenesis, orienting glucose-6-phosphate (G-6-P) into the monophosphate hexose shunt. Montague and Taylor (1968) have shown that short-chain fatty acids cause an accumulation of citrate which inhibits phosphofructokinase and consequently glycolysis. According to these authors, G-6-P is then forced to proceed towards the pentose pathway, an orientation which is, in addition, facilitated by an insulin secretion. We attribute also the protective action of GHB against high pressure O_2 toxicity, which we have demonstrated (Barthelemy *et al.*, 1960), to this G-6-P orientation toward the pentose pathway, with corresponding reducing power originating from NADP reduction. This metabolic action of GHB, which we have suspected on the ground of indirect evidence, and later demonstrated on red blood cells by Sonka and Sochorova (1967), has now received direct confirmation by Taberner *et al.* (1972) in brain *in vivo* and *in vitro* with labelled glucose. *In vivo* in mice there is a 300% increase in the $1\text{-}^{14}C/6\text{-}^{14}C$ ratio in the expired air. An identical result is obtained with slices of cortical grey matter of animals treated with GHB, in which the $1\text{-}^{14}C/6\text{-}^{14}C$ ratio increased from 1.72 to 3.63, a phenomenon not seen with homogenates. In the opinion of these authors, GHB, when administered i.p. (500 mg/kg), increases specifically by 27% glucose-6-phosphate dehydrogenase activity *in vivo* in the rat and mouse brains. This effect occurs simultaneously with the hypnotic effect. The same authors observed an increase in O_2 consumption on cerebral cortex slices, as had already been demonstrated earlier by Ornellas and Laborit (1966) on liver slices, although GHB, in itself, cannot behave as a respiratory substrate (Kerkut *et al.*, 1972).

It should also be noted that according to Margolis (1969), GHB increases the aspartic acid content of the brain and restores glutamine level when it has been lowered by fasting. Marcus *et al.* (1971), on the other hand, consider that GHB activity is similar to that of imidazole acetic acid, and find that both these compounds have a central stimulating activity which potentiates convulsants such as pentamethylenetetrazol, picrotoxine and strychnine, although with Jouany (Jouany *et al.*, 1960b) we observed, on the contrary, that GHB possesses a definite protective action against convulsions induced by strychnine, cardiazol and isoniazide. Marcus *et al.* (1971) have found that both the actions of imidazol

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acetic acid and of GHB can be inhibited by caffeine, and they believe in an activation of phosphodiesterase which lowers the energy supply to neuronal membranes with subsequent depolarization.

2.5. GHB AND NEUROTRANSMITTERS

2.5.1. *GHB and ACh*

In the opinion of Giarman and Schmidt (1963), GHB increases cerebral acetylcholine (ACh) synthesis. This is important since such a synthesis requires the functional integrity of oxidative processes which are not spared by anaesthetics or hypnotics (Quastel and Wheatley, 1932). On the other hand, certain authors suspect a cholinergic mechanism to be at the origin of paradoxical sleep (REM). Domino and Stawiski (1971) have administered an ACh synthesis inhibitor, 3-hemicholinium (3-HC), intraventricularly to the cat with implanted electrodes. EEG activity was continuously monitored during 24 hr. 3-HC reduces REM sleep during 6–12 hr, while slow sleep (NREM) is increased and consciousness decreases. Bowers *et al.* (1966) have noted that rats subjected to paradoxical sleep deprivation for 96 hr show an important ACh decrease in the telencephalon, and Jasper and Tessier (1971) have demonstrated that the amount of ACh liberated on the surface of the cerebral cortex increases during REM and consciousness. But the variations in brain concentrations of ACh should be considered with much caution for they could be the result either of a decrease in its utilization, or of an increase in its synthesis.

2.5.2. *GHB and CA*

Gessa *et al.* (1968) have demonstrated that GHB increases the level of cerebral dopamine during sleep, whereas it returns to normal upon awakening. GHB, however, does not inhibit monoamine oxidase (MAO), nor does it activate dopa-decarboxylase (Gessa *et al.*, 1966). Vysotskaya and Shugina (1968) have observed that brain-stem norepinephrine (NE) does not increase after GHB administration. Aghajanian and Roth (1970) have shown, with a fluorescent technique, that GHB increases very selectively the dopamine concentration in the corpus striatum, and particularly in the caudate nucleus; and Roth and Suhr (1970) believe that the increase in cerebral dopamine is due to maintenance inside the granules. The NE level is not increased, and that of homovanillic acid decreases (Roth, 1971). On the other hand, L-dopa increases the hypnotic effect of GHB as Rizzoli *et al.* (1969) were able to show; an action which we have also confirmed. Gessa *et al.* (1968), in addition, have noted that 1,4-butanediol and other butyric acid derivatives increase brain dopamine content only if they have a depressive action on the central nervous system. On the contrary, however, Hutchins *et al.* (1972) have reached the conclusion that the increase in cerebral dopamine concentration of homovanillic acid and of acetic dihydroxyphenyl, which they had observed with GHB, implies an inhibition of the dopamine storage mechanism.

Some authors (Jouvet, 1967) believe that paradoxical sleep would be under the control of the NE present in the locus coeruleus. But Kings and Jewett (1971) were able with α-methyltyrosine (an inhibitor of cerebral catecholamine (CA) synthesis) to increase the amount of REM sleep in the cat at a time when CA depletion in the brain is at its highest. Wyatt *et al.* (1971) believe that CA decreases REM sleep in man. At any rate, GHB does not modify cerebral NE level, while it certainly increases that of dopamine. But Jones *et al.* (1969) were able to cause a semicomatose state in the cat by destroying the dopaminergic

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neurones in the *substantia nigra* although no EEG disturbances were observed, a phenomenon which in their opinion is an intervention of dopamine in awakening and in behavioural sleep.

2.5.3. *GHB and 5-HT*

GHB does not seem to interfere with the synthesis of cerebral serotonin (5-HT) (Tagliamonte *et al.*, 1971). But in the opinion of Rizzoli and Galzigna (1970), GHB and 1,4-butanediol (the latter being metabolized into GHB, according to Roth and Giarman, 1968) induce sleep by acting on the neuronal membrane. In the opinion of these authors, when serotonin is liberated at the nerve ending, its access to the synaptic receptors requires crossing of the post-synaptic membrane on whose phospholipids either butyrate or GHB would have been bound. Thus an electrostatic complex would be formed between serotonin and GHB. Therefore, additional serotonin molecules would have to be liberated at the synapse, otherwise, the serotoninergic action would be depressed. This interpretation does not seem to conform with the slow sleep induced initially by GHB which the majority of authors consider as being dependent upon serotonin localized in the neurones of the raphe area. The destruction of this area leads to a state of permanent consciousness, while the increases in raphe neurone 5-HT concentration, after injection of nialamine as well as of 5-hydroxytryptophane (5-HTP), produce an increase in synchronized sleep (NREM) (Jouvet *et al.*, 1965). By stimulating electrically the raphe nucleus which is rich in serotonin (Dahlström and Fuxe, 1964), Aghajanian *et al.* (1967) were able to liberate 5-HT in the forebrain and to increase the level of 5-hydroxyindolacetic acid (5-HIAA) which is its metabolite. The effect of 5-HT on synchronized sleep has also been recognized by Koella and Czicman (1966), and Dement *et al.* (1969) were able, with parachlorophenylalanine (pCPA) which inhibits 5-HT synthesis, to suppress REM sleep while causing the appearance of the ponto-geniculo-orbital (PGO) spikes. But opinions are sometimes contradictory, and Wyatt *et al.* (1970, 1971) believe that, on the contrary, 5-HT induces REM sleep.

2.6. ALTERNATING CA AND 5-HT STORAGE

It appears that CA and 5-HT could be stored in the same granules, and that an alternance in levels could, therefore, exist between CA and 5-HT levels at nerve endings. Jaim-Etcheverry and Zieher (1969) have observed that it is only when granules are depleted of NE that 5-HT can fill them. Ng *et al.* (1970) stress that their studies seem to support the hypothesis that part of the administered exogenous L-dopa can penetrate the serotoninergic nerve endings of the rat brain, and be subjected there to decarboxylation into amines and thus bring about a shift on the endogenous indolamines from their storage site. Everett and Borcherding (1970) have shown that in mice high levels of L-dopa do not modify the NE brain content, but produce a strong decrease in serotonin content. Aghajanian *et al.* (1970) observed that IMAOs increase 5-HT liberation and exhaust the serotoninergic neurones which then respond less to stimulation. Similar facts were reported by Shaskan and Snyder (1970). Jester and Horst (1972) and Chase *et al.* (1970) have observed that, on rat brain slices, L-dopa increases the liberation of tritiated serotonin that it then replaces. Hanig and Seifter (1971) have observed a 14% decrease of 5-HT in the chick after L-dopa. Conversely, Snipes *et al.* (1968) consider that 5-HT can take the place of NE once the cat has been treated with α-methyltyrosine, an NE inhibitor, and that 5-HTP administration reduces the cerebral NE level.

Since there can be no doubt that GHB increases cerebral dopamine level and that, on the other hand, it does not seem to inhibit 5-HT synthesis, the discovery of the balance phenomenon between the concentrations of dopamine and 5-HT in the storage sites leads to the conclusion that GHB undoubtedly accelerates 5-HT turnover. This is exactly what Tagliamonte *et al.* (1971) have observed.

2.7. PROTEIN SYNTHESIS AND SLEEP

A series of studies conducted by Oswald (1969) have shown that the return to normal sleep after drug administration or after cerebral damage requires several weeks. Paradoxical sleep, after depression by drugs, shows a secondary rebound following their discontinuation. The author believes that this rebound expresses the time taken for the resynthesis of the proteins involved in neurone metabolic mechanisms. In his opinion, REM sleep is connected with protein synthesis. Protein synthesis inhibitors (cycloheximide, actinomycin D) cause the disappearance of REM sleep, while conversely, intense training increases cerebral protein synthesis and REM sleep. Mentally deficient children have less paradoxical sleep (Feinberg, 1968). In Oswald's opinion, the discontinuation of drug administration (amphetamines, heroin, reserpine, barbiturates, etc.) is accompanied by increased 5-HT synthesis and REM sleep, and 5-HT induces paradoxical sleep, although, as we have seen, numerous authors believe that it is the main factor of slow wave sleep (NREM).

Protein synthesis requires an active pentose pathway since this pathway will supply the riboses required for RNA-messenger synthesis, ribosomal RNA and transfer RNA. Since GHB activates the pentose pathway, it may be presumed that it is capable of facilitating protein synthesis. If so, its ability to promote the appearance of REM sleep would seem logical. What relationship could there be, in fact, between the increased 5-HT synthesis observed by Oswald and protein synthesis? We have discovered the antioxidant properties of serotonin by showing its protective action against convulsions induced by oxygen under pressure in mice (Laborit *et al.*, 1957). The indole nucleus is shared by 5-HT and plant growth hormones, the auxines. At a certain level, 5-HT has a stimulating action on young oat shoot growth, as we showed with Niaussat (Niaussat *et al.*, 1958). The injection of growth hormones (STH) increases the urinary excretion of 5-hydroxyindolacetic acid in the rabbit (Laborit *et al.*, 1959). In addition, during slow wave sleep there is a marked liberation of growth hormone (Takahashi *et al.*, 1968; Sassin *et al.*, 1969). The relationship between 5-HT and STH was confirmed by Sirek *et al.* (1966) and by Pukal'skaya and Man'ko (1964). Oyama and Takiguchi (1970) have shown in man that GHB anaesthesia, as in natural sleep, is accompanied by an important increase in the plasma level of somatotropic hormone, and recently, Elkaim *et al.* (1972) observed that L-dopa administration—which we consider as capable of displacing 5-HT from its storage site—increases the plasma 5-HT level. Growth hormone is an anabolic hormone which promotes protein synthesis and amino-acid uptake. Boucek and Alvarez (1970, 1971) have demonstrated the stimulation by 5-HT of fibroblast growth in culture. It is true that they attribute this phenomenon to the increase in survival due to the antioxidant properties of 5-HT. Finally, Barbosa *et al.* (1971) show that 5-HTP plays a fundamental role in cerebral protein synthesis regulation by controlling cerebral polyribosome aggregation. Similar observations have been made by Sidransky *et al.* (1968).

There seems, therefore, to exist a definite relationship between protein synthesis and paradoxical sleep, protein synthesis and STH, protein synthesis and 5-HT, as well as between

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GHB and STH, between GHB, pentose pathway and protein synthesis, between GHB and REM sleep, and probably by means of dopamine storage stimulation and consecutive 5-HT turnover increase, between GHB and 5-HT.

3. Electroencephalographic Aspects

3.1. GHB

3.1.1. *Stereotaxic study and evoked potentials study*

Bertharion and Laborit (1962) have studied potentials evoked by stimulation of the peripheral nerve of the controlateral paws in rats following GHB administration (1 g/kg, i.p.). GHB depresses the surface negative component in all forms of cortical primary evoked potentials. At the level of the median centre, responses are markedly depressed, while at the level of the ventro-posterolateral nucleus of the thalamus (VPL), evoked potentials are not modified.

Responses may be modified or amplified on the reticular formation. It would appear, therefore, that the drug acts primarily at the level of the cortex associative pathways.

In the rabbit (Laborit, 1969), GHB brings the appearance of very high amplitude slow waves on all leads except in the hippocampus, which is the location of rapid and high amplitude waves with some spike-waves at the 1 g/kg dose level. With slow-frequency stimulation (8 Hz), recruitment is facilitated in the medium thalamus, while cortical desynchronization to rapid frequencies is inhibited. At the level of the hippocampus, on the contrary, these rapid frequencies, as those of the mesencephalic reticular formation, are accompanied by high amplitude fast reactions and spike-waves. The stimulation of the hippocampus is accompanied by a considerable prolongation of post-discharges, except on the caudate nucleus and the cortex where slow waves persist. Low- or high-frequency stimulation of the sciatic nerve is unable, even at high intensities, to cause the disappearance of the cortical slow waves, but it does cause the appearance of rapid waves on the other leads. A pseudo-affective violent reaction (vocalization or defence movements) is then produced. The injection of eserine restores the initial EEG pattern. The study of the progressive modifications of the EEG in man produced with increasing doses of GHB was conducted by Schneider *et al.* (1963). There is first an overall wave flattening. Then theta waves appear followed by polymorphous delta waves on which the rapid frequencies are superimposed. Delta waves do not prove that the sleep is of the anaesthetic type. EEG awakening precedes comportemental awakening. We have already mentioned the numerous studies in which GHB induced sleep is described as being close to physiological sleep, and that with doses of 50 to 60 mg/kg it induces rapidly slow wave sleep followed by REM sleep, which then becomes predominant. According to Scholes (1966), GHB would specifically inhibit the axodendritic synapses, and Zakusov and Ostrovskaya (1971) have observed that it interferes more markedly with responses through the corpus callosum and associative responses of the cortex than with those caused by direct stimulation.

3.1.2. *Medullary action*

A decrease in amplitude of the monosynaptic reflex is induced by 300 mg/kg GHB i.v. (Drouet and Laborit, 1963). Local application inhibits monosynaptic reflexes. In rats, studying the lingual-mandibular reflex, Hampel and Hapke (1968) have shown that GBL inhibits interneuronal transmission at the level of the brain-stem and of the higher centres. High concentrations, however, are required to cause complete anaesthesia. On the contrary,

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Uspenskii (1965) observed, as we did, that GHB inhibits mono-synaptic reflexes. Kvasnoi and Kruglov (1967) did not observe any action on the direct or recurrent mechanisms at the level of the spinal cord, but they noted that it increases presynaptic inhibition. Besson *et al.* (1971) have made clear that GHB has no influence on presynaptic inhibition of infra-spinal origin in the cat, but it depresses segmental presynaptic inhibition markedly. It does not interfere with neuromuscular transmission, but since a certain similarity exists with muscle relaxation obtained with GABA, we suggest that GHB could affect the responsiveness of stretch receptor; an action on pulmonary stretch receptors would explain the decrease in frequency and increase in amplitude of ventilation under the action of GHB. The preservation of the respiratory centre's responsiveness to CO_2 would offer an explanation for the Cheyne-Stokes respiratory rhythm observed with high GHB doses.

The muscle-relaxing action of GHB may be related to dopamine concentration increase in the extrapyramidal system, but it is also possible that dopamine plays a role in the induction of behavioural sleep by decreasing proprioceptive stimulation.

3.2. SSA

3.2.1. *Evoked cerebral potentials*

The i.p. administration to the rat of a 30% solution SSA corresponding to 1 g/kg increases the amplitude of both the positive and negative components of evoked cortical potentials (Bertharion *et al.*, 1963). Identical observations were made at the level of the thalamus, whereas at the level of the VPL, the evoked potentials were unchanged. On the contrary, evoked potentials at the level of the mesencephalic reticular formation were depressed, while those of the hippocampus did not seem to be altered.

3.2.2. *Electroencephalographic recordings in the rabbit*

In the animal either under light Nembutal anaesthesia or awake, the intravenous infusion of 65.8 mg/kg to a total dose of 2.10 g/kg causes a discreet slowing of the basal rhythm without appearance of any high amplitude slow waves, as with GHB. The maximum effect obtained 30–40 min after injection in the conscious animal is characterized by a decrease or suppression of the alarm reaction to noise. In the unrestrained animal, a marked decrease in spontaneous motility is noted, with disappearance of the startle response to noise.

3.2.3. *EEG studies in man*

The i.v. infusion of 2–3 g of SSA as a 30% solution up to 6–8 g, or the i.v. injection of 6 g over 2–3 min do not induce any variations in pulse or arterial pressure. There is no tendency toward somnolence. Psychically, a sensation of constant euphoria is produced with disappearance of anxiety. The deeply disturbed psychosis of two patients was clearly improved, although transitorily, following a single injection. Memory, attention and mental calculation tests have shown that all functions are preserved in the mentally normal patients, and that they were improved in two mentally deficient patients. Simultaneously, an often considerable modification of the EEG tracing is observed. In general, eye opening is no longer accompanied by the disappearance of the α-rhythm and the alarm reaction seems less marked. If the tracing had been disturbed earlier, a considerable increase is noted in these disturbances with spilling over to other leads which until then had been normal. The deterioration of the tracings is in contrast with the maintenance or improvement of the psyche (Laborit, 1965).

These properties of SSA, shared also by other reducing agents, can lead to hypotheses with regard to their biochemical mechanism. It is enough to note that both from the study of EEG patterns as well as from the behaviour standpoints, SSA and GHB have very different pharmacologies.

4. Suggestion of a Neurobiological Model of the Anatomical-functional Aspects of Sleep

4.1. DIFFERENCE IN METABOLIC FUNCTION BETWEEN NEUROGLIA AND NEURONE

For several years now, we have suggested a metabolic hypothesis of the anatomical-functional aspects of sleep (Laborit, 1964a, c; Laborit and Laborit, 1965; Laborit, 1969). This hypothesis is based on the fact that the enzymatic make-up and metabolic activity of the neuroglia and neurones are very different. We cannot repeat here all the experimental data which, on the basis of histoenzymology, electronmicroscopy and general biochemistry, shows that the neuroglia (or more precisely, the astrocytes) is made up of structures with predominantly glycolytic as well as hexosemonophosphate shunt activities, while the phosphorylating oxidative activity and the mitochondrial system are relatively little developed. Conversely, the neurone has opposite characteristics. It is a predominantly oxidative system. Taking into account the fact that the neuronal extracellular space consists mainly of the neuroglia through which the majority of exchanges between the neurone and the circulating blood take place, the nutritional role of the neuroglia with respect to the neurones would be more active, so that during rest the neurones are forced to excrete a greater quantity of end products of their oxidative metabolism and restore their biochemical structural integrity, and in particular, their protein structure.

4.2. THE NEURONE/NEUROGLIA AS A METABOLIC AND FUNCTIONAL PAIR

The narrowest contiguity relationships existing between these two structures should logically manifest itself at the level of dendritic extensions, where the surface/volume ratio of the neuronal extension is at its highest. It is understandable that at this level a hyper-polarization of the neuroglia which surrounds closely the dendritic axon can form an effective diffusion barrier against propagation both of the axodendritic synaptic depolarization waves, as of the chemical substances liberated at the level of the synapses.

It appeared to us that the balance between neuronal and glial metabolic activities could be controlled through the intermediary role of transmembrane ionic movements (Laborit, 1964b, c). Numerous studies have brought experimental proof of this hypothesis (Trachtenberg and Pollen, 1970; Treherne *et al.*, 1969; Hartman, 1966; Lane and Treherne, 1969). Principally the work of Kuffler (1967), of Orkand *et al.* (1966) and of Cohen *et al.* (1968) have confirmed the role of potassium ejection resulting from neuronal activity, in the metabolic activation of its uptake by the glia and Cohen (1970) believes, as we do, that glial cell electrogenesis can contribute to the electroencephalogram. But in our opinion, this influence is mainly felt at the level of the dendritic and axodendritic synaptic extensions. Thus the slow waves recorded on the EEG are, in our opinion, the expression of glial metabolic activity and the rapid waves mainly the expression of neuronal activity. But if the potassium lost by the neurone during the action potentials plays a part at the origin of

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the depolarization, and later stimulates the metabolic activity of the neighbouring glia, it can also be suggested that this glial activity will secondarily lead to sodium excretion during its repolarization. It can also be suggested that the high activity of the pentose pathway in the glia guarantees the supply of an amount of reducing substances equivalent to the degree of oxidation of the neurone resulting from its prolonged activity. The neuronal oxidative activity could facilitate GABA synthesis in the shunt from which it originates, as well as that of GHB. GHB acts preferentially on the specific glial pentose pathway. The glycolytic glial metabolism could, however, supply lactic acid, an efficient substrate for the neuronal tricarboxylic acid cycle. We cannot go into details here concerning the experimental observations that support the hypothesis concerning the feedback relationships of neuronal and glial metabolism. These relationships stop us from considering separate neuronal functions, and force us to conceive a metabolic and functional unity: the neurone–neuroglia pair.

4.3. METABOLIC REGULATION OF THE ANATOMICAL-FUNCTIONAL ASPECTS OF SLEEP

If we accept the preceding hypothesis which suggests that the functional activity of the neurone produces a simultaneous and progressive increase of sodium, and a depletion of potassium; if we accept that the potassium excreted into the neurone–neuroglial intercellular spaces will stimulate the metabolic activity of the neuroglia, then we can understand why sleep will start with the appearance of slow waves, an expression of the glial metabolic activity from its contact with dendritic extensions, and principally at the level of the superficial cortex layers. This is the NREM sleep phase which temporarily sets the neurone at rest. The neurone restores its ionic gradients and synthesizes proteins to replace those broken down during neuronal activity. It is possible that the activity of the glial pentose pathway is the origin of the supply of required ribose or even RNA. The progressive glial repolarization will lead to the rejection of sodium which could then act again as a stimulant of the metabolically restored neurone, while glial hyperpolarization will prevent the cortical diffusion of information in the associative systems. This is the REM phase of sleep.

Thus it can be understood why fatigue after exercise increases synchronized activity and momentarily suppresses the REM phase (Hobson, 1968). Matsumoto *et al.* (1968) have noted also that the synchronized phase of sleep is the first to return.

5. GHB and Metabolic Regulation of Sleep

5.1. PENTOSE PATHWAY AND SLOW WAVE SLEEP

The observations described above would lead to the view that pentose pathway stimulants should facilitate the appearance of slow wave sleep. This had been observed in our group by Weber and Gerard (1961), who showed that a deep behavioural sleep developed in the rat following an injection of hypertonic glucose and insulin after a 48-hr fast. A few months later, Tepperman and Tepperman (1961) showed with ^{14}C-labelled glucose that under the same experimental conditions, glucose is indeed preferentially used in the pentose pathway, probably because of a decrease in the [NADPH] : [NADP] ratio caused by fasting. Again under the same experimental conditions, Sudakov (1963) showed in the cat the appearance of slow waves on the EEG. Thus it appears that the increase in pentose

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pathway activity induced by GHB can be considered as the factor responsible for the appearance of behavioural and slow wave sleep resulting from neuroglial hyperpolarization. GHB suppresses the secondary negative phase of evoked cortical potentials that we consider as being of neuroglial-dendritic origin (Laborit, 1964b), and Scholes (1966) believes that it mainly blocks cortical axodendritic synapses. In addition, it can be added that, as any other pentose pathway stimulant, GBH reduces kaliemia without increasing kaliuria (Laborit *et al.*, 1960b), which would lead to the cellular build up of this cation which accompanies cellular hyperpolarization. This is also a known fact for the glucose–insulin association (Laborit, 1958).

5.2. GHB, NEUROTRANSMITTERS AND SLEEP

Fernstrom and Wurtman (1971) have shown that the ingestion of a carbohydrate diet after fasting, or the injection of a non-convulsing insulin dose, increases, on the one hand, the 5-HTP blood level and the 5-HT brain level, while on the other, it decreases the plasma level of the other amino acids. Thus serotonin could take part in the integration by the brain of information concerning cellular metabolic homeostasis which it would convert into behaviour. Tagliamonte *et al.* (1971) have shown in the rat after GHB administration an increase in 5-HT brain level, and specially in 5-HIAA, which would indicate an accelerated 5-HT turnover.

We have seen that 5-HT was probably connected with slow wave sleep. But the opinion of Taborsky (1971), already mentioned, should not be overlooked either, i.e. 5-HT initiates NREM sleep only through the action of its metabolite, 5-hydroxytryptophol. It is tempting to establish a relationship between the alcohol and other alcohols in general which, by reducing NAD under the action of alcohol dehydrogenase, stimulate the utilization of glucose-6-phosphate in the pentose pathway.

5.3. FEEDBACK REGULATION OF THE NREM AND REM PHASES OF PHYSIOLOGICAL AND GHB-INDUCED SLEEP

The question why prolonged wake and sleep deprivation preferentially increase cerebral serotonin level remains to be answered. Possibly, in this case too, the alternation between CA and 5-HT for occupying storage sites could be involved. Since wake is connected with a catecholamine liberation from their hypothalamic and reticular storage sites, the space would become free for serotonin to occupy it. From a certain level on, this serotonin while still favouring the appearance of slow sleep, would also stimulate recovery and anabolic activity. CA synthesis would be one aspect of this activity. Temporarily, these CAs would actually be better protected from oxidation (Vanderwende and Johnson, 1970) by a high serotonin level whose antioxidant properties we have described (Laborit *et al.*, 1957). Their stability in their storage sites would, therefore, be increased, their reintegration being facilitated, till a high enough level would permit the appearance of paradoxical sleep. This paradoxical sleep would therefore and indeed be the expression of metabolic restoration.

Slow wave sleep would thus be also the initiator of paradoxical sleep since it expresses the functional activity of the neuroglia, an activity which is essential for neuronal ionic, metabolic and protein recuperation. The properties of GHB, a pentose pathway stimulant, would accelerate this recuperation by inducing slow wave sleep without disturbing the mitochondrial oxidative processes of the neurone, as hypnotics, sedatives and general

anaesthetics are liable to do. This interpretation of a dynamic phenomenon progressing with time is based on the functional and metabolic association between the neuroglia and the neurone. It would seem to offer a coherent explanation for many biological and pharmacological aspects of the mechanisms of sleep which still remain unclear, and for the often paradoxical experimental results.

6. GHB and Therapeutic Sleep

6.1. GHB AS A HYPNOTIC

Drug-induced sleep is generally obtained through a functional depression of the activating reticular formation, but we have seen that GHB does not depress the reticular formation. Under the action of GHB, the potentials evoked by peripheral stimulation at its level are increased even in amplitude while the electrocorticogram shows slow waves, and behavioural sleep is observed (Bertharion and Laborit, 1962).

The reticular formation will be put in its resting state generally by pharmacological agents which depress neuronal oxidative phosphorylation. Obviously, their action will be that much more marked since it exerts itself on a multisynaptic network. It is very unlikely, however, that this action on oxidative processes can be favourable to neuronal protein restoration which appears to be the essential function of sleep. On the contrary, if we accept the direct action of GHB on the neuroglia and its indirect action through the latter on neuronal restoration, it is, indeed, capable of promoting the appearance of paradoxical sleep. GHB will deepen sleep, and often cause a relaxation of the radix linguae, myoclonias and Cheyne-Stokes rhythm. It can be assumed that it will facilitate as much somatic recuperation (muscle relaxation, potassium reintegration), as cerebral recuperation (repolarization, more rapid excretion of oxidation by-products, and resynthesis of neurotransmitter and proteins). But it is logical that GHB-induced sleep is of shorter duration, and this is observed in normal subjects. It would thus appear—and there is supporting clinical evidence—that the use of GHB to obtain sleep should apply primarily to subjects whose insomnia is caused by prolonged environmental stimulation (prolonged work, physical and intellectual efforts), for this type of sleep is paradoxically enough a true *antidepressive* therapy, both from the neuronal–neuroglia point of view, and from that of restoration of cerebral neurotransmitter content. This is once more supported by clinical evidence. The association of GHB with other drugs such as reticular formation inhibitors, either by a direct action (benzodiazepines), or at the level of the collateral extralemniscal junction with the reticular formation (phenothiazines), or limbic formation inhibitors (antineurotics or minor tranquillizers) leads to the possibility of a much greater variety of indications.

Paradoxical sleep plays perhaps an important role in sleep therapy since it coincides with its dream phase. It can, indeed, be assumed that this symbolic manifestation of the unconscious leads to a beneficial release of certain repressions. Since this symbolic manifestation of the unconscious must rest upon a memorized experience, it is tempting to consider that its appearance is of major importance for limbic activity. It is known that GHB considerably lowers the threshold of excitability in those areas which, under its action, could be the site of induced or spontaneous paroxystic activity. Beneficial results have been obtained by Appia (1967) following awakening during the dream phase induced by GHB and immediate psychotherapy.

6.2. GHB AND ANAESTHETIC SURGICAL SLEEP

Although widely used in anaesthesia, it may be suggested that GHB in itself is not an anaesthetic and it does not decrease the mitochondrial oxidative processes. When used alone at doses from 4 to 6 g i.v. in the adult, it induces a sleep which only appears to be anaesthetic, but which, in fact, will not be sufficient for any surgical intervention. This is not so in the case of the infant and child, since here it can be used without any additional drug to perform any type of surgery. This may be due to a relatively high proportion of neuroglia.

In the adult, therefore, an association with reticular formation inhibitors (neuroleptics) and with small doses of analgesics (potentiated anaesthesia) (Laborit, 1950) will be required to obtain a stable general anaesthesia. Its advantages are: (1) its low toxicity; (2) the absence of any respiratory depression and the maintenance of the respiratory centre response to CO_2; (3) its muscle relaxant action which allows the use of only light doses of or even no curare, since tracheal intubation can be performed on spontaneously ventilating patients; (4) the absence of any hypotensive action and its bradycardiac effect; (5) the absence of venous irritation; (6) a pleasant induction; (7) a more easily mechanically controlled ventilation since the patient does not fight against the imposed rhythm and amplitude; (8) easier hypothermia, if it is required; and (9) finally, its antishock properties (Laborit *et al.*, 1960c, 1965).

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Mode of Action of Gamma-Butyrolactone on the Central Cholinergic System

Herbert Ladinsky, Silvana Consolo, Alberto Zatta, and Annamaria Vezzani

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, I-20157 Milan, Italy

Summary. Gamma-butylactone (GBL), a drug depressing the central nervous system, produced marked increases in acetylcholine contents in rat brain hemispheric regions (striatum, hippocampus, cortex) and in striatal choline content without modifying choline acetyltransferase or acetylcholinesterase activities.

In the hippocampus GBL also strongly decreased the acetylcholine turnover rate and inhibited the high affinity uptake of choline. Its increase in acetylcholine content was prevented by an acute electrolytic lesion of the medial septum but not by a wide array of drug treatments designed to interfere with neurotransmission in various pathways. The results are taken to indicate that GBL directly depresses the cholinergic septal-hippocampal afferents by interrupting impulse flow.

In the striatum, too, GBL markedly depressed the acetylcholine synthesis rate but had no effect on the high affinity choline uptake process. Such dissociation of the two phenomena had previously been observed using other drugs and may denote that acetylcholine synthesis in this region is regulated differently from that in the hippocampus.

By comparison, gamma-hydroxybutyric acid (GHBA), an active metabolite which shares with GBL the capacity to produce a somnolent state and depress impulse flow in the dopaminergic nigroneostriatal pathway, had no effect on either striatal acetylcholine content or on hippocampal high affinity choline uptake. The results suggest that GBL can be distinguished from GHBA in its neuropharmacological central cholinergic effects.

Key words: Gamma-butyrolactone – Gamma-hydroxybutyric acid – Acetylcholine – Choline uptake – Brain – Sleep

Introduction

Gamma-butyrolactone (GBL), a drug depressing the central nervous system is transformed in vivo to an active metabolite, gamma-hydroxybutyric acid (GHBA) (Giarman and Roth 1964), a congener of GBL and a naturally-occurring compound in the brain (Bessman and Fishbein 1963; Roth and Giarman 1970). This substance in turn, according to the in vitro studies of Mitoma and Neubauer (1968), De Feudis and Collier (1970), Andén and Stock (1973) and Andén (1974), forms gamma-aminobutyric acid (GABA), an important putative inhibitory neurotransmitter within the CNS. Whether GBL acts per se, or through GHBA or GABA has been the object of intensive study. The local injection of GHBA into the substantia nigra raised the dopamine (DA) content in rat brain to almost the same level as the systemic treatment with the drug and a similar rise in brain DA was obtained by the local application of GABA to the same structure (Andén and Stock 1973).

The mechanism by which GABA or GBL causes the increase in striatal DA appears to be related to the ability of the drugs to block impulse flow in the nigrostriatal dopaminergic neurons (Roth and Suhr 1970; Roth et al. 1974; Stock et al. 1973; Menon et al. 1974). Stoppage of nerve impulses produces a decrease in DA release from storage sites, a decrease in 3-methoxytyramine (Di Giulio et al. 1978) and an increase in DA synthesis (Roth 1971; Roth et al. 1974; Andén 1974).

However, the depressant effect of GBL on the dopaminergic neurons does not fit with the property of the drug to increase rat striatal acetylcholine (ACh) content (Sethy et al. 1976). Rather, such depression should lead to a decrease in the striatal ACh level as the removal of inhibitory dopaminergic tone results in disinhibition of the cholinergic neurons intrinsic to the striatum (Consolo et al. 1975; Ladinsky et al. 1975; McGeer et al. 1974; Guyenet et al. 1975; Rommelspacher and Kuhar 1975). Nevertheless, the findings of Chéramy et al. (1978), who reported that GABA and GABAergic agonists, as well as GHBA infused into the substantia nigra stimulated (^{3}H)-DA release in the caudate nucleus could better explain the increase in ACh induced by GBL. These authors suggested that unidentified nigral interneurons could exert an inhibitory influence on the dopaminergic neurons; their inhibition by a descending GABAergic pathway projecting to the pars reticulata would result in activation of the nigroneostriatal dopaminergic neurons. The consequence of such an activation would result in an increase in striatal ACh content.

On the other hand, it has been considered that GBL might act directly on cholinergic neurons to block impulse flow although actual evidence to this effect has not been forthcoming and has been postulated solely on the basis of the drug's capacity to alter ACh levels in the CNS (Sethy et al. 1976).

Therefore, we attempted to investigate whether a direct or an indirect mechanism underlies GBL's cholinergic actions in rat brain.

Materials and Methods

Animals. Female CD-COBS rats (Charles River, Italy), body weight 175–220 g, were used. The animals were given free

Send offprint requests to H. Ladinsky at the above address

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access to water and food and were housed in groups of six in makrolon cages under standard conditions of humidity (70%), room temperature (23°C) and 12 h light and 12 h dark cycles for at least 4 days prior to the experiment.

The rats were killed by fast focussed microwave irradiation to the head (1.3 kW at 2.45 GHz for about 4 s) using an adapted commercial microwave oven (Medical Engineering Consultants, Peabody, MA, USA). The rat's head was fixed in place in a plastic animal holder by tightening two blunt plastic screws placed 180° apart in a horizontal plane until just enough pressure was exerted to discourage the rat from turning its head.

In ACh turnover studies, which required intravenous infusion of tritiated choline for 4 and 6 min, the plastic holder was further adapted by affixing to it a long and narrow cylindrical plastic holder for the tail which permitted intravenous placement of the needle but prevented the tail from even minimally moving and dislodging the needle. This assured that all of the label was infused into the rat over the time period desired.

Assay for Acetylcholine and Choline. The brain was quickly removed and dissected into different areas (striatum, hippocampus, hemispheric residuum and midbrain-hindbrain). After weighing, the tissues were homogenized in a mixture of 15% 1 N formic acid-85% acetone before proceeding to the measurement of ACh and choline by the radioenzymatic method of Saelens et al. (1970) with modifications (Ladinsky et al. 1976).

Acetylcholine Turnover. For the measurement of the acetylcholine turnover rate (TR_{ACh}), a tracer amount of (methyl-^{3}H)choline chloride (80 Ci/mmol, Radiochemical Centre, Amersham, UK) was infused into the tail vein of the rat at a constant rate of 0.84 nmol/80 µl/min for 4 min or 6 min. At the end of these times the rats were killed by focussed microwave irradiation to the head. The striatum and hippocampus were removed bilaterally and homogenized in 15 volumes of a cold mixture of 15% 1 N formic acid and 85% acetone. After low speed centrifugation, the acetone in the supernatant was removed by partitioning into heptane-chloroform. A known volume of the aqueous extract was lyophilized and the acidic residue was resuspended in 25 µl distilled water before applying 20 µl of this to the paper strip for low voltage electrophoresis (500 V, 60 min). The (methyl-^{3}H)choline and the acetyl(methyl-^{3}H)choline formed were identified by their relative positions to tetraethylammonium on each strip of paper. As the quantitative separation of these two labelled quaternary amines is critical to the results obtained, their overlapping was measured as previously described (Ladinsky et al. 1972). It was found that less than 1% of the tritiated choline overlapped onto the acetylcholine band and less than 6% of the tritiated ACh overlapped onto the choline band.

The bands were cut out and disintegrated in 1 ml soluene before adding 0.5 ml distilled water and 10 ml of toluene base phosphor for liquid scintillation counting. The turnover calculation was made as described by Racagni et al. (1976) and done by computer.

Measurement of Choline Acetyltransferase, Acetylcholinesterase and High Affinity Choline Uptake Activities. Choline o-acetyltransferase activity was measured by a modified radiochemical method of McCaman and Hunt (1965) and acetylcholinesterase activity was determined by the method of McCaman et al. (1968). Sodium-dependent high affinity uptake of choline (SDHACU) was measured by the method of Atweh et al. (1975) followed by filtration through 0.65 µ Sartorius cellulose nitrate filters.

Chemical Lesion. Kainic acid, 0.5 µg in 0.5 µl was dissolved in saline and injected bilaterally into the dorsal and ventral hippocampus (coordinates from bregma: −3.5, 2.5, 4.0 mm and −6.5, 5.5, 7.5 mm, respectively) of ether anaesthetized rats. Sham lesions were carried out in an identical manner but saline was injected. The experiment was performed 2 weeks after the treatment.

Acute Electrolytic Lesion. The animals were lesioned by electrocoagulation of the medial septal nucleus, using a stainless steel electrode (0.5 mm tip, 0.3 mm diam.). The stereotaxic coordinates were: AP 8.2, LO, H 4.8 mm. Sham lesions were carried out in an identical manner but no current was passed. Lesion placement was verified histologically in each animal after removal of the experimental tissue samples.

Treatments. The following drugs, their solvents, routes of administration, dosage and time schedules were used: GBL was diluted with distilled water and administered intraperitoneally at the doses and times shown in the Tables; GHBA, sodium salt was dissolved in distilled water and administered intraperitoneally at the doses and times described in the Tables or text; atropine sulfate 5 mg/kg i.p., 30 min dissolved in saline; reserpine, 5 mg/kg, i.p., 16 h, dissolved in 0.3% citric acid and 2% benzyl alcohol in 25% polyethylene glycol in saline; picrotoxin 2.5 mg/kg, i.p., 30 min, dissolved in saline; phenoxybenzamine · HCl, 20 mg/kg i.p., 240 min and haloperidol · HCl, 1 mg/kg i.p., 60 min, were dissolved in saline with the dropwise addition of 1 N HCl; propranolol · HCl 10 mg/kg i.p., 30 min, dissolved in saline; pimozide, 1 mg/kg i.p., 240 min, dissolved in 0.2 N tartaric acid; naloxone, 5 mg/kg i.p., 30 min, dissolved in saline; pentylenetetrazol, 60 mg/kg, i.p., 45 min, dissolved in saline; alphamethylparatyrosine methyl ester HCl (αMpT) dissolved in saline and administered as two consecutive doses of 300 mg/kg, i.p. and 200 mg/kg, i.v. respectively, 20 h and 4 h before GBL; prazosin, 62.5 mg/kg, p.o., 180 min, suspended in 0.5% carboxymethylcellulose.

Drugs and Reagents. Drugs were obtained from the sources indicated below: gammabutyrolactone, atropine sulfate and alphamethylparatyrosine methyl ester HCl (Aldrich-Europe, Beerse, Belgium); gamma-hydroxybutyric acid sodium salt and picrotoxin (Sigma Chemical Co., St. Louis, MO, USA); pimozide (gift from Janssen Pharmaceutica, Beerse, Belgium); reserpine (gift from Ciba-Geigy, Basel, Switzerland); phenoxybenzamine · HCl (gift from Smith, Kline and French, Welwyn Garden City, Herts, England); haloperidol · HCl (gift from Lusofarmaco, Milan, Italy); propranolol · HCl (gift from Icpharma, Milan, Italy); naloxone (gift from Endo Labs, Garden City, NY, USA); pentylenetetrazol (gift from Knoll, Milan, Italy); prazosin (gift from Pfizer Italiana, Latina, Roma, Italy).

All reagents of high purity grade were purchased from commercial sources.

Results

The effect of GBL on the brain regional ACh content is shown in Table 1. At 90 min following the intraperitoneal adminis-

Table 1. Effect of gamma-butyrolactone on the acetylcholine content in rat brain regions

Region	Acetylcholine (nmoles/g)	
	Controls	Treated
Striatum	66.6 ± 3.7	108.9 ± 8.0*
Hippocampus	22.1 ± 1.3	33.0 ± 0.7*
Hemispheric residuum	18.5 ± 0.7	28.4 ± 1.3*
Midbrain-hindbrain	23.9 ± 0.8	21.7 ± 1.1

The data are the means ± S.E.M. (n = 7)
* $P < 0.01$ vs control group, Student's t-test
The rats were killed 90 min after the intraperitoneal administration of GBL, 1.25 g/kg

Table 2. Dose-dependence of the increase in striatal acetylcholine content by gamma-butyrolactone

Dose of GBL (g/kg)	Acetylcholine (nmoles/g)	
	Striatum	Hippocampus
Controls	67.6 ± 1.9 (14)	21.6 ± 0.8 (7)
0.5	68.5 ± 3.3 (6)	23.5 ± 1.0 (7)
0.75	68.5 ± 3.2 (6)	25.3 ± 1.0 (7)*
1.0	71.0 ± 6.5 (5)	26.3 ± 1.0 (7)*
1.25	102.0 ± 6.4 (9)**	28.2 ± 0.8 (7)**
1.5	133.4 ± 9.3 (6)**	–

Data are the means ± S.E.M. (n)
The animals were killed 90 min after the intraperitoneal administration of GBL
*$P < 0.05$ and **$P < 0.01$ vs controls; Dunnett's test

tration of 1.25 g/kg, GBL increased ACh content by 64%, 49% and 54% in the striatum, hippocampus, and hemispheric residuum, respectively, but did not produce any effect in the midbrain-hindbrain region. The choline content was measured in the striatum and hippocampus and was found to be increased by about 45% in striatum and unchanged in the hippocampus (the data are given in Table 5).

The dose-dependence of GBL on the ACh content of the striatum and hippocampus 90 min after administration is shown in Table 2. In the striatum the drug gave no significant effect from 0.5 g/kg to 1.0 g/kg and then produced an increase of 51% at 1.25 g/kg and doubled the ACh content at 1.50 g/kg. The hippocampus was slightly more sensitive to GBL as it responded with a 17% increase at the doses of 0.75 g/kg and 1 g/kg followed by a 31% increase at 1.25 g/kg.

All the drug doses induced a sleep-like state within 5 min of their administration and high mortality arose at the dose of 1.5 g/kg from respiratory arrest. Thus, still higher doses of GBL could not be used although a plateau in the ACh content had not been attained. The dose of 1.25 g/kg has been used in the subsequent experiments.

The time course showed that the drug significantly increased the level of striatal ACh within 30 min of its administration (34%) and that the maximum increase of 51% was achieved at 90 min. This increase persisted until 180 min (Table 3) after which time most animals died. By comparison, GHBA did not significantly affect striatal ACh content at doses of 0.5, 0.75, 1 and 2 g/kg, 120 min after its administration (data not shown). The time course also showed lack of effects of the drug at 2 g/kg although there was a tendency towards a decrease at 15 min after its administration (Table 3). GHBA, like GBL induced a sleep-like state within 5 min of its intraperitoneal administration which suggests that the hypnotic effect of GBL is neither the result of, nor responsible for the increase in brain regional ACh content.

The specific activities of striatal and hippocampal choline o-acetyltransferase and striatal acetylcholinesterase activities were affected neither by the in vitro incubation of the 500 × g supernatant with GBL or its metabolite GHBA at a final concentration of 5×10^{-5} M, nor 90 min after the i.p. administration of GBL at 1.25 g/kg (data not shown). The control values for the enzymic activities of striatum were 112.6 ± 6 μmole ACh synthesized/h/g prot and 18.1 ± 9 mmole ACh hydrolyzed/h/g prot, respectively.

GBL produced a marked inhibition of the sodium-dependent high affinity uptake of choline (SDHACU) by a crude hippocampal synaptosomal preparation both at 30 and

Table 3. Time course of effects of gamma-butyrolactone and gamma-hydroxybutyric acid on rat striatal acetylcholine content

Time (min)	Striatal acetylcholine (nmoles/g)	
	GBL	GHBA
Controls	66.1 ± 2.8	65.2 ± 2.0
15	–	56.3 ± 3.1
30	88.6 ± 4.9*	63.4 ± 1.2
60	85.7 ± 2.0*	61.6 ± 1.0
90	99.7 ± 4.8**,***	–
120	106.5 ± 10.5**	60.5 ± 4.9
150	107.9 ± 4.6**	–
180	107.8 ± 4.3**	–

Data are the means ± S.E.M. (n = 5–6) · GBL, 1.25 g/kg i.p., GHBA, 2 g/kg, i.p. Both drugs induced sleep within 5 min
*$P < 0.05$ and **$P < 0.01$ vs controls; ***$P < 0.05$ vs 60 min group, ns vs 120 min group; Duncan's test

90 min after its administration of 1.25 g/kg, but had no effect on SDHACU by striatal synaptosomes (Table 4). By contrast, GHBA did not affect hippocampal SDHACU either after its in vivo administration of 2 g/kg (Table 4) or when incubated in vitro up to a concentration of 10^{-4} M (data not shown). Similarly, in vitro, GBL had no effect on hippocampal SDHACU when incubated up to a concentration of 10^{-3} M (data not shown).

The synthesis rate of ACh was measured by the i.v. infusion of (methyl-^{3}H)choline. It was found that GBL, at the dose of 1.25 g/kg and 90 min decreased the radioactivity of acetyl(methyl-^{3}H) choline both in the striatum and in the hippocampus by approximately 50% (Table 5) and decreased the specific radioactivity of ACh by about 70% in these two brain regions. The radioactivity of (methyl-^{3}H)choline and the specific radioactivity of choline was increased approximately twofold by GBL in both brain areas. Using the equation proposed by Racagni et al. (1976), the turnover rates of ACh were calculated to be 9.97 ± 0.3 and 3.53 ± 0.1 pmole ACh synthesized/min/mg wet wt in striatum and hippocampus for the controls. GBL reduced the turnover rate by about 70% in both the striatum and the hippocampus.

Pretreatment with drugs designed to interfere with cholinergic, dopaminergic, noradrenergic and GABAergic neurotransmission did not alter the GBL-induced increase in ACh of the striatum as determined by ANOVA two-way factorial

Table 4. Effect of gamma-butyrolactone and gamma-hydroxybutyric acid on sodium-dependent high affinity choline uptake in vivo

In vivo administration			Sodium-Dependent high affinity uptake of choline (nmoles/g prot/min)			
Drug	Dose (g/kg)	Time (min)	Hippocampus		Striatum	
			Control	Treated	Control	Treated
GBL	1.25	30	0.66 ± 0.04 (6)	0.43 ± 0.03 (5)*	–	–
	1.25	90	0.67 ± 0.04 (6)	0.41 ± 0.02 (5)*	1.37 ± 0.2 (8)	1.34 ± 0.06 (8)
GHBA	2	30	0.66 ± 0.05 (5)	0.65 ± 0.03 (6)	–	–

The data are the means ± S.E.M. (n)
*$P < 0.01$ vs control, Student's t-test

Table 5. Turnover rates of acetylcholine in rat striatum and hippocampus after a single injection of gamma-butyrolactone

Region	Treatment	Endogenous		Radioactive		Specific radioactivity		K_a (h^{-1})	ACh Turnover (pmoles/min/mg tissue)
		ACh	Choline	ACh	Choline	ACh	Choline		
		(pmoles/mg tissue)		(dpm/mg tissue)		(dpm/pmole)			
Striatum	Saline	68.0 ± 1.3	23.2 ± 0.9	209.6 ± 14.6	239.0 ± 15.5	3.08	10.3	8.8 ± 0.3	9.97 ± 0.3
	GBL	112.0 ± 8.0*	33.7 ± 1.1*	91.3 ± 12.3*	606.5 ± 23.5*	0.81	17.9	1.7 ± 0.1*	3.17 ± 0.1*
Hippocampus	Saline	22.1 ± 0.6	21.1 ± 0.4	114.5 ± 14.8	196.4 ± 31.3	5.18	9.3	9.6 ± 1.7	3.53 ± 0.1
	GBL	33.0 ± 1.1*	22.5 ± 0.9	56.8 ± 6.3	381.1 ± 22.4*	1.72	16.9	1.9 ± 0.1	1.04 ± 0.1*

The data are the means ± S.E.M. for 6 animals
*$P < 0.01$ vs the saline group, Student's t-test
GBL was administered i.p. at the dose of 1.25 g/kg. The rats were killed by focussed microwave irradiation to the head 90 min after GBL and 4 min after (methyl-^{3}H)choline i.v. infusion. This tracer dose did not affect either the ACh content or the choline content of the 2 brain regions

Table 6. Effect of treatment with various drugs on the GBL-induced increase in acetylcholine content in rat striatum

Drug in columns C and D	Striatal acetylcholine (nmoles/g)				Interaction
	A Vehicle	B GBL	C Drug	D Drug + GBL	
Reserpine	68.2 ± 2.0 (6)	131.6 ± 5.4 (6)*	59.0 ± 2.7 (6)	132.2 ± 5.9 (6)*	ns
Atropine sulfate	62.1 ± 1.5 (8)	107.9 ± 6.3 (8)*	57.7 ± 2.0 (8)	89.0 ± 11.1 (8)*	ns
Picrotoxin	68.5 ± 1.8 (6)	117.9 ± 6.7 (6)*	78.5 ± 2.0 (6)	128.3 ± 3.7 (6)*	ns
Phenoxybenzamine	66.5 ± 1.7 (8)	110.5 ± 6.5 (8)*	69.7 ± 1.6 (8)	101.3 ± 5.7 (8)*	ns
Propranolol	71.7 ± 2.7 (7)	92.5 ± 2.0 (7)*	61.9 ± 1.7 (7)	97.5 ± 3.2 (7)*	ns
Naloxone	63.0 ± 3.6 (6)	110.2 ± 11.2 (6)*	68.4 ± 3.9 (6)	121.6 ± 9.3 (6)*	ns
α-MpT	66.4 ± 2.6 (6)	100.1 ± 9.3 (6)*	61.2 ± 3.9 (6)	120.2 ± 5.2 (6)*	ns
Pimozide	70.9 ± 1.7 (7)	102.7 ± 5.0 (7)*	45.3 ± 1.8 (7)*	110.0 ± 9.6 (7)*	$F_{1,24} = 10.2$ $P < 0.01$
Haloperidol	67.0 ± 1.6 (7)	103.2 ± 8.3 (7)*	42.1 ± 1.3 (7)*	125.7 ± 5.6 (7)*	$F_{1,24} = 19.8$ $P < 0.01$
Pentylenetetrazol[a]	64.9 ± 1.5 (6)	120.0 ± 10.1 (6)*	58.0 ± 1.9 (6)	106.1 ± 6.7 (6)*	ns
Prazosin	68.3 ± 2.3 (8)	98.5 ± 7.9 (8)*	69.7 ± 2.6 (8)	92.2 ± 4.8 (8)*	ns

Reserpine, 5 mg/kg, i.p. 16 h; atropine sulfate, 5 mg/kg, i.p. 30 min; picrotoxin, 2.5 mg/kg, i.p. 30 min; phenoxybenzamine, 20 mg/kg, i.p. 240 min; propranolol, 10 mg/kg, i.p. 30 min; naloxone, 5 mg/kg, i.p. 30 min; alphamethylparatyrosine, 300 mg/kg, i.p. and 200 mg/kg, i.v., respectively 20 h and 4 h before GBL; pimozide 1 mg/kg, i.p. 240 min; haloperidol, 1 mg/kg, i.p. 60 min; pentylenetetrazol, 60 mg/kg, i.p. 45 min; prazosin, 62.5 mg/kg, p.o. 180 min
The rats were killed 90 min after the administration of GBL, 1.25 g/kg i.p. and at the times indicated for the drugs given above. The data are means ± S.E.M. (n). The data were analyzed by ANOVA two-way factorial analysis, Tukey's test and Tukey's test for unconfounded means
*$P < 0.01$ vs the vehicle group
[a] Mortality was 4/10 in the GBL plus pentylenetetrazol group; GBL did not prevent the convulsive symptoms manifested by the pentylenetetrazol-treated animals

analysis (Table 6). The drugs employed were reserpine, atropine sulfate, picrotoxin, phenoxybenzamine, propranolol, naloxone, alphamethylparatyrosine, pentylenetetrazol and prazosin. The powerful dopamine receptor blockers pimozide and haloperidol markedly decreased the level of striatal ACh by about 40% but despite this, the net increase in ACh induced by GBL was significantly greater in the neuroleptic-treated group than in the control group.

In the hippocampus and cortex none of the above drugs either affected the level of ACh by themselves or prevented the

Table 7. Effect of intrahippocampally-placed kainic acid lesion or acute medial septal electrolytic lesion on the increase in acetylcholine content induced by gamma-butyrolactone in hippocampus

Lesion in columns C and D	Hippocampal acetylcholine (nmoles/g)				
	A Control	B GBL	C Lesion	D Lesion + GBL	Interaction
Kainic acid lesion	21.5 ± 0.5 (11)	28.2 ± 0.7 (11)*	18.5 ± 0.4 (11)**	26.6 ± 0.8 (11)*	ns
Acute septal lesion	22.4 ± 0.6 (7)	31.3 ± 0.6 (7)*	27.9 ± 1.0 (7)*	29.5 ± 1.0 (7)*	$F1, 24 = 19$ $P < 0.01$

Control animals were sham operated in the septal lesion experiment. GBL was administered 90 min postlesion at the dose of 1.25 g/kg and the animals were killed 90 min after drug administration

Control animals were vehicle-treated in the kainic acid lesion experiment. The experiment was performed 15 days after the local application of kainic acid. The animals were killed 90 min after the administration of GBL, 1.25 g/kg. The data are the means ± S.E.M. (n). *$P < 0.01$ vs control; **$P < 0.05$ vs control

The data were analyzed by ANOVA two-way factorial analysis, Tukey's test and Tukey's test for unconfounded means

action of GBL (data not shown). In addition, 2 weeks after the intrahippocampal application of kainic acid, when the virtual complete destruction of perikarya had been achieved (Schwarcz et al. 1978), there was no blockade of the ACh increase induced by GBL (Table 7). Kainic acid lesion produced a small but significant decrease in hippocampal ACh content of 14%.

An acute electrolytic lesion of the medial septal nucleus was made in order to block impulse flow in the septal-hippocampal cholinergic pathway (Sethy et al. 1973). Such a lesion induced a rise in hippocampal ACh at 180 min (Table 7) probably as a result of a decreased release of this neurotransmitter. The choline content was not affected (data not shown). The increase in ACh content that GBL produced in the acutely septal lesioned rats was not additive with that of the lesion.

Discussion

The present study confirms that GBL causes an increase in the concentration of ACh in rat hemispheric regions. This effect cannot be ascribed to modifications in cholinergic neurotransmitter metabolism as the drug neither inhibited nor stimulated AChE or ChAT activities. The acute electrolytic lesion of the septal-hippocampal cholinergic pathway prevented the ACh rise induced by GBL suggesting that the increase in ACh, like that in DA (Roth et al. 1973) is impulse flow-dependent. It may be more broadly stated that GBL and the septal lesion share the property of slowing or interrupting impulse flow and as a consequence, they reduce ACh release, increase its level and then by some regulatory process depress the ACh synthesis rate. The high affinity uptake of choline is reduced in both types of manipulations, most likely secondarily to the buildup of intraneuronal ACh concentration, and could be the regulatory step in the synthesis of ACh as has already been postulated by Mulder et al. (1974) and Collier and Katz (1974). Such a hypothesis is consistent also with the in vitro work of Antonelli et al. (1981) who found that the content of ACh in cortical slices was inversely proportional to the rate of choline uptake by the synaptosomal fraction of these slices.

In the experiment in which the effect of GBL on ACh turnover was determined, it was established that besides the markedly reduced formation of acetyl(methyl-^{3}H)choline, there was a huge accumulation of (methyl-^{3}H)choline radioactivity in the hippocampus and striatum. The administration of the hypnotic chloral hydrate produced similar phenomena (Atweh and Kuhar 1976), and this drug's induced increase in (methyl-^{3}H)choline persisted when the hippocampal cholinergic afferents were destroyed by chronic septal lesion, denoting multiple pools of choline in the brain. If GBL, like chloral hydrate, increased the specific activity of choline in a "pool" distinct from the ACh precursor pool, then it can be understood how the GBL-accumulation of (methyl-^{3}H)choline could have occurred in the absence of a concomitant increase in acetyl(methyl-^{3}H)choline and in the presence of a strong block in SDHACU. It must be underscored at this point that the calculated value we gave for the ACh turnover rate following GBL treatment may be only a qualitative estimate if some of the (methyl-^{3}H)choline partakes of a pool unavailable for ACh synthesis. Should this turn out to be the case, then the parameter best correlated with cholinergic activity would be the level of acetyl(methyl-^{3}H)choline (Atweh and Kuhar 1976).

The process for the regulation of ACh synthesis appears to be different in the striatum and possibly is unrelated to choline uptake as GBL, despite the pronounced changes it produced in ACh content and ACh turnover, did not alter SDHACU. It is not clear as to where such a difference may lie (Jope 1979), but this characteristic of GBL is shared by other drugs affecting striatal ACh content such as bromocriptine (Consolo et al. 1981), oxotremorine (Ladinsky et al. 1981), neuroleptics (Sherman et al. 1978), minaprine and buspirone (unpublished data).

It can be derived from this information that GBL directly depresses cholinergic neurons through its interference with presynaptic events leading to the release of ACh. This hypothesis is substantiated inasmuch as the alteration of neurotransmission in several central pathways by drugs and neurotoxins failed to mitigate GBL's action. In particular, the possibility that GBL activates the nigroneostriatal dopaminergic neurons (Chéramy et al. 1978; see Introduction) to increase ACh appears to be ruled out as both haloperidol and pimozide were unable to block the GBL effect. Indeed, rather than blocking, these neuroleptics potentiated GBL's net increase in striatal ACh. As an explanation, it may tentatively be suggested that the GBL effect on ACh, although pronounced, was not maximal but rather reflected a net change resulting from the increase due to the direct action of GBL on the cholinergic neurons and the possible decrease in a limited population of cholinergic interneurons following blockade of impulse flow in the nigroneostriatal dopaminergic neurons.

As the dopamine receptor blockers act on the same dopaminergic pathway that GBL should have been affecting to deplete ACh, they made it possible to unmask the total extent of GBL's direct cholinergic action. The finding that there was a decrease in the striatal muscarinic receptor population following chronic treatment with GBL (Giorgi and Rubio 1981) is certainly in line with a disinhibition of cholinergic neurons induced by the drug indirectly through the dopaminergic system.

GBL and GHBA overlap in their pharmacological properties of inducing a sleep-like state and interrupting impulse flow in the nigroneostriatal dopaminergic neurons. Therefore, these effects of GBL may be indirectly due to GHBA, its active metabolite. In contrast, the cholinergic actions of GBL are not due to GHBA formation but due to GBL itself as indicated by the failure of GHBA to increase striatal ACh content or to inhibit hippocampal SDHACU. These data are consistent with the finding that GBL is not able to displace GHBA from its specific binding site in rat brain (Benavides et al. 1982). Because of the divergence in the actions of the two drugs, caution should be taken in attributing to GHBA the cholinergic effects of GBL, as has sometimes been done (Sethy et al. 1976; Snead 1977).

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Clinical Research

Gammahydroxybutyrate and Narcolepsy: A Double-Blind Placebo-Controlled Study

*§G. J. Lammers, †¶J. Arends, †A. C. Declerck, *M. D. Ferrari, ‡G. Schouwink and §J. Troost

**Department of Neurology, Leiden University Hospital, Leiden, The Netherlands*
†Department of Clinical Neurophysiology and Polygraphy, Kempenhaeghe, Heeze, The Netherlands
‡Department of Neurology, Hospital Rijnstate, Arnhem, The Netherlands
§Department of Neurology, Medisch Spectrum Twente, Enschede, The Netherlands
¶Janssen Pharmaceutica B. V., Tilburg, The Netherlands

Summary: We treated 24 patients with narcolepsy for 4 weeks with gammahydroxybutyrate (GHB), 60 mg/kg/night, in a randomized double-blind placebo-controlled cross-over trial. Both clinical and polysomnographic criteria were used to assess the results. Compared to placebo, GHB reduced the daily number of hypnagogic hallucinations (from 0.87 to 0.28; $p = 0.008$), daytime sleep attacks (from 2.27 to 1.40; $p = 0.001$) and the severity of subjective daytime sleepiness (from 1.57 to 1.24 on a 0–4 scale; $p = 0.028$). The number of daily cataplexy attacks was reduced from 1.26 at baseline to 0.56 after 4 weeks of GHB intake. This reduction, however, was not statistically significantly different from the difference between baseline and placebo. GHB stabilized nocturnal rapid eye movement (REM) sleep, i.e. it reduced the percentage of wakefulness during REM sleep ($p = 0.007$) and the number of awakenings out of REM sleep ($p = 0.016$), and tended to increase slow wave sleep ($p = 0.053$). Adverse events were few and mild. We conclude that GHB is an effective and well-tolerated treatment for narcolepsy. **Key Words:** Narcolepsy—Gammahydroxybutyrate—Polysomnography—Placebo-controlled clinical trial.

Narcolepsy is clinically characterized by excessive daytime sleepiness (EDS), a disturbed nocturnal sleep and the three rapid eye movement (REM) sleep-related phenomena: cataplexy, hypnagogic hallucinations and sleep paralysis (1). Polysomnographic findings of nocturnal sleep include instability of REM as well as non-rapid eye movement (NREM) sleep and shortened REM sleep latency (2–4). Standard drug treatment consists of psychostimulants for EDS and antidepressant drugs for the REM sleep-related symptoms (5).

Gammahydroxybutyrate (GHB) is a putative neurotransmitter in the human brain (6,7). After oral administration it has a hypnotic action and is considered to ameliorate (daytime) narcoleptic symptoms (8). To date, three open trials (9–11) and one double-blind placebo-controlled trial (12,13) testing this hypothesis have been published. A limitation for the interpretation of the polysomnographic effects in this last study was the previously defined duration of nocturnal sleep.

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Address correspondence and reprint requests to G. J. Lammers, M.D., Department of Neurology, Leiden University Hospital, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

To establish the efficacy of GHB in narcolepsy, we carried out a randomized double-blind placebo-controlled cross-over trial in an unselected group of patients. Results were assessed both clinically and with polysomnographic criteria.

PATIENTS

Twenty-four patients were selected on clinical criteria. All of the patients had a history of excessive daytime sleepiness and cataplexy.

The clinical and demographic characteristics are summarized in Table 1. Medication, if any, was continued and kept unchanged for at least the 4 weeks prior to the trial.

The protocol was approved by our local ethics committee and all patients gave their written informed consent.

STUDY DESIGN

A schematic outline of the trial is shown in Fig. 1.

Following recruitment, patients were entered into a baseline observation period of 1 week. At the end of

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TABLE 1. *Demographic and clinical characteristics of the study population*

Age mean (range)	36.0 (16–65)
Sex male/female	13/11
HLA DR2/DQW6	24
Complaints (history):	
EDS/cataplexy	24
Hypnagogic hallucinations	21
Sleep paralysis	17
Co-medication[a]	
None	12
Stimulants	9
Antidepressants	7
Hypnotics	1
Other	1

[a] Five patients used more than one co-medication because of narcolepsy.

this week the first polysomnographic recording (baseline 1) was made. Immediately afterward, patients were randomly divided into two groups and treatment was started for a period of 4 weeks. On the last treatment day a second recording was made. A washout period of 3 weeks followed. This period was considered to be sufficient because GHB has a very short plasma half-life (undetectable after 3–4 hours) and because its therapeutic efficacy is of short duration (1–2 days) (7,10). At the end of the second baseline week, a third recording (baseline 2) was made and cross-over took place. A fourth polysomnogram at the end of the second treatment period completed the study.

During both baseline and treatment periods, a diary (daily questionnaire; see below) was kept, and mood ratings were scored once a week. At the end of each treatment period the global therapeutic impression was assessed by the patient.

Co-medication was continued unchanged throughout the study.

METHODS

Patients noted the following items in their diaries: number of sleep attacks, awakenings at night, cataplexy attacks, hypnagogic hallucinations, sleep paralysis, a rating of daytime sleepiness (0 = no sleepiness, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe) and a rating of the feeling of being refreshed in the morning (0 = not refreshed, 1 = slightly, 2 = moderately, 3 = sufficiently, 4 = very). The global therapeutic impression was rated on a 0–3 scale: 0 = no effect at all, 1 = possibly beneficial, 2 = beneficial, 3 = strongly beneficial. For mood rating a visual analogue mood rating scale (VAMRS) (14) was used.

Each polysomnographic recording consisted of an ambulatory 24-hour polygraphic recording [eight-channel Oxford Instruments recorder; two channels were used for electrooculography (EOG), four for electroencephalography (EEG), one for electromyography (EMG), and one channel for electrocardiography (ECG)]. A multiple sleep latency test (MSLT) was carried out during each recording at the patient's home after careful instruction of the patient and the partner, but without supervision at home.

Sleep was scored according to standard criteria (15) in epochs of 1 minute.

The number of sleep attacks, cataplexy attacks and hypnagogic hallucinations were considered of primary importance in the judgment of clinical efficacy.

STUDY MEDICATION

Gammahydroxybutyrate was administered orally as a 10% watery solution. To minimize differences in taste between GHB and placebo we used flavors and salt. Two daily doses of 30 mg/kg each were given; the first shortly before nocturnal sleep, the second 4 hours later.

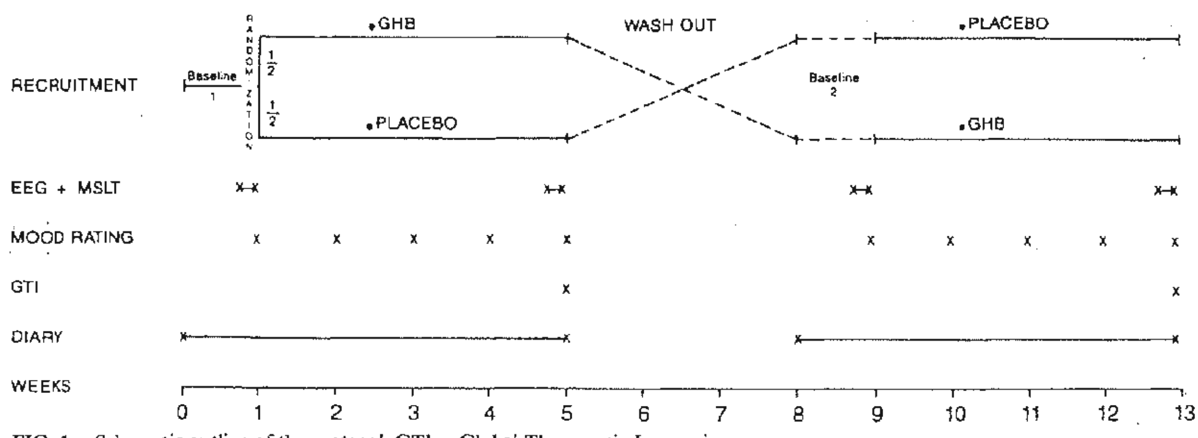

FIG. 1. Schematic outline of the protocol. GTI = Global Therapeutic Impression.

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STATISTICS

To analyze treatment effects on diary variables, mean daily values during baseline were compared with the mean daily values during the fourth treatment week. The difference from baseline obtained with GHB was compared with the difference obtained with placebo. If patients did not experience a specific diary item during either baseline period, then they were excluded in the analysis for that particular item.

Intrapatient differences over time were analyzed for each group with the Wilcoxon's signed-rank test. Intergroup differences were analyzed with the Wilcoxon's two-sample test. A probability of <0.05 (two-tailed) was considered statistically significant.

RESULTS

Study population

All 24 patients completed the trial. One patient was excluded from the diary analysis because he failed to keep his diary. One patient was excluded from the polysomnographic analysis because of technical disturbances in the recordings. Seven patients properly completed all four ambulatory MSLTs.

Treatment effects

GHB reduced the daily number of hypnagogic hallucinations (n = 12) from 0.87 ± 0.58 to 0.28 ± 0.40 (placebo: 0.88 ± 0.77–0.95 ± 0.86; p = 0.008), daytime sleep attacks (n = 23) from 2.27 ± 0.93 to 1.40 ± 1.17 (placebo: 2.22 ± 1.32–2.18 ± 1.17; p = 0.001), the severity of subjective daytime sleepiness (n = 23) from 1.57 ± 0.47 to 1.24 ± 0.68 (placebo: 1.55 ± 0.60–1.59 ± 0.63; p = 0.028), the number of cataplexy attacks (n = 21) from 1.26 ± 1.76 to 0.56 ± 0.84 (placebo: 1.56 ± 1.99–1.24 ± 1.38; ns) and awakenings at night (n = 23) from 3.30 ± 1.82 to 2.45 ± 1.73 (placebo: 3.32 ± 1.82–3.49 ± 1.87; ns). The feeling of being refreshed in the morning (n = 23) increased from 1.90 ± 0.57 to 2.13 ± 0.72 (placebo: 1.83 ± 0.72–1.92 ± 0.69; ns). The effect on sleep paralysis could not be assessed because of the low incidence of this item during the baseline weeks (n = 4).

The effects on polysomnographic variables are shown in Table 2. Compared to placebo, GHB significantly reduced the number of awakenings from, and the percentage of wakefulness during, REM sleep. The increase in the amount of nocturnal slow wave sleep (SWS) during GHB treatment was considerable and nearly significant (p = 0.053). The total amount of REM sleep, nocturnal REM sleep latency and other polysomnographic parameters were not influenced by GHB.

TABLE 2. *Polysomnographic data for nocturnal sleep (n = 23)*

Sleep structure (EEG)	GHB		Placebo		Effect
	Mean	SD	Mean	SD	p[a]
Sleep architecture					
Stage 1 (%)					
Before	18.8	11.7	17.7	9.3	
After	14.0	9.4	16.7	7.6	ns
Stage 2 (%)					
Before	40.2	8.7	40.5	10.4	
After	43.6	10.6	43.9	10.9	ns
Stage 3 + 4 (%)					
Before	24.9	9.0	25.5	9.4	
After	29.0	12.5	24.0	10.5	0.053
Stage REM (%)					
Before	16.1	4.7	16.3	6.6	
After	13.4	6.8	15.5	5.9	ns
Sleep stage shifts (total)					
Before	53.0	14.1	52.3	13.1	
After	42.5	13.8	50.8	14.2	ns
REM sleep variables					
Awakenings (no.)[b]					
Before	2.6	3.0	2.6	2.5	
After	1.9	2.4	3.8	3.4	0.016
Percentage wake[b]					
Before	19.6	39.6	11.4	19.7	
After	10.9	23.0	17.1	14.5	0.007
Sleep stage shifts[c]					
Before	6.5	2.1	8.0	4.1	
After	4.4	1.9	7.3	3.4	ns

[a] Wilcoxon's signed-rank test; ns = not significant.
[b] Awakenings/hour REM sleep and percentage of wake during REM sleep.
[c] Number of sleep stage shifts towards REM sleep/hour REM sleep.

In the seven patients who had correctly performed all four multiple sleep latency tests, GHB did not alter the mean sleep latency of the MSLT [GHB: 5.79 ± 5.03–3.67 ± 2.41 minutes; placebo: 5.22 ± 4.07–3.24 ± 1.73 minutes; p = 0.58 (ns)].

Mood ratings underwent no change [GHB: 204 ± 55–211 ± 63; placebo: 196 ± 51–205 ± 59; p = 0.67 (ns); n = 17].

The global therapeutic impression as rated by the patients was significantly more often in favor of GHB ("beneficial effect": 15 patients during GHB treatment versus 2 during placebo; "no beneficial effect": 9 during GHB versus 22 during placebo; p < 0.001).

Carry-over and period effects

Diary and polysomnographic baseline data did not differ significantly between the GHB and placebo groups, nor between the baseline periods 1 and 2 (= washout). Treatment responses showed no significant period effects.

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Tolerability

One patient reported a single period of protracted sleep paralysis in combination with a hypnagogic hallucination in the first week of treatment with GHB. Another patient reported loss of weight in the first 2 weeks of treatment with GHB. One patient reported stranguria during placebo treatment.

DISCUSSION

Gammahydroxybutyrate significantly reduced all narcoleptic symptoms compared to baseline, whereas placebo slightly reduced only the number of cataplectic attacks without influencing any of the other items. The placebo effect on the number of cataplectic attacks was short lasting and may have been related to the disproportionately high baseline frequency in the placebo group.

The clinical effects of GHB in our study are consistent with those observed in earlier open studies (9–11,16). Compared to the only other controlled clinical trial (12), we found milder baseline complaints and a more marked reduction of excessive daytime sleepiness. This may have been because in that particular study patients were selected on the frequency of cataplectic attacks, all anti-cataplectic medication was stopped prior to the study and virtually all patients also used stimulants during the trial.

Gammahydroxybutyrate stabilized nocturnal REM sleep and tended to increase nocturnal SWS. In this respect it seems to (partially) restore the presumed disturbed-state boundary control in narcolepsy (17). The reduction of cataplexy and hypnagogic hallucinations may be a reflection of the stabilization of nocturnal REM sleep, as has been suggested before (18). Compared to the earlier open trials (10,11), the polysomnographic effects in our study are more specific, especially on REM sleep variables.

In the only other double-blind study (13), REM sleep analysis was limited and the variables we found to have changed significantly were not measured. The reported pattern of changes in nocturnal sleep included an increased amount of SWS, a decreased amount of stage 1 sleep and fewer stage shifts and awakenings (13). We found a similar trend, although it was not statistically significant (Table 2).

Our observation that GHB, in contrast to stimulants (19), did not change the mood rating supports the hypothesis that GHB has a selective effect on nocturnal sleep.

Theoretically, the mechanism of action of GHB might have been a purely potentiating effect on co-medication, which was taken by half the study population. However, this possibility is unlikely because patients both with and without co-medication showed similar improvement.

Because the ambulatory MSLT at home was unsupervised, we were not able to reliably assess the effects of GHB on the sleep latency of the MSLT.

In summary, GHB is an effective and well-tolerated treatment for narcolepsy with a pronounced effect on (nocturnal) REM sleep. Although GHB produces hypnotic effects, it does not cause a hangover the next morning. Its clinical use is currently being limited because its short duration of action makes a second dose at night necessary, and because its long-term safety has not been determined (16).

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THE EFFECT OF GAMMA-HYDROXYBUTYRATE: A DOUBLE-BLIND STUDY OF NORMAL SUBJECTS

Odile Lapierre, Monique Lamarre, Jacques Montplaisir and Guy Lapierre
Sacré-Coeur Hospital, Sleep Disorder Center, Montréal, Québec, Canada

GHB is a drug commonly used to treat cataplexy in narcoleptics(1). In these patients, GHB was found to decrease REM latency and REM fragmentation and to increase stages 3 and 4 NREM sleep (SWS). GHB was also found to decrease REM latency and to increase SWS in a small group of insomniac patients of various etiologies, mainly affective disorders(2). In addition GHB also decreases REM latency in animals(3). However, the effect of GHB vs placebo on sleep of normal human subjects has never been documented in a sleep laboratory.

METHOD. Two groups of 6 subjects were studied. The first group (A) included subjects aged 23 to 32 years old ($\bar{X}$=25.1) while subjects of the second group (B), were in the age range of 41 to 63 years old ($\bar{X}$=48.7). Exclusion criteria were the presence of or a past history of any psychiatric illness, the use of psychotropic medications and the presence of any medical condition known to influence sleep. Sleep apnea and PMS were ruled out by all-night polygraphic recordings. All subjects were recorded for two consecutive nights; after the second night, a 2 hour nap was recorded at 10:00 a.m. This procedure was followed after placebo and GHB administration, given in a double-blind fashion. The order of drug administration was reversed in half the subjects of each group. Both the single oral dose of 2,25 gm GHB and the placebo were administered 15 minutes before each recording. Sleep was recorded and scored according to the standard method (Rechtschaffen and Kales 1968). Comparisons between placebo and GHB were performed on the recordings obtained from the second night and nap using paired t-tests.

RESULTS. Nocturnal sleep recording. As seen in Table 1, GHB increased REM efficiency but did not reduce REM latency nor did it increase the percentage of total REM time. However, following GHB administration SWS latency was significatively decreased and the time spent in these stages was significantly increased, especially in the first third of the night. Conversely, GHB decreased the time spent in stage 1 NREM sleep. Similar results were obtained for both groups of subjects except for the improvement of REM efficiency which was found only in subjects of group B.

Diurnal sleep. When GHB was given in the morning, the effect on sleep organization was similar apart from a significant decrease in REM latency in group B ($p<0,02$). No such effect was observed in group A.

TABLE 1 Effects of GHB on nocturnal sleep (group A + B = 12 s's)

		PLACEBO (X ± SEM)	GHB (X ± SEM)	Paired t-test
Total sleep time	(min)	440,2 ± 5,7	432,8 ± 8,3	0,39
Sleep latency	(min)	8,2 ± 2,2	5,3 ± 1,0	0,09
SWS latency	(min)	24,7 ± 3,6	16,7 ± 2,1	0,01*
REM latency	(min)	81,1 ± 10,8	84,3 ± 13,7	0,80
Sleep efficiency	(%)	94,3 ± 0,9	92,7 ± 1,8	0,32
REM efficiency	(%)	73,3 ± 3,5	80,5 ± 2,8	0,01*
Stage 1	(%)	10,1 ± 1,3	8,1 ± 0,8	0,02*
SWS	(%)	10,5 ± 2,2	13,6 ± 2,6	0,03*
REM	(%)	20,2 ± 1,4	17,7 ± 1,6	0,14

DISCUSSION. The effect of GHB on REM sleep. In contradiction to previous observations made in narcoleptic or depressed human subjects and in animals, GHB did not shorten REM latency at night. A significant reduction was found only during the morning recordings of the older group (B); as is known, REM latency shortens with age in normal subjects and REM propensity reaches an acrophase in early morning. These observations suggest that GHB promotes REM sleep in cases where it is already facilitated, such as in narcolepsy, depression and upon morning administration in older subjects.

GHB also improved REM efficiency. This effect was also seen in narcolepsy where it is positively correlated with the anticataplectic action of this drug.

The effect of GHB on SWS. The increase of SWS after GHB has already been reported(1). Some authors have postulated that it represents a drug-induced EEG pattern different from delta waves characterizing SWS. We have no data to further document or reject this hypothesis but it is interesting to note that the increase in delta sleep after GHB is not accompanied by a decrease in stage 2 but by a specific and significant decrease in stage 1 NREM, thus suggesting a shift toward deeper sleep.

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The Effect of Gamma-Hydroxybutyrate on Nocturnal and Diurnal Sleep of Normal Subjects: Further Considerations on REM Sleep-Triggering Mechanisms

O. Lapierre, J. Montplaisir, M. Lamarre, and M. A. Bedard

Centre d'Étude du Sommeil, Hôpital du Sacré-Coeur, Montréal, Québec, Canada

Summary: Gamma-hydroxybutyrate (GHB) is a drug currently used to treat narcolepsy. The present study documents its effect on sleep organization in healthy subjects. GHB and a placebo were given at bedtime and before a morning nap in a double-blind fashion. GHB administered before nocturnal or diurnal sleep increases stages 3 and 4 and decreases stage 1 non-rapid eye movement (NREM) sleep. In additiou, GHB improves REM efficiency at night and reduces wake time after sleep onset when administered before a morning nap recording. GHB also slightly decreases REM latency when administered in the morning, and this effect is correlated with age. Hypotheses regarding mechanisms of action of GHB and the involvement of hypothalamic structures in the regulation of REM sleep are discussed. **Key Words:** GHB—REM sleep—Nap—Hypothalamus.

Gamma-hydroxybutyrate (GHB), a naturally occurring metabolite of the mammalian brain (1,2), produces behavioral and biochemical effects when administered orally. GHB was first used clinically as an anesthetic agent (3). Its current clinical use is restricted to the treatment of narcolepsy.

The effect of GHB on sleep varies from species to species: it has a potent rapid-eye-movement (REM)–inducing effect in cats (4,5), but not in rats (6,7) or rabbits (8). In humans, a shortened REM latency occurs during nighttime sleep after GHB administration in subjects with affective disorders (9) or narcolepsy (10), two conditions in which short REM latencies already exist before treatment (11–13). In healthy subjects, REM sleep facilitation is reported after neither diurnal (14,15) nor nocturnal GHB administration (15).

The aim of the present study was to document further the effect of GHB on sleep in healthy subjects in a double-blind cross-over study. Special attention was given to the facilitation of REM sleep by GHB. REM sleep is known to follow a circadian cycle,

Address correspondence and reprint requests to Dr. Jacques Montplaisir, Centre d'Étude du Sommeil, Hôpital du Sacré-Coeur, 5400 boul. Gouin Ouest, Montréal, (Québec), H4J 1C5 Canada.

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with an acrophase in the morning (16). Furthermore, an inverse relationship between REM latency and age has been reported by several authors (17,18); the effect of age and of time of administration was therefore measured.

MATERIALS AND METHODS

Subjects

Twelve subjects, six men and six women, aged 23–63 years (mean 36.9 ± 3.9), entered the study. All were free of a past history or current symptoms of psychopathology, as well as of any medical condition known to influence sleep. None had taken psychotropic medication during the 6 months preceding the study, and none reported symptoms of narcolepsy or of any other sleep disorder. In addition, sleep apnea syndrome and periodic movements in sleep (PMS) were ruled out by all-night polysomnographic (PSG) recordings.

Drug administration and experimental-procedure

A single oral dose of 2.25 g GHB or placebo was administered in a double-blind fashion 15 min before each nocturnal and diurnal PSG recording. These PSG recordings were performed for two consecutive nights. After the second night, a nap was recorded from 10:00 a.m. to noon. This protocol was carried out under each drug condition, at an interval of 1 week. The order of administration (GHB versus placebo) was reversed for half the subjects.

During each recording session, central electroencephalogram (C3/A2), electrooculogram (EOG), and chin electromyogram (EMG) were recorded. In addition, oral and nasal airflow, respiratory movements, and EMG activity of right and left anterior tibialis muscles were monitored during the first placebo and GHB recording nights.

Sleep was recorded and scored according to a standard method (19) using 20-s epochs. Sleep onset was defined as the first occurrence of three consecutive epochs of stage 1 or one epoch of any other sleep stage. REM latency was measured from sleep onset, and stage REM periods were defined as subsequent epochs of REM sleep not separated by more than 15 min of another sleep stage or of waking. REM efficiency was defined as the percentage of the REM period spent in stage REM. Slow-wave sleep stage (SWS) was defined as stages 3 and 4 non-REM sleep.

Statistical analysis

The results of polygraphic recordings obtained after GHB administration were compared with those obtained after placebo administration using paired t tests. Only results of the second night of each PSG session and those of the nap recordings were considered for statistical comparison.

RESULTS

Nocturnal recordings (Table 1)

GHB had no effect on total sleep time (TST), but the percentage of time spent in SWS (SWS%) increased significantly, whereas the percentage spent in stage 1 (stage 1%) decreased and the percentage spent in stage 2 (stage 2%) remained unchanged. These changes were confined to the first third of the night. Sleep latency decreased, although not significantly, after GHB administration. On the other hand, SWS latency shortened significantly.

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TABLE 1. *Nocturnal recordings (mean ± SEM)*

	Placebo	GHB	*t*-Test
TST (min)	440.2 ± 5.7	432.8 ± 8.3	NS
Sleep latency (min)	8.2 ± 2.2	5.3 ± 1.0	NS
SWS latency (min)	24.7 ± 3.6	16.7 ± 2.1	[b]
REM latency (min)	81.1 ± 10.8	84.3 ± 13.7	NS
WASO (min)	26.4 ± 4.1	34.2 ± 8.6	NS
Stage 1%	10.1 ± 1.3	8.1 ± 0.8	[a]
Stage 2%	59.1 ± 2.4	60.5 ± 1.9	NS
SWS%	10.5 ± 2.2	13.6 ± 2.6	[a]
REM%	20.2 ± 1.4	17.7 ± 1.6	NS
REM efficiency (%)	73.3 ± 3.5	80.5 ± 2.8	[b]
REM 1 efficiency (%)	68.0 ± 5.2	87.5 ± 3.7	[b]
REM 2 efficiency (%)	84.4 ± 3.0	84.0 ± 2.9	NS
REM 3 efficiency (%)	76.0 ± 3.2	72.5 ± 4.6	NS

[a] $p \leq 0.05$.
[b] $p \leq 0.01$.
NS, nonsignificant.

No difference was found in REM latency and the percentage spent in REM sleep (REM%) after GHB administration. REM efficiency increased significantly after GHB; looking at each REM period separately revealed that REM efficiency improved for the first REM period only (REM 1 efficiency). No worsening of REM efficiency was seen during the following REM periods.

No pathological PMS or sleep apnea was found after GHB administration. None of the subjects reported sleep paralysis or hypnagogic hallucinations.

Nap recordings (Table 2)

Nap recordings revealed consolidation of sleep after treatment with GHB, with a significant decrease in wake time after sleep onset (WASO) and percentage of time spent in stage 1 sleep. A nonsignificant increase in percentage of time spent in SWS and reduced REM latency also occurred during morning nap recordings. Individual results revealed that all subjects over the age of 40 ($n = 6$) experienced a marked reduction of REM latency with GHB. Pearson correlation coefficients were calculated between age and changes induced by GHB on each sleep variable listed in Table 2. A coefficient

TABLE 2. *Morning nap recordings (mean ± SEM)*

	Placebo	GHB	*t*-Test
TST (min)	87.5 ± 7.6	106.0 ± 10.5	NS
Sleep latency (min)	10.0 ± 3.4	9.3 ± 3.8	NS
SWS latency (min)	59.6 ± 6.3	39.8 ± 7.5	NS
REM latency (min)	57.6 ± 9.9	41.3 ± 11.3	NS
WASO (min)	20.7 ± 5.6	7.3 ± 2.2	[a]
Stage 1%	19.1 ± 2.5	8.6 ± 2.4	[b]
Stage 2%	62.6 ± 4.2	39.8 ± 7.5	NS
SWS%	6.5 ± 2.2	9.2 ± 2.6	NS
REM%	11.6 ± 3.4	15.6 ± 3.1	NS
REM efficiency (%)	74.7 ± 8.5	78.1 ± 8.1	NS

[a] $p < 0.05$.
[b] $p < 0.01$.
NS, nonsignificant.

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correlation of 0.69 ($p < 0.05$) was found for REM latency, indicating that GHB decreases REM latency with advancing age. Changes of other sleep variables were not significantly correlated with age.

DISCUSSION

GHB and SWS

GHB increased the duration of nocturnal SWS at the expense of stage 1 non-REM sleep. This observation was made previously in normal (14,15), depressed (9), and narcoleptic (10) human subjects, although none of these studies used GHB and placebo in a double-blind fashion. It has been questioned whether delta activity induced by GHB represents physiological SWS or a drug effect, as delta activity was seen also in the waking state (10,14,15). In the present study, no delta activity was seen during wakefulness before sleep onset or upon awakening during the night. Further studies should include EEG spectral analysis during waking in subjects treated with GHB.

GHB and REM sleep

GHB significantly improved the efficiency of the first REM period during nocturnal sleep recordings. During morning nap recordings, there was no significant increase in REM efficiency due to the large standard deviation. GHB reduced REM latency during morning nap recordings, and this effect increases with advancing age. REM sleep shows a strong circadian distribution, with an acrophase in the morning. In subjects submitted to a 90-min sleep–wake schedule, REM periods appear chiefly from 7:30 a.m. to 2:00 p.m. (16). This circadian distribution is also shown by shorter REM latencies during morning sleep recordings (20).

REM latency is also known to decrease with age (17,18). In previous studies of GHB in healthy human subjects, age was either not mentioned (14) or ranged from 20 to 33 years old (15). The effect of GHB on REM sleep, restricted to older subjects upon morning administration thus seems to follow a physiological trend.

Brain mechanisms involved in the regulation of REM sleep

There is general agreement that the neural structures responsible for the major tonic and phasic events of REM sleep are located in the brainstem and that the cholinergic system plays an important role. Most substances known to influence REM latency work through cholinergic mechanisms (21–25). Injection of a variety of cholinergic agonists into various brainstem regions indicates that there is a localized region within the pontine reticular formation from which REM sleep can be most readily triggered (21,26–28). GHB has no cholinomimetic effect and even depresses the activity of cholinergic neurons whose terminals lie in the striatum and the hippocampus (29). Moreover, pretreatment with atropine, a muscarinic anticholinergic agent known to suppress REM sleep when administered alone (30), does not prevent the facilitation of REM sleep following GHB administration (31). REM sleep is also facilitated by a decrease of serotonin (5-HT) or norepinephrine (NE) neuronal activity in the brainstem. However, GHB has little effect on brain 5-HT or NE content in rats (32). More recently, a similar experiment (33) was conducted with γ-hydroxybutyric acid, and no changes in the concentration of 5-HT were observed in the hemispheres, the hypothal-

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amus, or the brainstem, and a decrease of NE content was seen only in the hypothalamus.

There are some indications that in addition to the brainstem cholinergic mechanisms, a more rostral control system, possibly located in the hypothalamus, may modulate REM sleep. Tumor of the rostral brainstem and hypothalamus (34,35), adenoma of the pituitary (36), and surgical lesion involving the perichiasmal hypothalamus (37) have all been found to produce sleep onset REM periods, muscular atonia, sleep paralysis, or hypnagogic hallucinations. These symptoms encountered in narcolepsy may represent dissociated manifestations of REM sleep. There are also physiological indications, based mainly on the work of Jouvet (38), showing a suppression of REM sleep in pontine cats (without "ilot hypothalamique") and restoration of REM sleep after daily administration of posterior and intermediate pituitary extracts. Jouvet proposed that hypothalamic structures, and particularly the arcuate nuclei, synthesize a factor responsible for REM sleep. Dopaminergic neurons are present in the hypothalamus (39) and may be involved in the regulatory mechanism of REM sleep at this level.

GHB mode of action

The exact site of action of GHB is unknown. Some observations favor the brainstem, e.g., the ability of GHB to induce REM sleep in pontine and mesencephalic cats (4) and the presence of GHB binding (40) and synthesis sites (41) in the brainstem. However, a more recent study (42) showed that the brainstem was practically devoid of high-affinity binding sites for GHB.

GHB binding (40,42) and synthesis sites (41) have also been found in the hypothalamus. In addition, intravenous administration of a low dose of GHB (2.5 g) increases the plasma prolactine level (43). This action could be due to the release of the inhibitory control of dopaminergic tuberoinfundibular neurons on prolactine secretion in the hypothalamus. Consequently, hypothalamic structures may mediate the effect of GHB. However, it should be stressed that the highest densities of GHB binding sites are found in various regions of the limbic structure, especially field CA-1 of Ammon's horn (42). This region may play a role in the physiopathology of cataplexy, a symptom triggered by specific emotions and controlled by GHB (10). Further research is necessary to clarify this issue.

The mechanism of action of GHB is not fully understood, but it is known that GHB depresses dopaminergic neurons in the extrapyramidal system (44) as well as in the hypothalamus (39). The exact role of dopaminergic neurons in sleep is still controversial. Several observations suggest that dopamine may have an inhibitory effect on REM sleep. Dopamine receptor agonists reduce or even abolish REM sleep in humans (45) and can prevent the REM rebound that normally follows REM sleep deprivation in cats (46). Intravenous infusion of L-DOPA during NREM sleep delays the onset of REM sleep in humans (47). Dopamine receptors, number of cell bodies, and level of neurotransmitter and biosynthetic enzymes all decrease with age (48). This reduced dopaminergic transmission, along with the decrease in DA release caused by GHB, may explain the shortened REM latency observed in older subjects after GHB.

GHB REM induction test

GHB has a facilitative effect on REM sleep, but this effect seems to be restricted to populations specifically sensitive to REM induction, namely, subjects with narcolepsy or major affective disorders (9,10). It is suggested that response to GHB may help in the

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diagnosis of these two conditions. It may reduce REM latency and even induce SOREMPs during diurnal sleep recordings in these patients.

Recently, GHB was administered to subjects with major affective disorder (MAD) in remission; it markedly reduced REM latency (49). If replicated in a larger sample, this response to GHB may become a diagnostic tool in MAD and may represent a trait marker for this condition.

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BIOCHEMICAL MEDICINE 17, 284–291 (1977)

Evidence for the β-Oxidation of Orally Administered 4-Hydroxybutyrate in Humans

C. R. LEE

M.R.C. Unit for Metabolic Studies in Psychiatry, University Department of Psychiatry, Middlewood Hospital, Sheffield, S6 1TP, England

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INTRODUCTION

γ-Hydroxybutyrate (GHB), a minor metabolite of 4-aminobutyrate (1), occurs in normal mammalian brain (2,3). GHB has hypnotic properties and it is used as an adjuvant in general anesthesia (4,5). ^{14}C-labeled GHB given to animals is converted very rapidly to respiratory $^{14}CO_2$, and it was originally suggested that the compound is catabolized by entry into the citric acid cycle via succinic semialdehyde (6). However, the incorporation into succinate of label from [1-^{14}C]- and [4-^{14}C]GHB given intravenously or intraperitoneally to rats and cats accounts for only a small proportion of the metabolized compound (7,8). This result led Walkenstein and co-workers (8) to propose a β-oxidative pathway (Fig. 1), but there has hitherto been only slight evidence for the formation of any of the postulated intermediates from GHB.

Contrary to earlier findings, Doherty and co-workers obtained substantial labeling of succinate and its amino acid metabolites in rats' brains after intraventricular administration of [1-^{14}C]GHB (9). Very recently, Möhler and co-workers have demonstrated that the labeling pattern in mouse brain after an intravenous injection of [1-^{14}C]GHB can be ex-

$HO\text{-}CH_2CH_2CH_2COOH$ (4-hydroxybutyrate) → $HO\text{-}CH_2(H)C{=}C(H)COOH$ (*trans*-4-hydroxy-2,3-butenoate) → $HO\text{-}CH_2\text{-}CH(OH)\text{-}CH_2COOH$ ((R)-3,4-dihydroxybutyrate) → $HO\text{-}CH_2\text{-}C(=O)\text{-}CH_2COOH$ (4-hydroxy-3-oxobutyrate) → $HO\text{-}CH_2COOH$ (glycollate) + CH_3COOH (acetate)

FIG. 1. Postulated pathway for the β-oxidation of 4-hydroxybutyrate. The intermediates probably react as the CoA esters.

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plained by oxidation of GHB via succinate, but not by β-oxidation (10). However, these findings do not rule out the possibility of β-oxidation occurring in other organs such as the liver.

Previously (11) we have described the occurrence in man of 3,4-dihydroxybutyrate (DHB), one of the intermediates of the postulated β-oxidative pathway of GHB. DHB is one of a number of tetronic and deoxytetronic acids present in human urine (12); Their origin is uncertain, but they are possibly derived from the metabolism of complex carbohydrates.

METHODS

Chemicals

γ-Hydroxybutyric acid (sodium salt) was a gift from Servier Laboratories Ltd., Greenford, Middlesex, and the lactone was obtained from Aldrich Ltd. Tetronic acid was a gift from Dr. J. V. Greenhill (13). *Trans*-4-hydroxy-2,3-butenoic acid was prepared by the method of Laporte and Rambaud (14). Significant peaks in the mass spectrum of the bis-trimethylsilyl derivative, apart from intense peaks of *m/e* 73, 75, and 147 were: *m/e* 246 (36%, M^+); 231 (95%, M^+-15); 230 (28%, M^+-16); 156 (100%, M^+-90); 133 (25%); 131 (12%); and 129 (37%). Other materials were prepared as described before (11).

Analytical Procedures

Details of instrumentation were as described earlier (11), except that the gas chromatograph–mass spectrometer was fitted with a selective ion detector (15). In addition, gas chromatographic profiles of the urine extracts were obtained using an open-tubular glass capillary column coated with OV-101 and fitted with a packed precolumn of 3% OV-101 on Gas-Chrom Q (16). The precolumn was maintained at 250° and the capillary column was programmed from 80–250° at 4°/min, after an initial isothermal period of 10 min.

Urinary acids were extracted and converted to trimethylsilyl derivatives as before (11), but methoxyamine was not used except where stated in the results section.

Experimental Procedure

Four normal adults (two male, two female) collected their urine hourly between 8:00 AM and 3:00 PM on each of 2 days. On one of the days, butyrolactone (1 g dissolved in water) was given at 11:00 AM and the other day was used as a control. The subjects ate the same breakfast on the experimental and control days, and fasted until the end of the day's

experiment. The subjects were healthy volunteers, and they were fully informed of the nature of the experiment.

RESULTS

Formation of Dihydroxybutyrate

Ingestion of GHB as the lactone produced substantial increases in urinary DHB excretion in all four subjects (Fig. 2). None of the subjects excreted more than 1.2 mg/hr on the control days. In a preliminary experiment, GHB given as the sodium salt to two subjects had a similar effect, but as this form of the drug made the urine strongly alkaline, the increased excretion of DHB could have been artifactual (11). The lactone of GHB is rapidly converted *in vivo* to the free acid (7,17).

The chiral configuration of the urinary DHB was determined on three samples collected while the excretion rates were 5–10 times baseline levels. The method (11) involves oxidation of DHB to malate, which is then estimated both enzymatically (for L-malate) and by gas chromatography–mass spectrometry (for total malate). Values of 85, 85, and 95% L-malate were obtained, which indicates that most, if not all, of the DHB derived from GHB has the same *S*-configuration as the endogenous compound. A small proportion of *R*-DHB would not be detected by this method, because approximately 10% of the compound is racemized during the oxidation. Experimental errors could also amount to about 10%.

Formation of Other Intermediates

Gas chromatographic profiles of the urinary acids showed increased excretion of three other compounds besides DHB (Fig. 3). Two of these

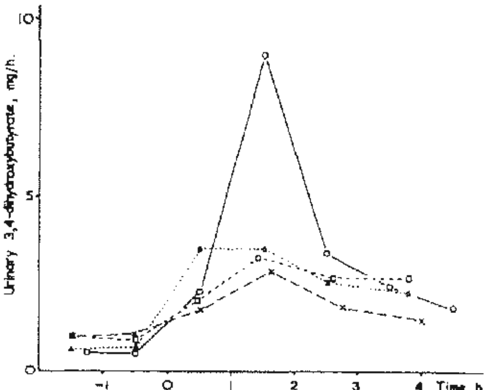

FIG. 2. Urinary excretion of 3,4-dihydroxybutyrate (DHB) by four subjects who received an oral dose of butyrolactone (1 g) at time zero.

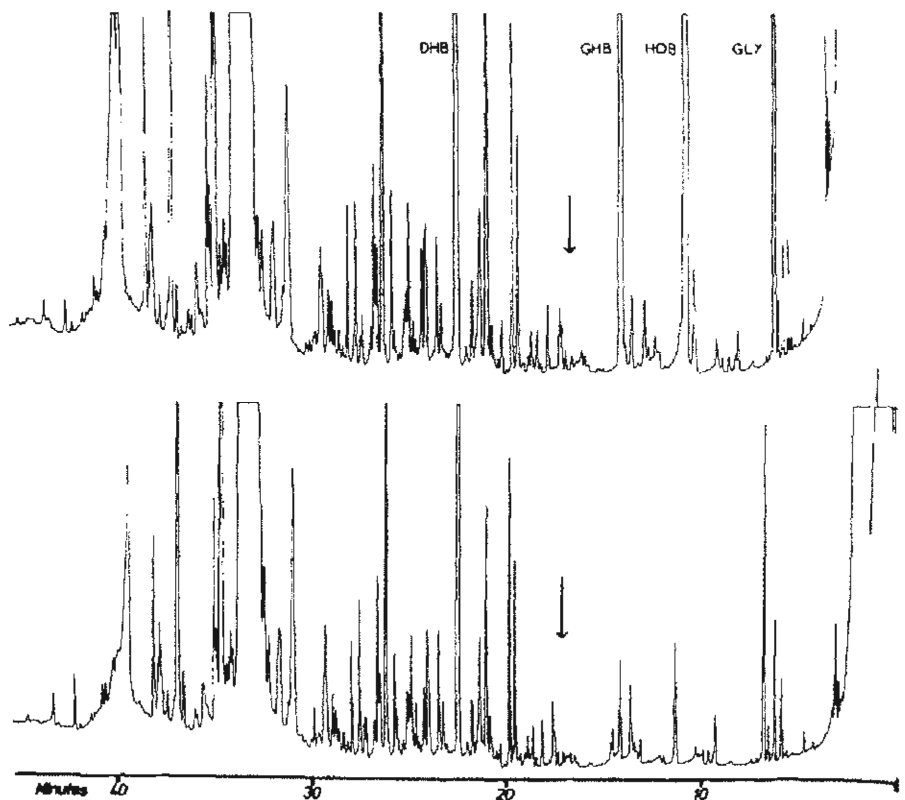

FIG. 3. *Upper trace:* gas chromatogram of urinary organic acids after taking butyrolactone. GLY, glycolate; HOB, 4-hydroxy-3-oxobutyrate (tentative assignment); GHB, 4-hydroxybutyrate; DHB, 3,4-dihydroxybutyrate. *Lower trace:* profile of control urine from the same subject. For conditions see methods section. The arrows indicate the position at which 4-hydroxy-2,3-butenoate was eluted when added to the extract.

were identified by their mass spectra and retention times as glycolic acid, and GHB itself.

The mass spectrum of the third peak is shown in Fig. 4. The ion at *m/e* 247 cannot be the molecular ion since the next fragment is only 14 amu lower. A bis-trimethylsilyl derivative of 4-hydroxy-3-oxobutyrate (HOB) (Fig. 5a) would have a molecular weight of 262 giving *m/e* 247 for $M^+ - 15$.

Supporting evidence for this assignment was obtained by treating a urine sample with sodium borohydride. The HOB peak disappeared, and the DHB peak increased by about 50%. Another sample which was evaporated to dryness at 35°, then taken up in D_2O before treatment with borohydride, gave dideuterated DHB. Similar treatments of a control urine did not change the concentration of DHB. Addition to the urine of methoxyamine HCl also caused the peak to disappear, but no new peak could be found.

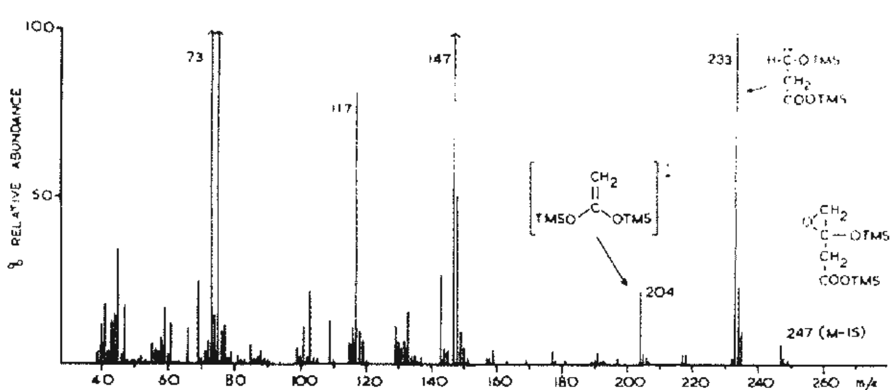

FIG. 4. Mass spectrum of peak HOB in Fig. 3, normalized with respect to *m/e* 233. The spectrum is interpreted in terms of the bis-trimethylsilyl derivative of the hydroxyepoxide tautomer of 4-hydroxy-3-oxobutyric acid.

HOB has not been described before. Its existence, except as a transient intermediate, would be rather surprising since the compound should rapidly and irreversibly lactonize to "tetronic acid"[1] (18, Fig. 5b). The unexpected stability of HOB could be due to formation of the tautomeric hydroxypoxide (Fig. 5c). Jacquier (19) has reviewed evidence that in neutral or acidic solutions, α-hydroxyketones exist only as the hydroxyepoxides, and that this form is quite stable. In Fig. 4 the mass spectrum of the trimethylsilyl derivative is tentatively assigned in terms of this structure, although the aldehyde tautomer of 3-hydroxy-4-oxobutyrate cannot be ruled out. The peak at *m/e* 233 (M-29) could be due to α-cleavage of an aldehyde, or (as illustrated) to transannular cleavage of the epoxide ring, with transfer of hydrogen. This process of cleavage associated with rearrangement occurs in aromatic epoxides and nonterminal epoxyalkanes (20). "Tetronic acid" could not be detected in any of the urines, using high voltage paper electrophoresis at pH 5.3, and spraying with Br_2 then starch/KI (21). A urine sample containing the authentic compound at a concentration corresponding to excretion at 2 mg/hr gave a strong blue spot in the appropriate position.

The unsaturated intermediate *trans*-4-hydroxy-2,3-butenoate was not detected in any of the urines. When added to a urine sample to simulate excretion at 0.1 mg/hr, the synthetic compound gave a well-resolved peak of 5% full scale deflection at the position indicated in Fig. 3. Close inspection of the traces suggests that the rate of excretion was very much less than this, and that the normal rate of production is less than 1 mg/24 hr.

[1] The nomenclature is confusing; The acids formed by oxidizing the aldehydic carbons of the tetroses are sometimes referred to as *erythro-* and *threo-*tetronic acids.

a) 4-hydroxy-3-oxobutyric acid, keto form.

b) "tetronic acid".

c) 4-hydroxy-3-oxobutyric acid, hydroxyepoxide form.

FIGURE 5

DISCUSSION

The increased excretion of glycolate after ingestion of GHB lactone strongly supports the suggestion (8) that GHB is metabolized by a β-oxidative pathway. Although thiolytic cleavage of the intermediate HOB–CoA could be prevented by formation of the hydroxyepoxide tautomer, the pathway would not be blocked completely, as the rate of tautomerization is likely to be lower than that of enzymatic reactions (22). Accumulation of the hydroxyepoxide form of HOB–CoA might account for the production of free HOB hydroxyepoxide.

The 3-hydroxyalkyl-CoA intermediates in β-oxidations invariably have the L-configuration (referred to 3-hydroxybutyrate), which implies that the DHB–CoA derived from GHB should be the R-enantiomer. (The DL notation is ambiguous for DHB (11).) Thus, the S-configuration of the urinary DHB suggests that it is not a primary intermediate of the oxidative pathway, but that it is derived from the reduction of HOB, by analogy with the formation of the "ketone body" D-3-hydroxybutyrate. However, speculation must be postponed until the HOB peak has been more fully characterized. Although the mass spectrum does not rule out the aldehyde 3-hydroxy-4-oxobutyrate, this compound seems less likely on biochemical grounds. Evidence for ring–chain tautomerism (19) is somewhat circumstantial, and investigations using modern physicochemical methods do not seem to have been carried out.

Failure to detect *trans*-4-hydroxy-2,3-butenoate, and R-DHB in the urine is in keeping with the results of studies on the β-oxidation of fatty acids, in which the intermediates have never been isolated as the free acids. On the other hand, in certain inborn errors of metabolism, where the metabolism of acyl–CoA derivatives is blocked, the free acids are excreted in large quantities. Possibly the formation of urinary glycolate is analogous.

The proportion of the ingested GHB lactone (1 g) which appeared as metabolites in the urine can be estimated only roughly, since HOB, glycolate, and GHB were not measured. It is unlikely, however, that the total excretion of these three compounds as well as DHB exceeded 100–200 mg in 5 hr. This agrees with the results of experiments on rats using 1-^{14}C- and 4-^{14}C-labeled GHB, in which most of the radioactivity was excreted as respiratory CO_2 (7,8,23). These results give no information about the percentage of exogenous GHB which is metabolized by β-oxidation, because respiratory CO_2 is the major metabolite of both acetyl–CoA and glycolate (24).

The contribution of the β-oxidative pathway to the turnover of endogenous GHB cannot be determined from these experiments. However, the chromatograms (Fig. 3) show that the urinary excretion of DHB and glycolate by normal adults is much higher than that of GHB. HOB is not detectable by this method, suggesting that urinary *S*-DHB and glycolate are probably derived from precursors other than GHB.

SUMMARY

Four normal adults who took 1 g of butyrolactone excreted increased amounts of *S*-3,4-dihydroxybutyrate and glycolate in their urine. A compound tentatively identified as the hydroxyepoxide tautomer of 4-hydroxy-3-oxobutyrate (the acid whose lactone is tetronic acid) was also excreted. These results provide strong evidence that 4-hydroxybutyrate is metabolized by β-oxidation. *S*-3,4-Dihydroxybutyrate has the same chiral configuration as D(−)-3-hydroxybutyrate, suggesting that its production resembles that of ketone body formation. Two intermediates of the postulated β-oxidative pathway, *trans*-4-hydroxy-2,3-butenoate and *R*-3,4-dihydroxybutyrate were not formed in measurable quantities.

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IMPROVED PHARMACOLOGICAL ACTIVITY VIA PRO-DRUG MODIFICATION: COMPARATIVE PHARMACOKINETICS OF SODIUM γ-HYDROXYBUTYRATE AND γ-BUTYROLACTONE

John Lettieri and Ho-Leung Fung
Department of Pharmaceutics, School of Pharmacy
State University of New York at Buffalo, Amherst, NY 14260

ABSTRACT

Although γ-butyrolactone (GBL) rapidly converts to γ-hydroxybutyrate (GHB) *in vivo*, the lactone gave significantly more prolonged hypnotic effects than GHB when equimolar doses were compared both parenterally and orally in rats. Plasma drug concentrations were higher after GBL administration through both routes, consistent with the observed differences in the pharmacological activity of these two compounds. Oral GBL was absorbed much faster than oral GHB, with the dual effects of decreasing potential first-pass metabolism and elevating plasma drug concentrations to the region where capacity-limited elimination is operative. Parenteral GBL produced a slower initial drug plasma clearance than parenteral GHB. In spite of the rapid metabolism of GBL to GHB, the apparent tissue distribution of these two compounds may be different.

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INTRODUCTION

Sodium γ-hydroxybutyrate (GHB) has been found to be a very useful intravenous anesthetic in man, particularly in obstetric and pediatric procedures (Hunter et al., 1971). When used intravenously, GHB has also been shown to be beneficial in Parkinson's Disease (Boncinelli et al., 1971). Oral dosing of this drug, however, was shown to give decreased and variable activity (Jenney et al., 1962; Metcalf et al., 1966; Laborit, 1964). No improvement in Parkinsonian symptoms was observed even when oral doses of GHB were increased to 8 g/day in humans (Papavasiliou et al., 1973).

Lettieri and Fung (1976, 1978) showed that the oral absorption of GHB is quite extensive in rats. The lack of oral activity was attributed to the relatively slow absorption of this compound. Even at high doses, the plasma and/or brain GHB concentrations did not reach sufficient levels to elicit reproducible and sustained pharmacologic effects after oral administration.

γ-Butyrolactone (GBL), a pro-drug of GHB, appears to have greater oral activity. Following oral administration of GBL to rats, Guidotti and Ballotti (1970) observed much higher blood levels of drug than were attained with orally administered GHB. They also reported that rats given the lactone orally slept for 60-90 minutes, whereas those dosed with oral GHB did not sleep at all. Root (1965) also reported a more rapid onset of sleep with oral GBL in children than with GHB. Jenney (1962) reported that 1.5 g of GBL given orally produced sleep for about one hour.

Evidence has been presented that the blood of various species, including man, contains a lactonase enzyme which catalyzes the

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hydrolysis of GBL to GHB. After intravenous dosing of GBL, Giarman and Roth (1964) attempted to isolate GHB and GBL simultaneously in blood but were unable to detect any significant levels of lactone. In vitro studies have indicated that the half-life of conversion in blood may be as rapid as one minute, but the lactonase activity in liver and brain was found to be less than that of blood (Roth and Giarman, 1966).

Interestingly, intravenously administered GBL also induced a more prolonged period of sleep in rats compared to an equimolar dose of GHB (Giarman and Roth, 1964; Guidotti and Ballotti, 1970; Bessman and Skolnik, 1964). This observation is somewhat surprising in view of the very rapid conversion of GBL to GHB in blood. Detailed comparisons of the pharmacokinetics of these drugs may be useful in understanding the differential pharmacological phenomena observed after GBL and GHB dosing. This study was aimed at characterizing the pharmacokinetics of GBL in relation to those of GHB, both as functions of dose and route of administration.

MATERIALS AND METHODS

Male Sprague-Dawley rats, 260-340 g, were used in all experiments. Prior to drug administration, the rats were fasted for approximately 15 hours. Two doses of GBL and GHB were given: 1.58 mmole/kg (equivalent to 136 mg/kg GBL and 200 mg/kg GHB) and 6.34 mmole/kg (equivalent to 546 mg/kg GBL and 800 mg/kg GHB). Oral doses were administered via gastric intubation to lightly anesthesized animals. Parenteral doses were given intracardially (1.58 mmole/kg) and intravenously (6.34 mmole/kg). Immediately after dosing, the animals were placed in

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restraining cages and blood was collected at various time intervals from the tail vein. Orbital puncture or cardiac puncture was used as an alternate means of blood sampling when tail vein collection did not provide enough blood. The blood was immediately centrifuged and the separated plasma frozen until it was assayed for total GHB according to the procedure previously described (Lettieri and Fung, 1978a). Each dosing group consisted of at least four animals. No animal received more than a single dose.

In a single rat dosed with 6.34 mmole/kg of GBL, blood samples were taken between 0 and 3 hours and assayed differentially for GHB and GBL (Lettieri and Fung, 1978a).

The area under the plasma concentration-time curve (AUC) was determined from the time zero to time infinity for each animal studied. The trapezoidal rule was used for the time period in which data points were collected. For the remaining period, an estimate was obtained based on the observed terminal elimination half-life.

RESULTS AND DISCUSSION

Pharmacokinetic differences between GBL and GHB. The plasma-concentration time profiles obtained after intracardial and oral dosing of 1.58 mmole/kg GBL and GHB are shown in Fig. 1. The concentrations reported were those of total GHB and GBL, because the assay procedure used did not distinguish between GHB and GBL. Since GBL rapidly degrades in blood, it is likely that these concentrations were essentially those of GHB. This point will be addressed to later in this communication.

At the 1.58 mmole/kg level, there was rapid and extensive absorption or oral GBL. The oral/intracardial AUC ratio was 0.85 compared to 0.59

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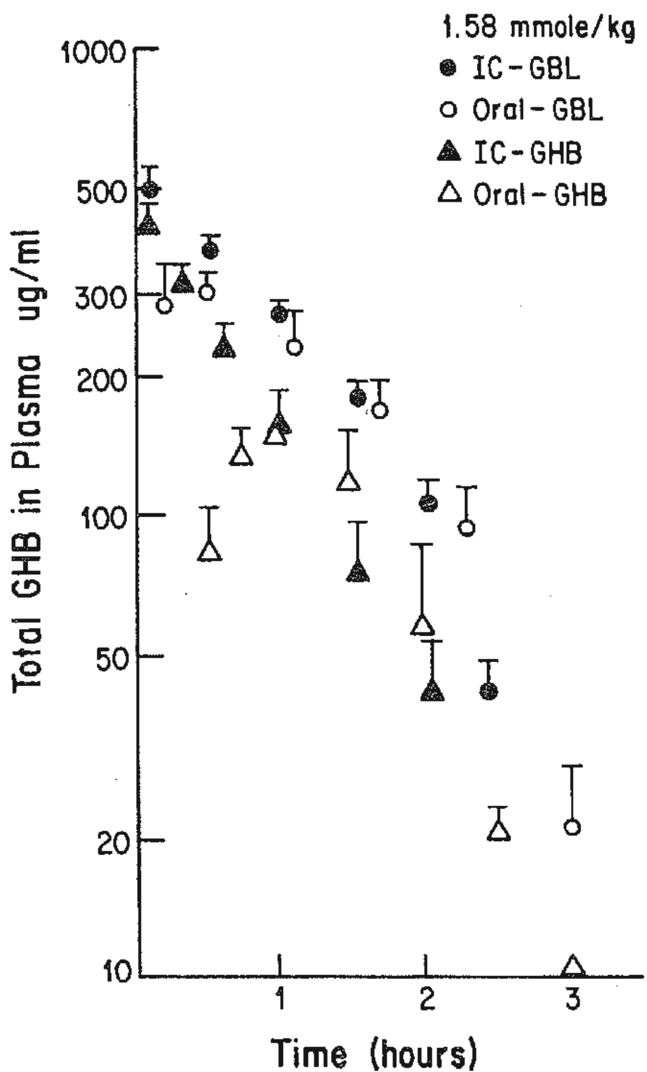

Fig. 1. Plasma concentrations of total GHB following oral and intracardial administration of 1.58 mmole/kg of GHB or GBL. Bars represent standard errors.

for a similar dose of GHB (Table I). The plasma-time curves obtained from two of the rats dosed with GBL actually resembled intravenous curves in that the initial sample had the highest concentration, indicating extremely rapid absorption. Peak levels after GBL were in the order of 350 μg/ml, whereas those following oral GHB at the same dose were never above 200 μg/ml. There was also considerably less variability in the total AUC after GBL than was observed after GHB dosing; the coefficient of variation of the areas was 10% following GBL compared to 33% found with GHB.

TABLE I

AUC ($0 \rightarrow \infty$) values after administration of GHB and GBL to rats

DOSE (mmole/kg)		AREA VALUES[a] (μg-hr/ml) x 10^{-2}	
		GHB	GBL
1.58	oral	2.2 ± 0.7	5.1 ± 0.5
	i.c.	3.7 ± 0.8	6.0 ± 0.2
6.34	oral	16.0 ± 3.2	61.8 ± 21.0
	i.v.	30.6 ± 2.6	59.1 ± 11.9

[a] Mean ± S.D.

The intracardial data also revealed a difference between the kinetics of GHB and GBL. Consistent with previous reports (Giarman and Roth, 1964), the lactone appeared to have a slower initial elimination. At later time points, the elimination half-life of GBL was approximately 0.3 hours, similar to that found with GHB. The AUC following intracardial

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GBL dosing was significantly higher than that calculated for an equimolar dose of GHB ($P < 0.05$).

Similar results were seen at the higher dose level; viz., 6.34 mmole/kg (Fig. 2). Oral administration of GBL at this dose resulted in extremely rapid and virtually complete absorption. The oral AUC relative to the intravenous AUC at this dose of GBL was essentially unity. This compares to an area ratio of 0.52 found with an equivalent dose of GHB (Table I). The comparable levels obtained between the two routes of administration of GBL are quite evident. The dramatic increases in plasma levels achieved by oral administration of GBL compared to oral GHB are also apparent from Fig. 2. In principle, these results concur with those of Guidotti and Ballotti (1970) in that GBL acted as a much more bioavailable and active compound than GHB. However, the actual levels and effectiveness of both GHB and GBL were quite different between their study and ours. After oral or intravenous administration of a 5.8 mmole/kg dose, Guidotti and Ballotti reported peak concentrations in blood of 600-700 μg/ml and sleeping times of 1 to 1½ hours. However, the plasma levels obtained in the present investigation using a 6.3 mmole/kg dose, were greater than 1000 μg/ml for almost three hours following dosing. Also, the rats slept for approximately four hours.

As with GHB (Lettieri and Fung, 1978), the nonlinearity in GBL elimination was very pronounced (Figs. 1 and 2). Increasing the dose from 1.58 mmole/kg to 6.34 mmole/kg resulted in a 2.5-fold increase in the AUC/dose ratio.

Several factors may contribute to the elevated and prolonged plasma levels obtained with GBL. Following oral administration, the absorption rate is so rapid that concentrations are high enough to

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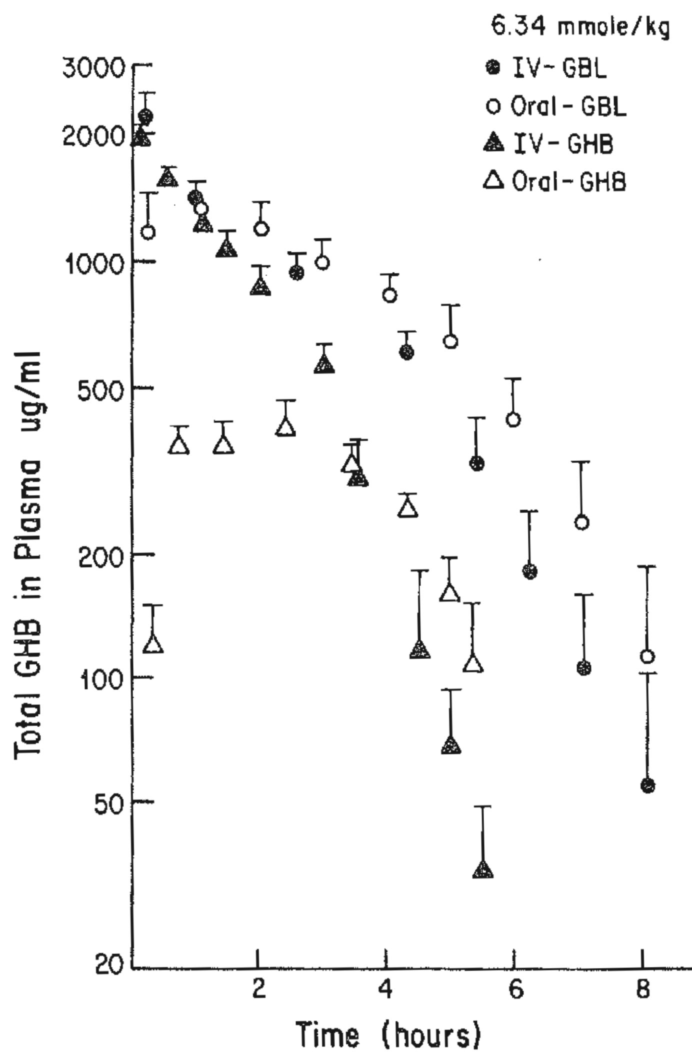

Fig. 2. Plasma concentrations of total GHB following oral and intravenous administration of 6.34 mmole/kg of GHB or GBL. Bars represent standard errors.

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enter the nonlinear range, thereby delaying the apparent elimination. The apparent biological availability in nonlinear systems is also dependent on the absorption rate (Jusko et al., 1976). This could also contribute to the dramatic increases in AUC seen upon oral administration of GBL. The chemical modification from the hydroxy acid to the lactone also apparently offered some protection versus the first-pass metabolism observed for GHB (Lettieri and Fung, 1976). Because absorption of GBL was just as rapid for the high dose as in the low dose, GBL absorption is apparently not impaired by the possible capacity-limited process suggested for GHB absorption (Lettieri and Fung, 1978).

TABLE II

Mean sleeping time (hrs) after GHB and GBL dosing

DOSE (mmole/kg)		GHB		GBL	
1.58	oral	0	(0/4)	0.7	(1/5)
	i.c.	0.1	(3/4)	0.5	(6/6)
6.34	oral	0.5	(1/4)	4.6	(5/5)
	i.v.	2.4	(4/4)	4.7	(4/4)

( ) - indicates fraction of rats which slept in that group

Pharmacological differences between GBL and GHB. Table II compares the sleeping times observed after administration of either GHB or the lactone pro-drug. As might be expected from the plasma levels, the lactone resulted in much more prolonged hypnotic activity relative to GHB at equivalent doses. This is especially notable following oral

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dosing. Even with the 1.58 mmole/kg dose of GBL, one of the animals slept for about 0.5 hours after oral dosing. Oral GHB was devoid of hypnotic activity at this dose. At the 6.34 mmole/kg dose level, all the rats given oral GBL slept for periods comparable to an intravenous dose of GBL. This contrasts with the results seen with GHB. In the case of the acid, 800 mg/kg orally (6.34 mmole/kg) was virtually ineffective as a hypnotic, and even a dose of 1600 mg/kg was only partially effective when given orally. Interestingly, GBL also exhibited enhanced activity relative to GHB even after intravenous dosing, indicating that increased absorption was not the only factor responsible for the pronounced activity of GBL.

In order to explore whether the prolonged activity of GBL might be due to the presence of intact lactone in the bloodstream, the blood from a rat dosed with GBL (6.34 mmole/kg) was assayed for GBL specifically. Most, if not all, of the lactone recovered could be accounted for by the artifactual conversion of GHB during the assay procedure (Lettieri and Fung, 1978a). Because of this uncertainty, it cannot be concluded that there were significant amounts of unchanged GBL in plasma, even at the earliest time points. These results are consistent with the reported rapid conversion of GBL to GHB in blood (Roth and Giarman, 1966).

It has been suggested that the increased activity of GBL might arise from its storage in a tissue depot from which release is relatively slow (Roth and Giarman, 1966). In spite of the rapid hydrolysis of the lactone in blood, the distributive pattern of GBL may be different from that of GHB. This suggestion is supported by several observations. For example, Giarman and Roth (1964) showed that brain levels of drug were about two times higher after GBL administration than after GHB.

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Guidotti and Ballotti (1970) reported similar findings. Roth and Giarman (1964) also found a greater concentration of total drug (GHB + GBL) in muscle when the lactone form was given. Bessman and Skolnik (1964) obtained levels in various tissues following GHB and GBL and found that GBL produced higher concentrations of drug in brain, muscle, heart, blood and kidney, and slightly lower levels in liver.

CONCLUSION

The pronounced hypnotic activity of GBL over that of GHB is consistent with the pharmacokinetic behavior observed for these two drugs. In the rat, the behavioral responses and plasma drug concentrations seen with parenteral GHB can be realized with oral administration of comparable doses of GBL. On a molar basis, the hypnotic activity of GBL is superior to that of GHB regardless of route of administration. GBL appears to be an excellent pro-drug for GHB in that it not only increases the bioavailability but also confers a sustained release characteristic for the drug. It will be of interest to see whether lactone pro-drug of this kind can be equally successful for other hydroxy acids, e.g., prostaglandins.

ACKNOWLEDGMENTS

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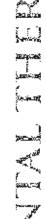

A Specific γ-Hydroxybutyrate Receptor Ligand Possesses both Antagonistic and Anticonvulsant Properties[1]

MICHEL MAITRE, VIVIANE HECHLER, PHILIPPE VAYER, SERGE GOBAILLE, CHRISTOPHER D. CASH, MARTINE SCHMITT[2] and JEAN-JACQUES BOURGUIGNON[2]

Centre de Neurochimie du Centre National de la Recherche Scientifique, 5 rue Blaise Pascal, 67084-Strasbourg Cedex, France

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ABSTRACT

Administration of γ-hydroxybutyrate (GHB) to animals induces electroencephalographic and behavioral changes that resemble petit-mal seizures. Furthermore, these GHB-induced electroencephalogram-behavioral changes can be blocked by anticonvulsant drugs, which are specific in their action against petit-mal seizures. These effects of GHB on electroencephalogram and behavior may well be due to an effect of exogenously administrated GHB on GHB-mediated systems in the brain. GHB has many properties of a neuromodulator including the existence of receptors with a specific affinity for this compound. A synthetic structural analog of GHB, NCS-382, possessed anticonvulsant activity against several animal models of seizure and, in particular, against that induced by GHB administration. NCS-382 was also shown to be an antagonist at GHB receptor sites and blocked the neuropharmacologic effects induced in the striatum and hippocampus by GHB administration. In particular, NCS-382 inhibited the increase in cGMP levels and in inositol phosphate turnover induced by GHB in hippocampus. Furthermore, *in vivo* dialysis demonstrated that NCS-382 blocked the increased release of dopamine in striatum after GHB administration *in vivo*. Thus, this ligand appears to be the first described antagonist substance for GHB receptor(s). These results suggest that NCS-382 may represent a harbinger for a new class of anticonvulsant drugs that most probably act by modifying the endogenous GHB system.

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GHB is a naturally occurring endogenous compound of mammalian brain (Roth and Giarman, 1970) where it has a discrete distribution (Doherty *et al.*, 1978; Snead and Morley, 1981; Vayer *et al.*, 1988a) located principally in the synaptosomal compartment (Snead, 1987) as is its synthetic enzyme (Rumigny *et al.*, 1980, 1981a, b; Weissman-Nanopoulos *et al.*, 1982). *In vitro*, GHB is released from brain tissue in a Ca^{2+}-dependent process by membrane depolarizing concentrations of K^+ or veratridine (Maitre *et al.*, 1983a; Vayer and Maitre, 1988).

These data suggest a role for GHB in neuronal transmission (Vayer *et al.*, 1987b), a hypothesis that is reinforced by the presence in both rat and human brain of specific high-affinity binding sites for this compound (Benavides *et al.*, 1982a; Maitre *et al.*, 1983b; Snead and Liu, 1984). These sites, which are heterogenously distributed in brain tissue, are densely located in the hippocampus and fronto-parietal cortex but are absent in the cerebellum (Hechler *et al.*, 1987). The binding sites appear to represent a class of receptors that mediates the pharmacologic and biochemical effects of GHB *in vivo* and *in vitro*.

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[2] Pharmaceutical chemistry department, Centre de Neurochimie du Centre National de la Recherche Scientifique.

The administration of GHB to various animals induces EEG abnormalities that closely resemble those seen during human absence epilepsy (Winters and Spooner, 1965; Godschalk *et al.*, 1977). This GHB-induced seizure activity is blocked by administration of the anti-petit-mal drugs valproate and ethosuximide (Godschalk *et al.*, 1976); thus it has been proposed that the GHB-treated animal represents a model for generalized absence epilepsy (Snead, 1988). Administration of GHB induces significant changes in the spontaneous firing of dopaminergic neurones in the substantia nigra and an increase in striatal tyrosine hydroxylase activity (Roth *et al.*, 1973; Morgenroth *et al.*, 1976; Roth *et al.*, 1980). In the hippocampus, GHB administration is associated with elevated cGMP levels together with an increase in inositol phosphate turnover, whereas cAMP levels are unchanged (Vayer *et al.*, 1987a; Vayer and Maitre, 1989). These modifications in cellular responses may be biochemical correlations of local depolarizations and could underly the EEG changes induced by GHB administration.

Although GHB possesses these diverse neuropharmacologic and neurophysiologic effects (Snead, 1977), the basic mechanism of action of this ubiquitous substance remains largely unknown. One of the reasons for this is that there was, until now, no specific GHB antagonist available. We now report the

ABBREVIATIONS: GHB, γ-hydroxybutyrate; NCS-382, 6,7,8,9-tetrahydro-5-[H]-benzocycloheptene-5-*ol*-4-ylidene acetic acid; DA, dopamine; Na^+ salt; IC_{50}, median inhibitory concentration; EEG, electroencephalogram; GABA, γ-aminobutyric acid; SWD, spike and wave discharges.

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characterization of such a compound and demonstrate that it is anticonvulsant in the GHB model as well as other models of seizures.

Methods

Synthesis of NCS-382. Equimolecular amounts of benzosuberone and glyoxylic acid hydrate were heated at 90°C in H_3PO_4 (1g of benzosuberone/ml of H_3PO_4) for 15 hr. Classic work-up afforded 6,7,8,9-tetrahydro-S-[H]-benzocyclohepten-S-oxo-4-yliden acetic acid, yield 77%, boiling point 232°C (AcOH). Reduction of the ketonic compound as described previously for the preparation of GHB analog (Bourguignon *et al.*, 1988) afforded the awaited alcohol boiling point 169°C (AcOH). The sodium salt of NCS-382 was prepared by adding to the compound in ethanol, one equivalent of 0.1 N Titrisol NaOH solution. Evaporation of solvents and trituration of the resulting solid with isopropyl alcohol afforded the pure salt of NCS-382 (sodium salt of NCS-382) (fig. 1).

Binding studies. Competitive binding assays were performed as described by Benavides *et al.* (1982a) with some modifications. Briefly, adult male Wistar rats were killed by decapitation and the brains were removed. Hippocampi and striata from 10 animals were rapidly dissected and crude membranes were prepared by homogenization with a Polytron of a crude synaptosomal-mitochondrial fraction (P_2 fraction) in ice-cold deionized water. Membranes were centrifuged at 50,000 × *g* for 20 min and stored overnight at −20°C. For binding assays, membrane suspensions were thawed and resuspended in the incubation medium, which consisted of 50 mM potassium phosphate, pH 5.5, at 0°C containing 5 mM EDTA. For the displacement curves, GHB and NCS-382 concentrations in the assay medium ranged from 10^{-8} to 10^{-4} M, with [^{3}H]GHB 200 nM (100 Ci/mmol). The bound radioactivity was determined after centrifugation and rapid washing of the pellet (Benavides *et al.*, 1982a). Na^+ was omitted in order to avoid interference with the GHB transport system, which is strongly Na^+ dependent (Benavides *et al.*, 1982b). Under these conditions, 500 μM unlabeled GHB displaces about 50% of the bound [^{3}H]GHB.

Hippocampal slice experiments. Hippocampal slices (300 μm) from adult Wistar rat brain were obtained and maintained in oxygenated physiologic medium (Krebs bicarbonate buffer) as previously described by Maitre *et al.* (1983a). The slices were preincubated with various concentrations of NCS-382 and after 15 min, stimulated by 500 μM GHB. Thirty minutes later, cGMP and inositol phosphate levels were measured as described by Vayer and Maitre (1989). Inhibition of hippocampal cellular responses due to GHB in the presence of NCS-382 were calculated as IC_{50} values, computerized by the affinity spectra method of Tobler and Engel (1983).

Inhibition of cGMP increase *in vivo*. GHB causes a time- and dose-dependent accumulation of cGMP in hippocampus (Vayer *et al.*, 1987a). Thus, the effect of NCS-382 on the GHB-induced increase in cGMP level was also tested *in vivo*. Male adult Wistar rats (about 300 g) were administered 2.3 mmol/kg NCS-382 by i.p. injection 60 min before injection of 4 mmol/kg GHB in the same manner. Other animals were treated with either GHB alone or NCS-382 alone; 45 min after the GHB treatment or 105 min after injection of NCS-382 alone, the animals were sacrificed by exposing the head to focused microwave irradiation (7.5 kw, 1.6-sec exposure), which prevents postmortem

OH

O

HO

Fig. 1. Structural formula of NCS-382 (sodium salt of 6,7,8,9-tetrahydro-5-[H]benzocycloheptene-5-*ol*-4-ylidene acetic acid).

changes in cyclic nucleotide levels (Lenox *et al.*, 1982). The dissected hippocampi were placed in liquid nitrogen, weighed frozen, and homogenized in 10 volumes of ice-cold perchloric acid. Protein was removed by centrifugation (20,000 × *g*; 25 min). The supernatants were neutralized with 3 M K_2CO_3 and cGMP contents were determined with the radioimmunoassay kit from Amersham. Protein contents of the different pellets were measured by the method of Lowry *et al.* (1951) after solubilization in 2 N NaOH.

***In vivo* dialysis.** Adult male Wistar rats (250–300 g) were anesthetized with ketamine and mounted in a stereotaxic frame (Narishige). The guide was inserted at coordinates AP 0.5, ML 3.0 and DV 1.8, with Bregma as the reference (flat skull position, according to Paxinos and Watson, 1986) so that after implantation, the 4-mm probe protruded 4 mm beyond this guide, into the right anterior caudate-putamen. The guide cannula was secured to the cranium using dental cement. At the same time, a 2-cm-high and 1.5-cm-diameter cut plastic tube was placed around the guide in order to protect the inlet-outlet tubing from the probe.

Three days after surgery, the rat was lighly anesthetized with ether and the microdialysis probe (polycarbonate-polyether, 20,000 daltons, 4-mm length and 0.52-mm diameter; Carnegie Medicin) was implanted using the guide cannulae. The *in vivo* microdialysis of the striatum was then carried out as described by Ungerstedt and Hallström (1987), the rat being allowed to move freely within a hemispherical bowl (55 cm diameter). The perfusates were collected in small tubes containing 5 μl of 0.5 M perchloric acid and 5 pmol of internal standard (3–4 dihydrocinnamic acid). Samples were collected at 20-min intervals and at least seven control samples were taken before drug administrations. At the end of each experiment, the site of the dialysis tube was verified histologically after fast blue-cresyl violet staining.

The perfusates were assayed for DA and its acid metabolites dihydroxyphenylacetic acid and homovanillic acid by high-performance liquid chromatography as described by Sharp *et al.* (1987). The sensitivity limit of these assays was approximately 10 to 20 fmol.

In order to estimate the recovery of the dopamine and metabolites through the membranes, dialysis probes were perfused *in vitro* at adequate flow rates and placed in physiologic Ringer's solution containing DA, dihydroxyphenylacetic acid and homovanillic acid at 10^{-6} M. The amount of substance in the perfusate was compared with the amount outside the dialysis tube and expressed as percent recovery. The same procedure was used to estimate the amount of GHB perfused into the brain through the probe using [^{3}H]GHB.

Experiments were carried out in parallel to measure the diffusion space of [^{3}H]GHB in the striatum during *in vivo* dialysis: 1 mM [^{3}H] GHB (80 μCi/mmol) was perfused through the dialysis probe during 20 min (1 μl/min). The animal was killed, and the brain was removed and frozen by immersion in isopentane at −40°C for 1 min. The frozen brains were cut in serial sections of 25 μm with a cryostat microtome at −15°C. Anatomical characterization of sections, autoradiographic exposure and computerized image analysis (BIOCOM 500 system) were carried out as described previously (Hechler *et al.*, 1987). The results showed that 1 mM [^{3}H]GHB diffused *in situ* in a volume of about 40 μl around the probe with a yield of about 16%.

Effect of NCS-382 on GHB-induced seizure. GHB administration to rats induces epileptiform, nonconvulsive seizures (Winters and Spooner, 1965; Godschalk *et al.*, 1977). Thus, this model has been used in order to test the antagonistic properties of NCS-382 on the GHB system.

Male Wistar rats (250–350 g) were given implants of three epidural stainless-steel screw electrodes (two for fronto-parietal derivation of the EEG and one as reference) under pentobarbital anesthesia. The electroencephalographic recordings and the administration of GHB (3.2 mmol/kg i.p.) were carried out as described by Depaulis *et al.* (1988). In some experiments, the rats were pretreated with 1.5 or 2.3 mmol/kg i.p. of NCS-382, 45 min before administration of GHB.

Effect of NCS-382 in a genetic model of petit-mal epilepsy. NCS-382 has been tested in the Wistar rat model of spontaneous generalized nonconvulsive seizures (Vergnes *et al.*, 1982). Experiments

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were carried out out as described by Depaulis *et al.* (1988). EEG were recorded for a 2-hr reference period, then NCS-382 (300–800 mg/kg) was injected and the EEG was recorded for up to 2 hr.

Effect of NCS-382 on audiogenic seizures in Swiss Rb mice. The mice of this strain, which are congenitally prone to audiogenic seizures (Frings and Frings, 1953) were given i.p. injections of saline or various dosages of NCS-382 up to 2.3 mmol/kg. After 30 min and at later time intervals, the animals were subjected to an acoustic stimulus of 100 db, 8000 Hz.

Results

In Vitro Studies

IC_{50} for GHB and NCS-382 were determined in binding experiments using crude hippocampal and striatal membranes. Under these conditions, NCS-382 displacement curves exhibit affinity for two populations of binding sites, as does GHB itself (Benavides *et al.*, 1982a; fig. 2, A and B) (best goodness of fit, GRAPHPAD program). For both striatum and hippocampus, NCS-382 showed lower affinity than GHB for the high-affinity population of sites, but greater affinity for the low-affinity component (table 1). NCS-382 (1 and 10 μM) displays no affinity for GABA binding sites as measured under the conditions described by Enna and Snyder (1975) using [^{3}H]GABA as ligand.

The presence of GHB in the incubation medium of rat hippocampal slices led to an increase of intracellular cGMP and inositol phosphates (Vayer and Maitre, 1989). This phenomenon is time- and dose-dependent and might be the result of the stimulation of GHB receptor sites, which are abundant in rat hippocampus (Hechler *et al.*, 1987). Incubation of rat brain hippocampal slices with NCS-382 alone (10^{-7}–10^{-3} M) has no effect on cGMP levels or inositol phosphate accumulation. However, preincubation with this compound dose-dependently antagonized the GHB-evoked increase in cGMP level (fig. 3A) with an IC_{50} value of 30 μM (nonlinear regression fitting, Graphpad program, $r = 0.99$) and blocked the increase in inositol phosphate accumulation (IC_{50} value of 8.6 μM, $r = 0.99$, fig. 3B).

In Vivo Studies

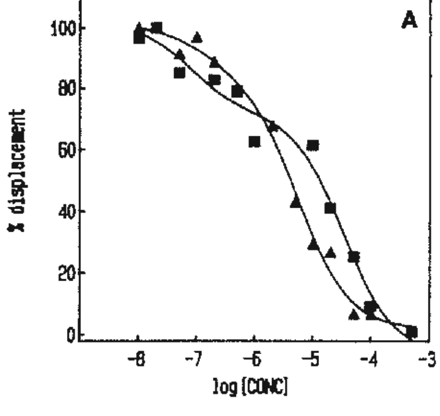
Inhibition of cGMP increase. Administration of NCS-382 alone (2.3 mmol/kg) has no effect on hippocampal cGMP levels compared with animals treated with saline, whereas GHB pretreatment alone (4 mmol/kg) doubled the brain level of this cyclic nucleotide. Pretreatment of the animals with NCS-382 (2.3 mmol/kg) before administration of GHB completely abolished the cGMP increase evoked by GHB alone (fig. 4).

Effects of NCS-382 on GHB-induced release of DA in striatum. Using the *in vivo* microdialysis technique in the caudate nucleus, it was confirmed that the release of DA was increased by local application of GHB through the dialysis probe. By measuring the local [^{3}H]GHB level in tissue after dialysis, it could be calculated that a local cerebral concentration of 120 μM GHB induced a 60% increase in DA release as compared with control rats perfused with physiologic saline. This release could be stimulated by increasing the local concentration of GHB up to 240 μM (fig. 5A). Pretreatment of the animals subjected to *in vivo* dialysis by i.p. injection of 2.3 mmol/kg NCS-382 totally abolished the dopaminergic response induced by local application 60 min later of 120 μM GHB (fig. 5B). Previous experiments had shown that 120 and 240 μM NCS-382 applied directly *in situ* had no effect on DA release.

Prevention by NCS-382 of the electroencephalographic abnormalities induced by administration of GHB. Figure 6 shows the EEG pattern recorded after i.p. injection of 3.2 mmol/kg of GHB in the Wistar rat. After a lag time of about 5 min, numerous epileptiform spikes occur in the EEG (Godschalk *et al.*, 1977). These abnormalities induced by GHB administration are blocked by prior treatment with antipetit-mal drugs (valproate, ethosuximide, trimethadione) (Godschalk *et al.*, 1976). When the rats were pretreated with 2.3 mmol/kg i.p. NCS-382, 45 min before GHB administration, no abnormalities in the EEG pattern were observed (fig. 6D). When the dose of NCS-382 was reduced to 1.5 mmol/kg, GHB administration induced only slight modifications of the EEG (fig. 6C), compared with those obtained with GHB alone (fig. 6B). Thus NCS-382 administered at a sufficient dosage completely antagonizes the hypersynchronous electrocorticogram pattern induced by GHB administration.

Effects of NCS-382 on audiogenic seizures in Swiss Rb mice. NCS-382 (2.3 mmol/kg) fully protected these mice against audiogenic stimuli for approximately 3 to 4 hr. Half of this dosage induced a total protection for 2 to 3 hr. However, 0.57 mmol/kg totally protected only 15% of the animals. Each group receiving one dosage is constituted of 15 animals tested every 30 min after injection (fig. 8).

Attenuation of petit-mal-like seizures spontaneously occurring in a strain of Wistar rats. NCS-382 also reduced the intensity of generalized nonconvulsive seizure in a model of genetically seizure-prone rats. Figure 7 shows the mean



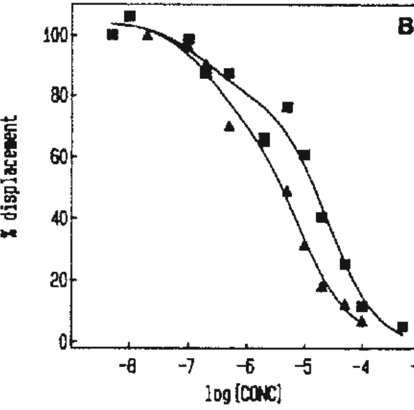


Fig. 2. Displacement curves of [^{3}H]GHB by GHB (■) and by NCS-382 (▲). A: Membranes prepared from rat hippocampus. B: Membranes prepared from rat striatum. Data are derived from three separate experiments performed in triplicate at each concentration (variation <5%). Statistical fitting with GRAPHPAD program.

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TABLE 1
IC_{50} for GHB and NCS-382

IC_{50}	GHB	NCS-382
Striatum		
High-affinity component (nM)	15.5 ± 2.0	134.1 ± 17.4
Low-affinity component (μM)	26.9 ± 0.7	5.5 ± 0.2
r Value	0.99	0.99
Hippocampus		
High-affinity component (nM)	7.1 ± 0.9	201.3 ± 17.4
Low-affinity component (μM)	25.4 ± 0.6	8.5 ± 0.4
r Value	0.98	0.99

The IC_{50}s were calculated from displacement curves using [³H]GHB as ligand (10^{-8}–10^{-4} M). In both cases, NCS-382 is a ligand for the two populations of sites (high and low affinity) described for GHB (Benavides *et al.*, 1982a) (Fitting with GRAPHPAD program calculated from results in fig. 2, A and B.).

duration of SWD during seven successive 20-min periods after i.p. injection of 2 mmol/kg NCS-382. Control EEGs were recorded for 2 hr on each animal just before injection (zero time). The results are expressed in mean accumulative SWD duration for each 20-min period. Experimental *vs.* control conditions (zero dose) were compared for the 2-mmol/kg dose using the Wilcoxon test (fig. 7). After a lag time of about 20 min, a maximal decrease of about 80% in the duration of SWD per 20-min period was observed. This decrease lasted about 60 min, then the duration of spiking episodes slowly returned to control values. In this model, various dosages of NCS-382 were tested (0.75–4 mmol/kg). However, no clear dose effects were observed, the 2-mmol/kg dose being the most effective.

Discussion

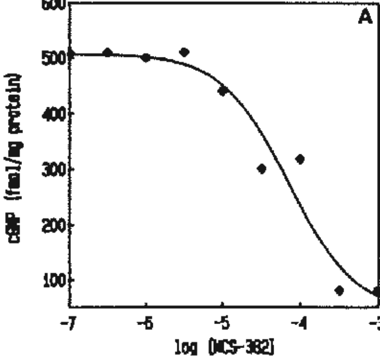
Administration of γ-hydroxybutyrate to animals induces several neuropharmacologic and neurophysiologic modifications (Snead, 1977). In the striatum, the control of dopaminergic terminals activity by γ-hydroxybutyrate consists of a perturbation of cell firing with a modification of DA release and an activation of tyrosine hydroxylase together with an accumulation of this transmitter (Walters *et al.*, 1973; Morgenroth *et al.*, 1976; Roth *et al.*, 1980). In parallel, γ-hydroxybutyrate induces a hypersynchronization of the EEG with numerous epileptiform discharges (Winters and Spooner, 1965; Godschalk *et al.*, 1977). These modifications, which are blocked by anti-petit mal drugs (Godschalk *et al.*, 1976), resemble human petit-mal epilepsy (Snead, 1988). Finally, γ-hydroxybutyrate, at a relatively high dosage, causes a profound sedation with loss of righting reflex and anesthesia (Laborit, 1964).

These modifications, obtained at pharmacologic dosages of GHB, led several authors to consider a possible implication of this substance, which is present endogenously in brain, as a neuromodulator at several types of synapses (Vayer *et al.*, 1987b). The successive findings of specific neuronal synthesis (Rumigny *et al.*, 1981a, b; Weissmann-Nanopoulos *et al.*, 1982), specific transport (Benavides *et al.*, 1982b), release (Maitre *et al.*, 1983a; Vayer and Maitre, 1988) and binding (Benavides *et al.*, 1982a; Snead and Liu, 1984; Hechler *et al.*, 1987) for GHB in brain, in addition to elements of cell responses (modifications in cGMP and inositol phosphates accumulation; Vayer *et al.*, 1987a; Vayer and Maitre, 1989) support the concept of an endogenous synaptic system, using GHB as a neuromodulator that acts on several neuronal populations *via* specific receptors. However, until recently, the lack of a specific antagonist to the effects of GHB was a major hinderance in the search for definitive clues about the GHB system. We have now reported the existence of such a substance that can be considered as a semi-rigid compound structurally related to GHB.

This compound displaces [³H]GHB with a good affinity, but depending on the class of sites considered (high- or low-affinity classes). Because γ-hydroxybutyrate has been considered in the past as a GABA site ligand, we have shown that GHB and NCS-382 bind in fact to specific sites, and possess no capacity to displace GABA binding. NCS-382 is a good ligand for both populations of GHB binding sites but with higher affinity for the low-affinity component. However, the requirement of [³H] NCS-382 is evident in order to precisely estimate these parameters.

The cellular response following GHB stimulation occurs in hippocampus by an accumulation of cGMP and of inositol phosphates *in vitro* (Vayer and Maitre, 1989). The effects on cGMP levels have also been found *in vivo* (Vayer *et al.*, 1987a). These modifications, which do not exist in the other brain regions investigated, are probably the results of local depolarizations induced by GHB (Vayer *et al.*, 1988b). Various epileptic drugs increase cGMP levels before convulsions (Lust *et al.*, 1978) and hippocampus has been implicated in the genesis of various experimental seizures (Wong and Traub, 1983). In addition, iontophoretic applications of GHB in various brain areas frequently induce depolarization phenomena (Kozhechkin, 1980), which could be the basis of GHB-induced EEG abnormalities and of cGMP and inositol phosphate accumulation.

Pretreatment with NCS-382 completely blocks simultaneously the EEG perturbations and the modification of cGMP



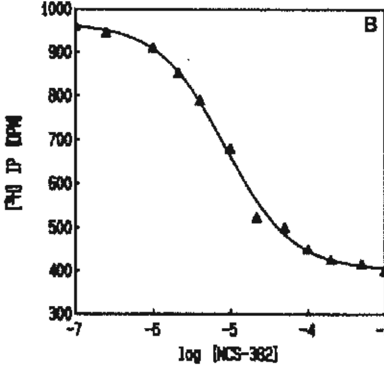


Fig. 3. A: Inhibition by NCS-382 of GHB-induced increase of cGMP in rat brain hippocampal slices. B: Inhibition by NCS-382 of GHB-induced increase in inositol phosphate turnover in rat brain hippocampal slices. Curves were calculated using nonlinear regression fitting (GRAPHPAD program).

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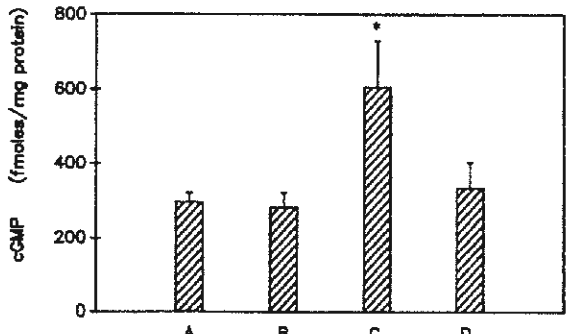
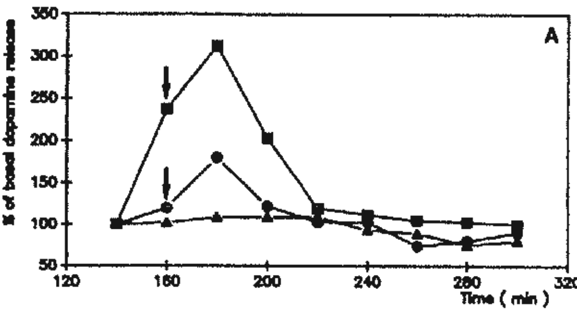


Fig. 4. Effects of NCS-382 on the cGMP increase induced by GHB in rat hippocampi. Animals injected with saline (A); with NCS-382, 2.3 mmol/kg (B); with GHB, 4 mmol/kg (C) and pretreated with NCS-382, 2.3 mmol/kg, (D) 60 min before GHB injection (4 mmol/kg). Each value is the mean of four different determinations ± S.E.M. *P < .005 *vs.* A, B and D (Student's *t* test).



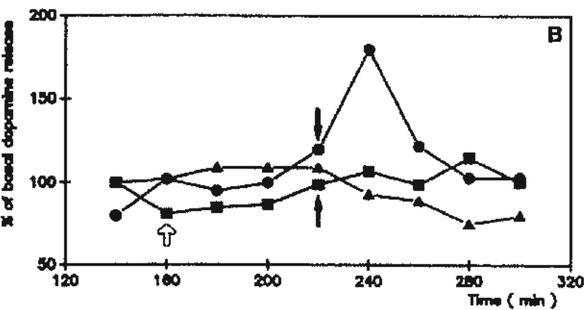


Fig. 5. A: Effects of local GHB administration (▲——▲, control; ●——●, 120 μM; ■——■, 240 μM) on the level of DA in perfusates from the striatum. The results are given as percent of basal DA release (32 ± 2 fmol/min) and are the means of the last three measurements before drug administration. Each value is the mean of three experiments with the S.E.M. not greater than 10% (not indicated). Black arrow: *in situ* administration of Ringer solution containing GHB. B: Effects of NCS-382 administration on the increased dopamine levels in perfusates induced by local GHB administration. The experiments were carried out and represented as in A. ●——●: Effects of GHB local administration (120 μM) at the time indicated by the black arrow. ■——■: Same experiments, but the rats are pretreated i.p. by administration of 2.3 mmol of NCS-382 at the time indicated by the open arrow, then stimulated *in situ* by GHB (black arrow). ▲——▲: Control rats perfused only with Ringer solution.

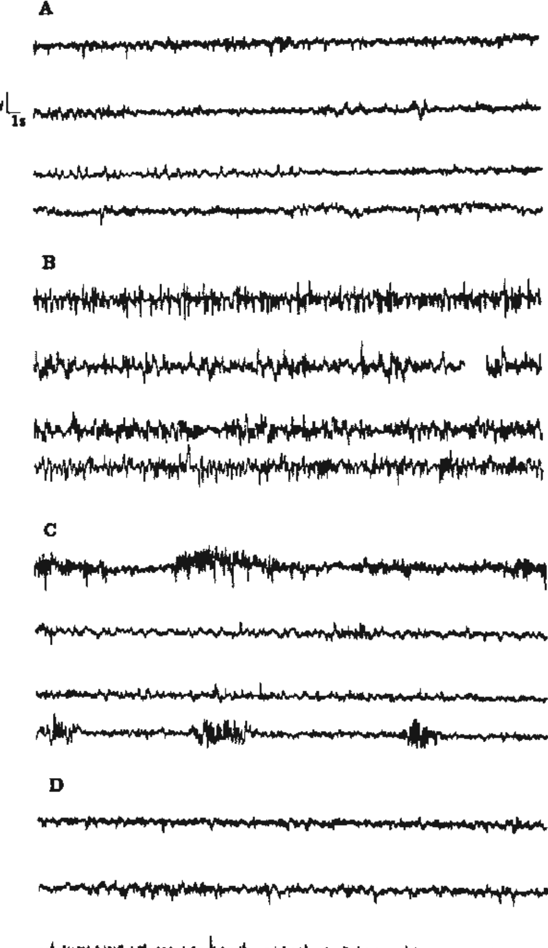


Fig. 6. Effects of GHB (3.2 mmol/kg i.p.) and of NCS-382 (1.5 and 2.3 mmol/kg i.p.) on the EEG of the rat. Each trace represents an individual rat. A: Baseline EEG patterns; B: EEG response to GHB (3.2 mmol/kg i.p.) administered 10 min previously; C: EEG response to GHB (3.2 mmol/kg i.p.) administered 10 min earlier in rats pretreated with NCS-382 (1.5 mmol/kg i.p.) 45 min before GHB injection; D: EEG response to GHB (3.2 mmol/kg i.p.) administered 10 min previously in rats pretreated with NCS-382 (2.3 mmol/kg i.p.) 45 min before GHB injection. The injection of NCS-382 alone had no influence on the EEG.

and inositol phosphate levels in hippocampus. Thus, it could be speculated that NCS-382 antagonizes local depolarization phenomena in hippocampus and thus abolishes EEG spiking.

The IC_{50}s for NCS-382 in inhibiting the cGMP and inositol phosphate increases are within the range of GHB concentration used to induce these phenomena (Vayer and Maitre, 1989). As discussed elsewhere, this rather large concentration of GHB and the high IC_{50} for NCS-382 might be explained by the existence of intermediate circuits mediating the cGMP and inositol phosphate responses after GHB receptor stimulation (Vayer *et al.*, 1988b). These results might also indicate that the low-affinity binding site for GHB is implicated in mediating these effects.

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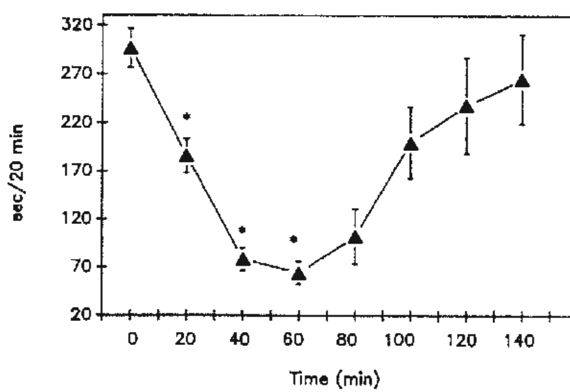

Fig. 6. Effects of various dosages of NCS-382 on audiogenic seizures in Swiss Rb mice.

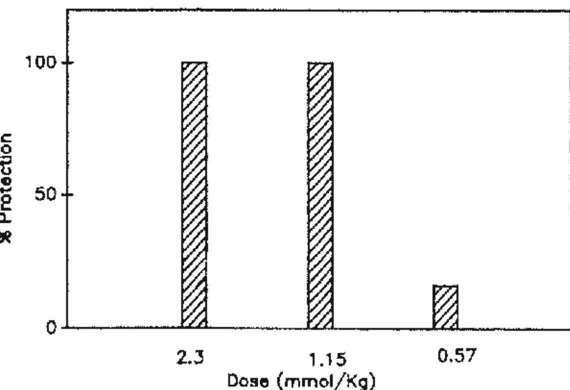

Fig. 7. Effects of NCS-382 in a model of petit-mal epilepsy (Vergnes et al., 1982). Mean duration of SWD in sec during 8 successive 20-min periods, before (0) and after i.p. injection of 2 mmol/kg NCS-382 (20–140 min). For each animal, the SWD duration in sec during the 20-min postinjection periods was measured. The results were expressed in mean accumulative SWD duration for each 20-min period. Experimental vs. control conditions (dose 0) were compared for the 2 mmol/kg dose using the Wilcoxon test (*$P < .01$). Results are expressed as mean ± S.E.M.

Thus, GHB-induced depolarizations appear to be the result of specific receptor stimulation, probably related to the low-affinity binding sites (micromolar range). The availability of specific ligands for both categories of sites (high- and low-affinity) will further help characterization of effects linked to stimulation of these different types of recognition sites.

In addition, NCS-382 reduced the period of spiking episodes in a genetic petit-mal epilepsy model in Wistar rats and completely blocked audiogenic seizures in Swiss albino mice. The petit-mal epilepsy of Wistar rats has been proposed as a model for human absence seizures, and a GHBergic mechanism has been suspected as the basis of these EEG abnormalities (Snead, 1988). However, the rather weak effect of NCS-382 in this model seems to indicate other biochemical mechanisms at the origin of these seizures, or at least, additional mechanisms.

GHB could also be implicated in some grand-mal models of epilepsy, as indicated by its strong action on audiogenic seizures in mice. However, these kinds of seizures appear to be abolished by a great variety of drugs, acting by several biochemical mechanisms (Schlesinger *et al.*, 1968). In striatum, low doses of GHB induce a diminution of impulse flow in the nigrostriatal dopaminergic system with a decrease in cell firing and transmitter release (Roth *et al.*, 1973). However, after this first phase, which is shorter with higher amounts of GHB, a second period begins with tyrosine hydroxylase stimulation (Morgenroth *et al.*, 1976), dopamine accumulation in striatum (Gessa *et al.*, 1968) and a subsequent increase of transmitter release (Cheramy *et al.*, 1977). In our present experiments, the second phase is very rapidly reached, due to local administration of GHB in relatively high concentration (tissue concentration of about 120 μM). Pretreatment of the animals under study by NCS-382 completely blocked the increase in dopamine release observed in control animals administered with GHB *in situ*. Thus the stimulation of dopaminergic activity induced by high doses of GHB (100 μM range) is most probably due to stimulation of GHB specific receptors mediating the dopaminergic response. In this case also, the low-affinity class of GHB receptor could be implicated in the pharmacologic effect of GHB administration.

However, the sedative/hypnotic effect of GHB obtained at a high dosage (7–20 mmol/kg i.p.) do not appear to be significantly attenuated by NCS-382 pretreatment (2–4 mmol/kg i.p.). This effect is possibly mediated *via* other routes, different from the GHB sites under study. Furthermore, the possible interferences of NCS-382 with other neurochemical mechanisms cannot be completely ruled out, because no tests of specificity (except a possible interference with GABA binding) have been carried out. This problem is too great to be dealt with in the present study.

In summary, a specific ligand for GHB binding sites exhibits antagonistic properties against several pharmacologic phenomena due to GHB administration. These results are in favor of specific GHB receptors mediating these effects, which are blocked by administration of NCS-382. This substance could represent a model for a new class of antiepileptic drugs, acting with priority on seizures in which the endogenous GHB neuromodulatory system is implicated.

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Send reprint requests to: Michel Maitre, Centre de Neurochimie du CNRS, 5 rue Blaise Pascal, 67084-Strasbourg Cedex, France.

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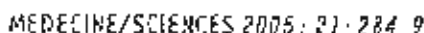
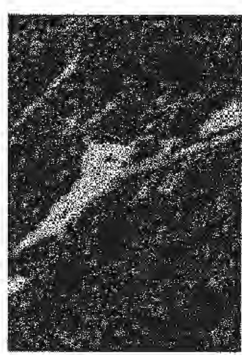


Mécanismes d'action d'un médicament détourné : le γ-hydroxybutyrate

Michel Maitre, Jean-Paul Humbert, Véronique Kemmel, Dominique Aunis, Christian Andriamampandry

> Le γ-hydroxybutyrate (GHB), synthétisé dans les années 1960 pour ses propriétés GABAergiques (GABA, acide γ-amino-butyrique), est une substance qui pénètre facilement et rapidement dans le cerveau. Il induit un sommeil proche du sommeil physiologique, avec un réveil de bonne qualité, et possède des indications en anesthésiologie et dans le traitement des troubles narcoleptiques et de l'addiction à l'alcool. Ses propriétés sédatives, anxiolytiques et euphorisantes ont détourné ce composé de ses indications en thérapeutique, pour une utilisation à des fins récréatives et une consommation illicite. Le GHB à doses pharmacologiques interagit avec les récepteurs $GABA_B$ cérébraux et avec une famille de récepteurs spécifiques, exprimés principalement par le système nerveux central. Cette dernière famille représente l'un des constituants essentiels d'un système GHB endogène qui aurait comme rôle principal de contrôler l'activité de certaines synapses GABA du système nerveux central. <



Institut de Chimie biologique
et Inserm U 575,
Faculté de médecine,
11, rue Humann,
67085 Strasbourg Cedex, France.
maitre@neurochem.u-strasbg.fr

Le γ-hydroxybutyrate (GHB), connu en raison de sa consommation illicite et des intoxications qu'il peut entraîner, est devenu populaire dans les années 1980 auprès des *body builders* californiens, du fait d'actions supposées trophiques et lipolytiques. En réalité, l'absorption de GHB à doses pharmacologiques (plusieurs grammes) entraîne une forte sédation ou un sommeil profond de stade IV qui s'accompagnent de la sécrétion d'hormone de croissance. En raison de ses propriétés sédatives, anxiolytiques et euphorisantes, la consommation de GHB s'est répandue chez les participants aux *rave parties* ; à doses toxiques, les médecins urgentistes et les toxicologues ont pu constater des comas plus ou moins profonds avec, dans de rares cas, une issue fatale liée à son association avec d'autres substances telles que l'alcool, les barbituriques ou la MDMA (méthylène dioxyméthamphétamine, *ecstasy*) [1].

Pour le médecin et le biologiste, l'histoire du GHB commence beaucoup plus tôt, dans les années 1960, date à laquelle le laboratoire de H. Laborit [2] synthétise le GHB dans le but d'obtenir un GABA mimétique capable de franchir facilement la barrière hémato-encéphalique. Ce composé a rapidement été utilisé, surtout en France, dans certaines indications en anesthésiologie. Actuellement supplanté par d'autres médications dans ce domaine, le GHB est utilisé officiellement aux États-Unis et en Europe pour diminuer les attaques de sommeil diurne et les épisodes de cataplexie chez le narcoleptique [3]. En Italie et dans certains autres pays européens, le GHB est employé pour aider au sevrage et à l'abstinence chez le sujet alcoolodépendant. Il fait l'objet d'études cliniques pour le traitement d'autres assuétudes et pharmacodépendances, et pour la réduction des symptômes chez le patient atteint de fibromyalgie.

Le GHB est aussi et avant tout une molécule endogène, présente dans plusieurs organes et dans le sang circulant en quantité micromolaire. Du fait de ses effets psychotropes, ce sont surtout les effets du GHB endogène cérébral qui font l'objet de cet article.

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Le GHB : une molécule signal de la communication synaptique

Dans le cerveau, le GABA est désaminé, puis oxydé ou réduit pour conduire soit à l'acide succinique dans la mitochondrie, soit au GHB dans le cytosol. Il semble que cette dernière transformation soit l'apanage de certains neurones dont la grande majorité expriment également la glutamate décarboxylase (GAD), qui fabrique le GABA à partir de l'acide glutamique. Dans les neurones qui produisent du GHB cohabitent donc la GAD et la SSR (acide succinique semi-aldéhyde réductase), l'enzyme de synthèse du GHB [4]. De localisation cytosolique comme la GAD, la SSR est donc le marqueur des neurones GHB (*Figure 1*). Il semble exister plusieurs isoformes de cette SSR, dont plusieurs ont été clonées. À part le GABA, d'autres précurseurs mineurs endogènes du GHB ont été identifiés (γ-butyrolactone et 1,4 butanediol), mais il semble que ces voies aient peu d'importance fonctionnelle *in vivo*.

Le GHB synthétisé dans le cerveau possède un temps de renouvellement rapide (entre 9 et 30 minutes suivant les régions cérébrales) [5] et est distribué de façon hétérogène en quantité micromolaire (1 à 20 µM). Il a été récemment démontré que le GHB était capturé de façon active par une population de vésicules synaptiques et que ce transport était inhibé par le GABA et la glycine [6]. Cela conforte l'idée que le GHB est synthétisé et accumulé dans les vésicules synaptiques de neurones GABAergiques exprimant à la fois la GAD et le VIAAT (*vesicular inhibitary aminoacid transporter*).

Le GHB est présent dans le milieu extracellulaire cérébral à des concentrations encore inconnues. Des études ont montré qu'il était libéré dans ce milieu du fait de la dépolarisation neuronale induite par la vératridine ou les ions potassium, par des mécanismes inhibés par la tétrodotoxine ou les agents complexant les ions Ca^{2+} [7]. Participant à la régulation des concentrations extracellulaires de GHB, une capture active de cette substance a été mise en évidence dans des lignées neuronales ou des synaptosomes. Ce transport possède un Km de 35 à 46 µM et est dépendant du gradient de Na^+/Cl^- [8]. Différents analogues structuraux du GHB, dont le GABA lui-même, interfèrent avec ce transport; la sélectivité de ces inhibiteurs n'a toutefois pas encore été testée [9].

Le GHB endogène du cerveau possède une famille de récepteurs propres

Depuis 1982, on sait qu'il existe pour le GHB, dans le cerveau de rat, un ou plusieurs sites de fixation membranaire, saturable et réversible [10]. Les valeurs des affinités du GHB radioactif pour ces sites sont compatibles avec les concentrations de GHB endogène. Ces sites de fixation sont de distribution hétérogène, avec un maximum dans les couches superficielles du cortex, dans l'hippocampe et dans les bulbes olfactifs de l'animal. Le striatum, le thalamus et certains noyaux dopaminergiques du mésencéphale expriment une densité moyenne de sites [11]. En revanche, il n'a pas été possible de mettre en évidence une fixation de GHB radioactif de forte affinité dans l'hypothalamus et les régions caudales du cerveau (à l'exception d'une expression très faible dans le cervelet). Durant le développement chez l'animal, ces sites de fixation pour le GHB apparaissent après les sites récepteurs $GABA_B$ et possèdent une pharmacologie spécifique [12]. Seuls les analogues structuraux synthétiques du GHB déplacent le GHB radioactif de ses sites, les ligands $GABA_A$ et $GABA_B$ n'ayant pas d'effets.

La fixation du GHB sur ses sites membranaires semble dépendre du couplage de ces sites avec des protéines G, car l'affinité pour le GHB est significativement altérée par un traitement des membranes par la toxine pertussique ou un analogue non hydrolysable du GTP [13]. Un premier récepteur GHB a été cloné récemment à partir d'une banque d'ADNc d'hippocampe de cerveau de rat [14]. Les algorithmes utilisés pour prédire sa structure proposent 5 à 6 domaines transmembranaires, et 7 domaines si on impose quelques faibles contraintes de modélisation. Par ailleurs, la séquence

25 µm
GAD + SSR

Figure 1. *Localisation de la SSR et de la GAD dans la substance noire réticulée chez le rat.* La détection effectuée par immunofluorescence indirecte marque la SSR (acide succinique semi-aldéhyde déshydrogénase) en vert (CY3) et la GAD (glutamate décarboxylase) en bleu (CY5). Les cellules SSR positives sont dans 70 % des cas également GAD-positives. Un réseau dense de fibres GAD-positives coexiste avec un réseau plus discret de prolongements contenant de la SSR. Dans les cellules qui n'expriment que la SSR, la synthèse de GHB pourrait se faire à partir du GABA transporté de l'espace extracellulaire.

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du récepteur comporte un site consensus pour la fixation de protéines G sur une boucle intracellulaire. Enfin, des expériences de *patch-clamp* réalisées sur des cellules CHO transfectées par le récepteur et stimulées par le GHB expriment une activation irréversible sous l'effet du GTP-γ-S : il semble donc s'agir d'un récepteur couplé à une protéine G (RCPG). Une partie de la molécule présente une forte homologie avec la tétraspanine de type 5 issue du cerveau de rat *(Figure 2)*. La stimulation par le GHB induit une réponse en *patch-clamp* sur des cellules hétérologues transfectées, avec une EC_{50} de 4 μM. Néanmoins, ce récepteur ne possède pas d'homologie significative avec les autres classes de RCPG connus, et n'a pas été retrouvé chez d'autres espèces comme l'homme ou la souris. De plus, il n'est pas sensible à l'antagoniste NCS-382, qui est le seul antagoniste actuellement connu capable de bloquer certains des effets électrophysiologiques et neuropharmacologiques du GHB *in vivo* ou en culture de cellules. De ce fait, il a été postulé l'existence de deux grandes classes pharmacologiques de récepteurs GHB, l'une sensible et l'autre insensible au NCS-382. Notre équipe a d'ailleurs isolé, à partir du cerveau humain, une autre famille de récepteurs GHB dont certains membres sont sensibles à l'action inhibitrice du NCS-382.

Les sites récepteurs cérébraux du GHB se désensibilisent facilement. La plupart des résultats dans ce domaine ont été obtenus en explorant l'évolution des densités de sites de liaison pour le GHB après hyperstimulation. La présence de fortes concentrations de GHB (500 à 1000 μM) soit chez l'animal, soit dans le surnageant d'une culture de neurones, induit très rapidement une diminution de la densité des sites de fixation membranaire pour le GHB [15]. Cette baisse est réversible après élimination du GHB. Du point de vue de la réponse cellulaire, les mouvements de ^{86}Rb induits par de faibles concentrations de GHB (5 à 25 μM) dans la lignée NCB-20 disparaissent rapidement dès que les concentrations de GHB atteignent 50 à 100 μM [16]. Enfin, des expériences de *patch-clamp* réalisées sur certains des récepteurs GHB déjà clonés ont montré un épuisement de la réponse au GHB dès la deuxième application de cette substance. Ces résultats pourraient expliquer qu'aux doses thérapeutiques ou récréatives de GHB utilisées chez l'homme, une partie au moins des récepteurs GHB évoluent vers une activité fonctionnellement très réduite.

Quel rôle physiologique pour le GHB cérébral ?

Compte tenu des connaissances actuelles, le GHB endogène du cerveau, en quantité micromolaire, est suffisant pour solliciter les récepteurs décrits précédemment, à l'exclusion de tout autre type de récepteurs. Bien que l'on ne connaisse pas les concentrations de GHB dans le milieu extracellulaire, les expériences de neurochimie ou de neuropharmacologie fonctionnelle pratiquées *in vivo* ou sur des coupes de cerveau en survie ont montré que l'application de quantités micromolaires de GHB induisait des modifications de certains seconds messagers. Des inhibitions d'adénylate cyclase [17] et des stimulations de la cascade Ca^{2+}/NO/GMPc [18, 19] ont notamment été décrites. La lignée de neuroblastome NCB-20 a été particulièrement étudiée à cet égard, car elle exprime la plupart des marqueurs du système GHB (enzyme de synthèse, transporteur[s], certains sites récepteurs, libération dépendante du Ca^{2+} de GHB induite par la dépolarisation...)

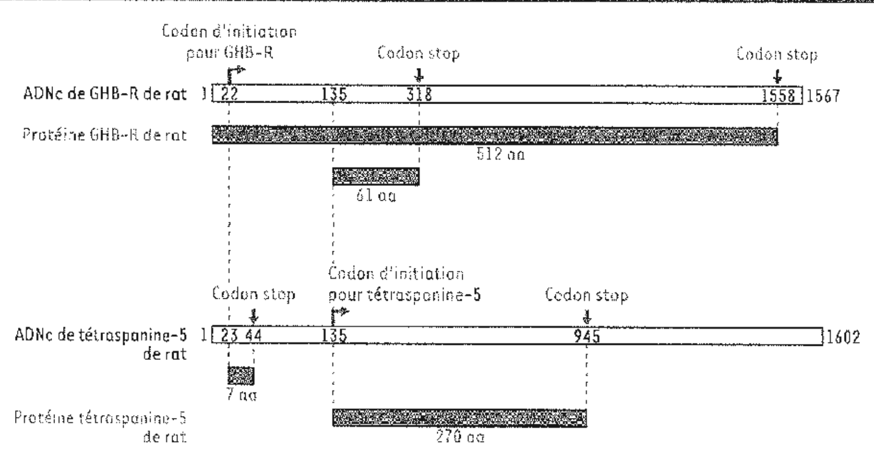

Figure 2. ***ADNc de GHB-R et de la tétraspanine, et protéines GHB-R et tétraspanine.*** L'homologue humain du récepteur GHB (GHB-R) de rat n'a pas encore été identifié. Il existe, cependant, une très forte homologie (sur 105 acides aminés) entre le GHB-R et la tétraspanine-5 (4TMSF) de rat. Un certain nombre d'insertions et de délétions dans la séquence nucléotidique conduisent à un décalage de la phase de lecture et, par conséquent, à la production de deux protéines différentes (512 aa pour GHB-R contre 270 aa pour la tétraspanine-5). En effet, si l'on considère le codon d'initiation pour GHB-R (→) pour les deux ADNc (position 22 pour le GHBR et 23 pour la tétraspanine-5), cela conduit à la synthèse d'une protéine de 512 aa (GHB-R, en rouge) et un peptide de 7 aa (en rouge). Mais si le codon d'initiation pour la tétraspanine-5 (→) est considéré (position 135 pour les deux ADNc), il y a formation d'un peptide de 61 aa (en bleu) et d'une protéine de 270 aa (tétraspanine-5) (en bleu) ; aa : acides aminés.

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lorsqu'elle est différenciée par l'AMP cyclique [20]. Dans cette lignée, l'application de faibles quantités de GHB module des perméabilités membranaires pour des ions Ca^{2+} et K^+, en fonction du potentiel de membrane. Ces modifications de conductance sont bloquées par l'antagoniste NCS-382 [21].

D'une façon générale, l'application de quantités micromolaires de GHB dans divers modèles induit une hyperpolarisation neuronale. Ce phénomène est confirmé par les expériences de microdialyse menées *in vivo* chez l'animal éveillé. Lorsque les quantités de GHB administrées soit localement (*via* la sonde de dialyse), soit à la périphérie (voie intrapéritonéale généralement) sont faibles (de telle façon que les concentrations de GHB cérébral ne s'élèvent pas durablement à des niveaux supérieurs à environ 100 μM), on observe généralement une inhibition de la libération des transmetteurs étudiés (GABA, glutamate, dopamine surtout) [22, 23]. Cela est cohérent avec l'installation d'une hyperpolarisation neuronale et une inhibition de l'entrée présynaptique des ions Ca^{2+} dans la terminaison dépolarisée.

Compte tenu des arguments formulés en faveur de la synthèse et de la libération du GHB par des neurones synthétisant et libérant également du GABA, ce GHB, co-libéré avec le GABA, pourrait participer à la régulation de l'activité de la synapse GABAergique *via* un récepteur GHB présynaptique, la sollicitation de ce récepteur ralentissant l'activité de la synapse (*Figure 3*).

Un mécanisme pour les effets thérapeutiques et récréatifs du GHB

Que ce soit pour ses effets thérapeutiques ou récréatifs, l'induction d'un effet neuropharmacologique par le GHB nécessite l'absorption d'une grande quantité de cette substance (200 mg/kg au minimum chez le rat, 2 à 3 grammes chez l'homme). Ces doses de GHB se répartissent en quelques minutes dans l'organisme, y compris dans le cerveau. Malgré un métabolisme rapide, les concentrations de GHB cérébral atteignent très vite au minimum 300 μM pendant plusieurs dizaines de minutes. Pourquoi faut-il des concentrations de GHB aussi élevées pour obtenir un effet neuropharmacologique, alors que les récepteurs GHB sont saturés à ces concentrations? Trois explications sont possibles à ce niveau, et il est possible que toutes les trois concourent au mécanisme d'action du GHB.

De fortes concentrations cérébrales de GHB sont nécessaires pour désensibiliser les récepteurs correspondants

Les expériences de microdialyse *in vivo* conduites chez le rat montrent que, à la suite de l'administration de quantités de GHB nécessaires et suffisantes pour induire un effet neuropharmacologique, les concentrations de glutamate, de GABA ou de dopamine (pour se limiter à ce qui a été étudié) dans le milieu extracellulaire fluctuent selon un mode biphasique. Après une première période courte (10 à 20 minutes) pendant laquelle la libération de ces transmetteurs est réduite par le GHB, une seconde période s'installe où, pendant 60 à 120 minutes (selon la dose de GHB

Figure 3. *Organisation d'une synapse mixte GABA/GHB.* Le neurone présynaptique contient à la fois la glutamate décarboxylase (GAD), qui synthétise le GABA, et l'acide succinique semi-aldéhyde réductase (SSR), qui synthétise le GHB. Le GABA et le GHB sont accumulés dans les vésicules synaptiques par le même transporteur (VIAAT, *vesicular inhibitory aminoacids transporter*). Il existe donc une co-libération des deux transmetteurs, le GHB, se liant avec un récepteur peut-être présynaptique, exerçant un rétrocontrôle de l'activité de la synapse. L'administration périphérique de grandes quantités de GHB augmente considérablement les concentrations de GHB extracellulaire, ce qui stimule les récepteurs $GABA_B$ et désensibilise les récepteurs GHB. Une désensibilisation des récepteurs $GABA_B$ n'a jamais été rapportée jusqu'à présent. Ces modifications retentissent sur l'activité post-synaptique (ici un neurone dopamine). αKG: α-cétoglutarate ; GDH : glutamate déshydrogénase; Glu: glutamate; GABA-T: GABA transaminase ; SSADH : acide succinique semi-aldéhyde déshydrogénose; SA: acide succinique.

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administrée), la concentration de ces mêmes neurotransmetteurs augmente largement au-dessus des concentrations basales [24, 25]. Ce rythme biphasique semble indiquer que, après une période courte d'hypersollicitation des récepteurs GHB (qui installent une hyperpolarisation neuronale et une chute de la libération des neurotransmetteurs étudiés), succède une période de désensibilisation de ces mêmes récepteurs GHB avec dépolarisation neuronale et libération accrue de GABA, dopamine et glutamate. Ce phénomène se produit dans la plupart des régions cérébrales étudiées. Le rôle bénéfique du GHB en thérapeutique ou son utilisation comme substance récréative serait donc la conséquence d'une adaptation des circuits GHB endogènes contrôlant la libération de certains neurotransmetteurs à la suite de l'augmentation importante des taux de GHB cérébral.

À fortes concentrations, le GHB cérébral active également les récepteurs $GABA_B$

Plusieurs études font état de la participation des récepteurs $GABA_B$ au mécanisme d'action neuropharmacologique du GHB. Cette conviction repose sur deux types de résultats : d'une part, le GHB à des doses variables (IC_{50} = 150 µM pour la plus faible valeur, jusqu'à IC_{50} = 3 à 5 mM pour les valeurs les plus fortes) [26] déplace *in vitro* le GABA ou le baclofène radioactif de leurs sites de fixation $GABA_B$. D'autre part, de nombreuses situations expérimentales (*in vivo* ou *in vitro*) montrent que les effets neuropharmacologiques du GHB sont exclusivement bloqués par les antagonistes $GABA_B$ et n'existent pas chez les souris dont les gènes codant pour ces récepteurs ont été invalidés [27]. Sur la base de ces résultats, le rôle des récepteurs GHB dans la médiation de ses effets pharmacologiques apparaît obscur. Il semble néanmoins que l'on puisse mettre en évidence des effets proprement GHB (*via* des récepteurs GHB) en bloquant au préalable les sites $GABA_B$ [28]. Le caractère massif de l'effet $GABA_B$ masquerait les effets du GHB *via* ses propres récepteurs. De plus, ces derniers participent très probablement à la réponse pharmacologique puisque des ligands spécifiques (dont l'antagoniste NCS-382) possèdent des effets propres alors qu'ils n'ont pas d'affinité pour les récepteurs $GABA_B$ [29]. Une autre hypothèse, déjà mentionnée plus haut, serait de postuler une participation des récepteurs GHB dans la régulation de l'activité de certains circuits GABAergiques stimulant des récepteurs $GABA_B$ *(Figure 3)*. La disparition de ces derniers récepteurs abolirait les effets pharmacologiques du GHB. Enfin, il faut constater que les effets pharmacologiques du GHB et du baclofène ne sont pas identiques et qu'ils possèdent des indications thérapeutiques bien distinctes [30].

L'administration de GHB à fortes concentrations peut-elle servir de précurseur à un compartiment de GABA endogène ?

La dégradation du GHB cérébral comporte son oxydation en acide succinique semi-aldéhyde (SSA). Le devenir de cette substance est classiquement son oxydation en acide succinique (SA). Certaines études proposent qu'une partie de ce SSA serve à la fabrication de GABA *via* la GABA transaminase [31]. Le rôle de ce compartiment de GABA formé à la suite de la prise de GHB pourrait être la stimulation de certaines classes de récepteurs GABA, dont les $GABA_B$ *(Figure 4)*.

Conclusions et perspectives

Les données disponibles semblent indiquer que le GHB, *via* ses propres récepteurs et les récepteurs $GABA_B$, participe à la régulation des influences GABAergiques dans le cerveau. Le système GHB endogène

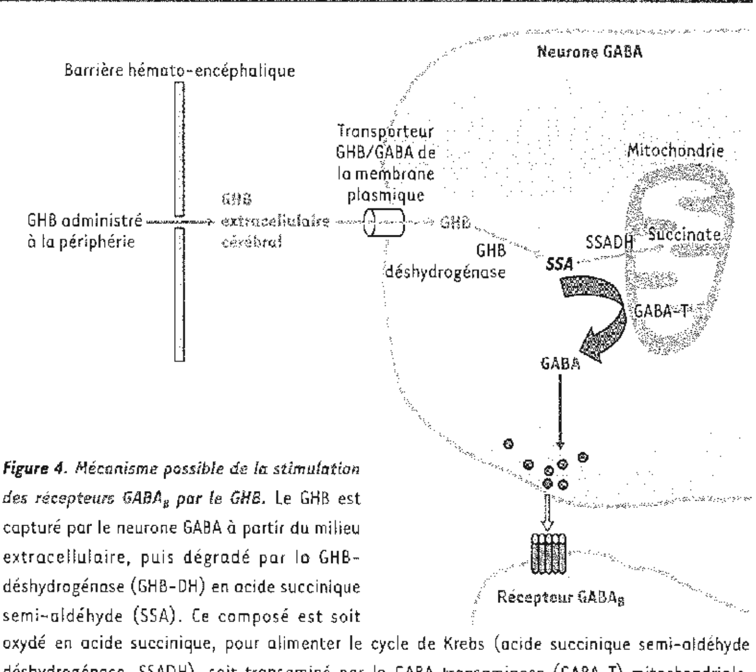

***Figure 4.** Mécanisme possible de la stimulation des récepteurs $GABA_B$ par le GHB.* Le GHB est capturé par le neurone GABA à partir du milieu extracellulaire, puis dégradé par la GHB-déshydrogénase (GHB-DH) en acide succinique semi-aldéhyde (SSA). Ce composé est soit oxydé en acide succinique, pour alimenter le cycle de Krebs (acide succinique semi-aldéhyde déshydrogénase, SSADH), soit transaminé par la GABA transaminase (GABA-T) mitochondriale, pour produire un compartiment de GABA spécifique qui pourrait être utilisé pour la stimulation de récepteurs $GABA_B$.

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constitue un ensemble de cibles nouvelles pour des substances modulant l'activité GABA du cerveau. Il est intéressant de signaler à cet égard que des ligands synthétiques, ligands exclusifs des récepteurs GHB, modulent la vigilance, l'anxiété et le sommeil chez le rat. ◊

SUMMARY

A mechanism for γ-hydroxybutyrate (GHB) as a drug and a substance of abuse

γ-hydroxybutyrate (GHB) is mainly known because of its popularity as a drug of abuse among young individuals. However this substance increases slow-wave deep sleep and the secretion of growth hormone and besides its role in anaesthesia, it is used in several therapeutic indications including alcohol withdrawal, control of daytime sleep attacks and cataplexy in narcoleptic patients and is proposed for the treatment of fibromyalgia. GHB is also an endogenous substance present in several organs, including brain where it is synthesized from GABA in cells containing glutamic acid decarboxylase, the marker of GABAergic neurons. GHB is accumulated by the vesicular inhibitory aminoacid transporter (VIAAT) and released by depolarization *via* a Ca^{2+} dependent-mechanism. A family of GHB receptors exists in brain which possesses hyperpolarizing properties through Ca^{2+} and K^{+} channels. These receptors - one of them has been recently cloned from rat brain hippocampus - are thought to regulate GABAergic activities *via* a subtle balance between sensitized/desensitized states. Massive absorption of GHB desensitize GHB receptors and this modification, together with a direct stimulation of $GABA_B$ receptors by GHB, induce a perturbation in GABA, dopamine and opiate releases in several region of the brain. This adaptation phenomenon is probably responsible for the therapeutic and recreative effects of exogenous GHB. ◊

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TIRÉS À PART

M. Maitre

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operation or post-mortem examination. The histology was available in 48 patients, and 10 others with malignant cytology, together with radiological bone metastases and raised serum-acid-phosphatase with no histological confirmation, were all grouped together as having a definitive diagnosis. There was no correlation between cytological and histological reports in 12 patients (20·7%) and details are shown in the accompanying table. Despite accurate reporting by the cytologist and histologist, the diagnosis of prostatic carcinoma was "missed" using the Franzen technique in 7 patients, as it was in 3 patients undergoing prostatic transurethral resection (T.U.R.). Franzen-needle cytological reports should be viewed with reservation, as should benign prostatic material obtained from a T.U.R. In such situations both techniques could be used to complement each other. 8% of the 152 cytological preparations were considered technically unsuitable or inadequate for diagnostic consideration. In this series, any one Franzen-needle prostatic biopsy was 85% reliable in providing an accurare diagnosis.

St. Bartholomew's Hospital,
London EC1. JOHN P. WARD.

ACUTE RENAL FAILURE

SIR,—I am worried by your editorial of July 21 (p. 134), which presents an unusually narrow view of the pathogenetic mechanisms. Two aspects are stressed—that renin plays an important role and that diuretics exert a protective effect. As for the former theory, the evidence presented is circumsrantial. I submit that there is no concrete evidence that renin activation in shock is other than a secondary effect. Few practising renal physicians support the view that in man diuretics prevent the condition—what they may do is stimulate further renin release. This action has previously been cited as a contraindication to their use! A recent article [1] emphasises that the circulatory type of acute renal failure, as produced in animals by glycerol (which is the popular technique used in many of the articles you cite), is actually worsened by frusemide pretreatment.

The unifying mechanism that is common to all types of shock which result in acute renal failure and tubular necrosis is disseminated intravascular coagulation (D.I.C.). This is a particular feature of traumatic, septic, and obstetric shock, whether in animals or man, and the fact that it is not found in simple hæmorrhagic shock, which is usually reversible without causing renal failure is, I believe, very significant. D.I.C. has been found to accompany those cases of acute renal failure with the worst prognosis.[2] More refined studies show that it is a feature of almost all types of acute renal failure.[3] It is also important that inhibition of fibrinolysis, damage to renal vascular endothelium, and often reticuloendothelial blockade accompany shock and acute renal failure. This means that fibrin deposition will be more than transient. Furthermore, inhibition of fibrinolysis can be shown to precipitate renal cortical necrosis.[4,5] It should also be realised by those who are ignorant of this particular theory that D.I.C. is a functional event which is accompanied by the production of powerful vasoconstrictor products, such as serotonin, that it is facilitated by the metabolic acidosis of shock, and that it is an inevitable accompaniment of sympathetic nervous activation.

I have shown that infusion of various procoagulant substances into the renal arteries of rabbits—for example, thrombin, the inevitable mediator of intravascular coagulation—results in acute renal shut-down, and that there follows a lysozymuria indicative of tubular damage. That thrombin causes impairment of renal blood-flow has also been shown by other groups.[6–8]

The observation that acute tubular necrosis is by no means the rule is based on morphological evidence. In human studies the histologist is at the mercy of capricious timing and limited sampling. It is of interest that Harrison et al.[9] have shown that lysozymuria indicative of tubular damage is more common than histological studies might have indicated.

Wellcome Research Laboratories,
Royal Victoria Infirmary,
Newcastle upon Tyne NE1 4LP. E. NIGEL WARDLE.

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SLEEP-INDUCING EFFECTS OF GAMMAHYDROXYBUTYRATE

SIR,—We were interested in the report by Brezinova and her colleagues on the use of tryptophan as a hypnotic for psychiatric inpatients.[10] The use of such naturally occurring metabolites is appealing because these compounds may prove safer to prescribe for impulsive suicidal patients and may be less addicting than the drugs now in use. Furthermore, currently used hypnotics do not bring on normal sleep, but reduce the proportion of both R.E.M. and delta sleep.[11] An ideal hypnotic would be safe and non-addicting and would induce the characteristic sleep stages. These considerations led us to study gammahydroxybutyrate (G.H.B.). This substance ('Gamma-OH') was introduced into medicine in 1960 by Laborit.[12] To date, it has proved remarkably safe, and animal studies have failed to demonstrate the development of rolerance with chronic use.[13] It is a normal constituent of the mammalian nervous system from which it has been isolated, in highest concentrations, from the midbrain and hippocampal areas.[14]

We have studied the sleep-inducing effects of this drug on 5 insomniacs.

Each patient was studied for eight consecutive nights with all night recordings of the E.E.G., E.O.G., E.M.G., and E.C.G. The patients, 3 males and 2 females, ranged in age from 35 to 60. All had past histories of psychiatric illness, but none was acutely ill at the time of the study. All complained of periodic insomnia. The first 3 nights were placebo nights followed by 3 drug nights on which each patient was given 1–3 g. of G.H.B. Nights 7 and 8 were again placebo nights. The records were scored according to international criteria.[15]

All patients responded similarly. A typical response pattern is illustrated in the accompanying figure. G.H.B. regularly induced the natural stages of sleep. There was a pronounced increase in the proportion of delta sleep. An early and prolonged initial R.E.M. period was consistently observed and the amount of R.E.M. sleep in the first third of the night was greatly increased. However, the total R.E.M. sleep per night was unchanged. There was no rebound or withdrawal effect on nights 7 and 8. The quality and restorative effect of G.H.B. sleep was consistently well rated.

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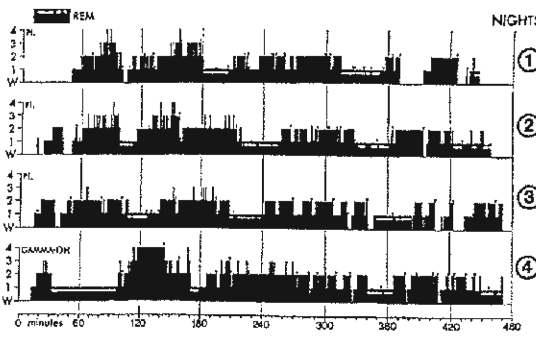

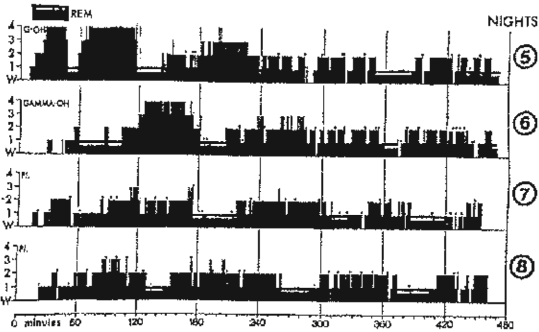

Effect of G.H.B. on sleep.

The vertical scale refers to the stages of N.R.E.M. (non-R.E.M.) sleep. W indicates wakefulness, and the arrows indicate movement epochs.

In doses greater than 3 g., the electrical signs of sleep are replaced by anæsthesia.[12] G.H.B. is rapidly metabolised and disappears from the bloodstream in two to three hours.[16] The increase in R.E.M. and delta sleep observed in the first third of the night is thus induced while G.H.B. is still present in the blood. The role of G.H.B. in the regulation of sleep and wakefulness is not yet known, but it has been implicated in the metabolism of four major neurotransmitter systems concerned with this process: dopamine, acetylcholine, serotonin, and G.A.B.A.[17,18] Its metabolic, electrical, and behavioural effects make it an attractive candidate for the long-sought sleep humour.[19]

We thank Laboratoire Egic, of Paris, for providing the gamma-OH.

Department of Psychiatry,
Sunnybrook Hospital,
University of Toronto,
Toronto, Ontario, Canada.

M. MAMELAK
J. M. ESCRIU
O. STOKAN.

LOW-RESIDUE DIETS AND HIATUS HERNIA

SIR,—Burkitt and James[20] convey the impression that straining at stool is the all-important factor in the cause of hiatus hernia (type unspecified). Frequent intermittent raising of abdominal pressure to unnatural levels, whether by straining to expel fæces or urine, or by the cough of the cigarette smoker or the chronic bronchitic, increases the tendency to herniation of abdominal contents through vulnerable sites such as the inguinofemoral region and the œsophageal hiatus in the diaphragm. This tendency increases as the years pass because of progressive decline of the containing powers of the abdominal walls (which include the thoracic and pelvic diaphragms). It is indeed possible that one only has to live long enough to get a hernia at one site or another. To single out one predominant cause for one type of acquired hernia seems unwise.

Burkitt and James are to be commended for including among the X-ray series from Africa and Asia, in which the disorder is rarely revealed, the figures from Vellore, in which the frequency is 38%, but their reason for casting doubt on the significance of this recording—" either the methods or the diagnostic criteria may have differed " —is unacceptable. One wonders what were the methods or criteria used for the others. Advocacy is not strengthened by accepting evidence which is favourable and rejecting that which is not. One welcomes the plea for " prospective [X-ray] studies in different communities using the same techniques and diagnostic criteria ".

Barton,
Cambridge.

HAROLD EDWARDS.

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COMPLETE DEFICIENCY OF BRUSH-BORDER LACTASE IN CONGENITAL LACTOSE MALABSORPTION

SIR,—Adults with specific hypolactasia usually do not give any history of milk intolerance in their early childhood, indicating that they have once been able to digest the large amounts of lactose present in their mother's milk. Therefore they must have had a high activity of brush-border lactase during the suckling period.

It is well documented that there remains a residual lactase activity in jejunal biopsies from adults with specific hypolactasia. This has generally been believed to represent an unspecific intracellular enzyme without ability to hydrolyse dietary lactose. By using recently developed specific methods,[1] however, we have demonstrated significant residual activities of brush-border lactase in jejunal biopsies from adults with hypolactasia. The mean activities found ranged from 1·8 units per g. protein in Zambians[2] to 4·1 units per g. protein in Finns,[3] compared with 40 units per g. protein in Finns with persistently high lactase.[3] The residual enzyme was also characterised and found to have the same enzymatic properties as brush-border lactase isolated from adults with persistently high activity of the enzyme.[3] Thus adults with specific hypolactasia are in fact able to synthetise brush-border lactase and the decrease in enzyme activity to the adult level, which is inadequate for hydrolysis of significant amounts of dietary lactose, is obviously a regulatory phenomenon. A similar switch down of lactase production occurs generally in mammals at weaning.

With this background we have now studied different enzyme activities in jejunal biopsies from 4 children with congenital lactose malabsorption:

Patient	*Sex and age (yr.)*	*Maltase (units per g. protein)*	*Brush-border lactase (units per g. protein)*
1	M 8	440	<0·3
2	F 7	497	<0·3
3	F 5	384	0·5
4	F 3	478	<0·3

Symptoms of milk intolerance, watery diarrhœa, appeared after the first meal of breast-milk. Diarrhœa persisted and

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Biological Psychiatry, Vol. 12, No. 2, 1977

The Effects of γ-Hydroxybutyrate on Sleep

Morty Mamelak,[1] Joseph M. Escriu,[1] and Olga Stokan[1]

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Sodium γ-hydroxybutyrate (GHB) is a remarkably safe and nontoxic hypnotic agent which is reported to be free of addicting properties. It is also a normal metabolite of the mammalian nervous system. We examined its effects on the sleep-EEG of eight patients with histories of impaired sleep, as a prelude to a more detailed study of its clinical potential. Sleep induced with GHB was indistinguishable subjectively from natural sleep as well as by behavioral and electroencephalographic criteria. Unlike most synthetic hypnotics, GHB increased delta sleep and did not suppress REM sleep. It shortened the REM sleep latency and shifted REM sleep into the first third of the night. On one occasion it induced a sleep onset REM period which was experienced as an attack of sleep paralysis. Withdrawal was simple; there was no REM sleep rebound and sleep patterns immediately returned to their pre-drug form. Its major clinical drawback was its short duration of action: its hypnotic effect lasting only 2 to 3 hr. We suggest that GHB may serve as the prototype for a new class of hypnotic compounds derived from natural sources and capable of activating the neurological mechanisms of normal human sleep.

INTRODUCTION

This study was undertaken to explore the usefulness of sodium γ-hydroxybutyrate in the treatment of insomnia. γ-Hydroxybutyrate (GHB) is a naturally occurring soporific; it is a normal constituent of the mammalian nervous system where it is concentrated in the midbrain and hippocampal areas (Roth, 1970). Its metabolic origin is uncertain, but it may be derived from γ-aminobutyric acid (Roth and Giarman, 1969).

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[1] Sunnybrook Medical Center, University of Toronto Clinic, Toronto, Ontario.

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GHB's attractiveness as a potential clinical hypnotic is based on a number of factors. First, it is a remarkably safe and nontoxic substance (Vickers, 1969). Its LD_{50} is 5 to 15 times the coma-inducing dose, and death, when it occurs, is thought due to sodium intoxication rather than to the active principle. Second, development of tolerance to its hypnotic effect has not been demonstrated in long-term animal studies (Vickers, 1969). Finally, in doses of approximately 30 mg/kg, it induces the natural stages of sleep (Yamada *et al.*, 1976). When given to healthy human subjects at bedtime, the normal sequence of NREM and REM sleep occurs; delta sleep tends to be prolonged, and REM sleep appears after a normal latency.

In contrast, the usefulness of most synthetic hypnotics is limited by the development of tolerance and by their high potential for abuse and self-poisoning. REM and delta sleep are usually suppressed, and the rebound of REM sleep upon drug withdrawal is associated with disturbed, nightmarish sleep — a factor which likely discourages attempts to discontinue the use of these drugs (Oswald and Priest, 1965).

These potential clinical advantages led us to study the effects of GHB in a heterogenous group of eight patients with long-standing histories of impaired sleep. Each patient was studied by means of subjective sleep reports and consecutive all night EEG-sleep recordings. Our study was designed as a preliminary to a more detailed evaluation of the effectiveness of GHB in the treatment of the individual forms of insomnia.

METHODS

Five men and 3 women, ranging in age from 34 to 60 years (mean age = 51 years), were studied. A resume of each subject's clinical history is given in the section on results. All had previously been treated for insomnia, but with the exception of one narcoleptic subject who continued on 10 mg t.i.d. of *d*-amphetamine, all were drug-free for at least 3 weeks before they were studied. Informed consent was obtained from each subject after the nature of the procedure had been fully explained.

Each patient was studied for eight or nine consecutive nights in the sleep laboratory with all-night recordings of the EEG, EOG, EMG, and EKG. The patients were asked not to sleep during the day and to refrain from all alcoholic beverages during the study. The first 3 nights were placebo nights. On the following 3 or 4 nights, each patient was given 1.0 to 4.5 g of γ-hydroxybutyrate orally (15.0-55.0 mg/kg). The last 2 nights were again placebo nights. Most often, on the first drug night, a 3-g dose was given, and depending on the electroencephalographic response, the dose of the drug was varied on the following nights. Our objective was to induce sleep as defined by the appearance of normal EEG-sleep patterns and to minimize the duration of the moderate to high-

voltage theta and delta rhythms induced by high doses of GHB (see Fig. 1). These slow-wave patterns have been previously described (Schneider *et al.*, 1963; Metcalf *et al.*, 1966; Ohye *et al.*, 1966). We arbitrarily scored these EEG patterns as stages X, Y, or Z depending upon the abundance of theta or delta frequency activity exceeding 75 μv (C_4-ear). In stage X, 50% or more of each epoch was occupied by moderate to high-voltage theta rhythms. This merged with stage Y in which the theta waves were progressively replaced by moderate to high-voltage delta waves. When more than 20% of each epoch, but less than 50% was occupied by such moderate to high-voltage delta waves, the epoch was scored as stage Y. When more than 50% of each epoch was occupied by moderate to high-voltage delta waves, the epoch was scored as stage Z. Stage Z was scored only when it occurred in the sequence X, Y, and Z. Otherwise it was often difficult to distinguish it from stage 4. Scoring according to international criteria (Rechtschaffen and Kales, 1968) commenced when well-formed sleep spindles or REM sleep appeared.

The drug or placebo was given at lights out, usually about 11.00 PM, and sleep was recorded continuously until 7.00 AM. The patients were not told on which nights they were given the drug. γ-Hydroxybutyrate was obtained from Laboratoire Egic of Paris, France, who market this drug as a banana flavored syrup, Gamma-OH, for oral use. The placebo consisted of 5 cc of banana flavoring in water.

About ½ hr after awakening, each patient was asked to assess the quality of his previous night's sleep. The sleep self-rating scale of Platman and Fieve (1970) was used. The quality of sleep was rated on a scale of zero to six: zero indicated a very poor night of sleep and six a very good night. Each patient was also asked to guess if his sleep had been drug induced.

Recordings were done with a Grass Model 6 electroencephalograph. Paper speed was 15 mm/sec and scoring was done on each 20-sec epoch. The total recording time was measured from the time the drug or placebo was given, i.e., lights out, until 7.00 AM. The total sleep time was calculated by subtracting the period of wakefulness from the total recording time. Sleep latency was calculated as the time from lights out until initial stage 2 of 1 min or more in duration. REM latency was from initial stage 2 until the first REM sleep period of 1 min or more in duration. On some drug nights REM sleep occurred before stage 2. On these nights, sleep latency was measured from lights out until the beginning of the first REM sleep period of 1 min or more in duration. The sleep latency was not measured in the narcoleptic patient who often fell asleep as the electrodes were being applied. The sleep latency could also not be measured accurately on one night in another subject after a 3-g dose of GHB obscured the normal EEG-sleep patterns (Fig. 1, night 4). REM density was measured as the percentage of 20-sec REM sleep epochs containing one or more rapid eye movements. Delta sleep was calculated by summing stage 3 and 4 sleep. The time spent in each sleep stage in each third of the night was measured after first dividing the total recording time into three equal periods.

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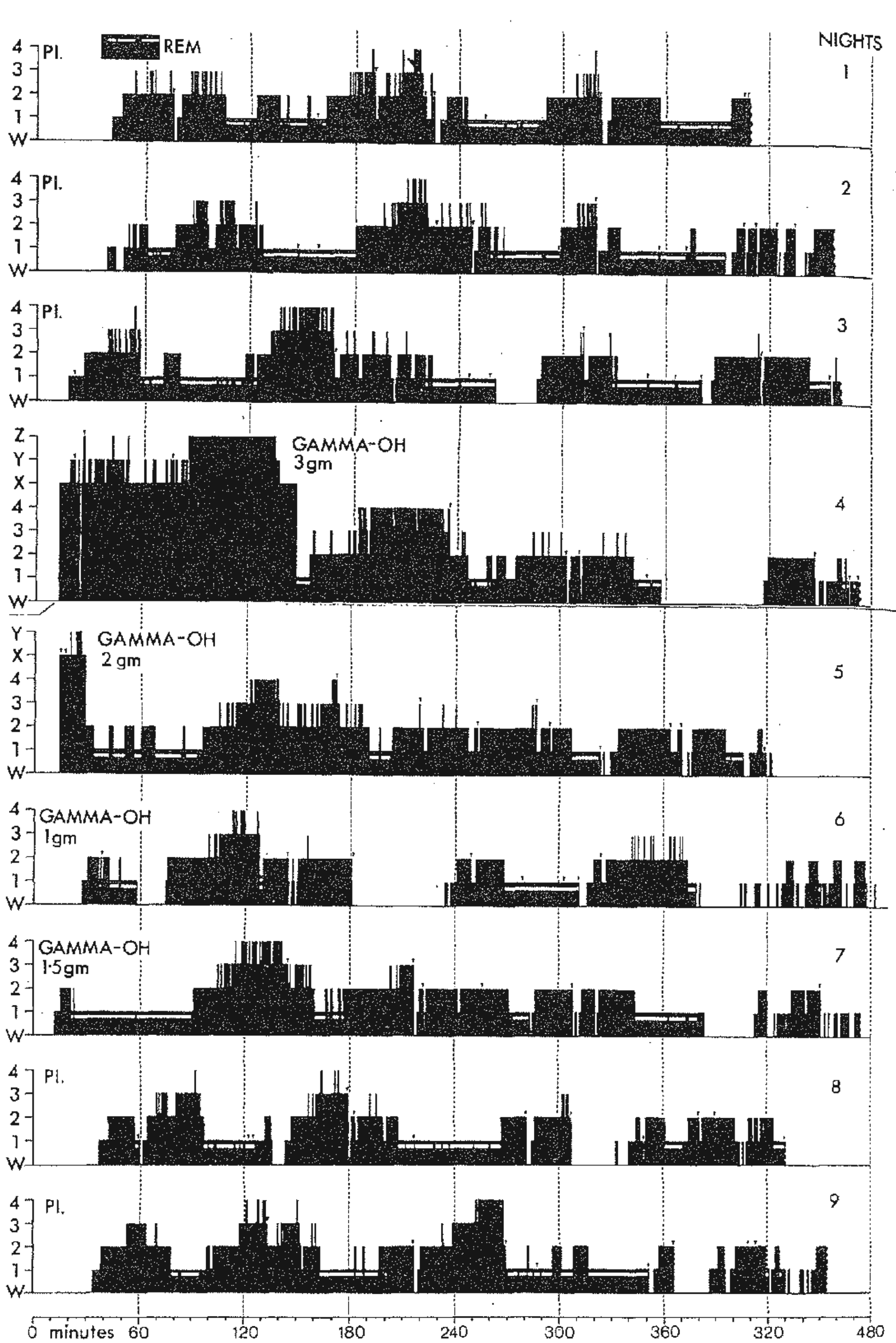

Fig. 1. Case I sleep patterns. In Figs. 1 and 2 the vertical axes indicate the stages of sleep. The horizontal white bars at the level of stage 1 indicate REM sleep and arrows above each night's sleep pattern indicate movement arousals. Consecutive nights of sleep are shown in decending order.

RESULTS

A. Clinical Data

Case I

A 34-year-old woman with an 8-year history of recurrent depressions. For these she had received ECT, tricyclic antidepressants, neuroleptics, and numerous hypnotics. She was withdrawn from daily doses of chlorprothixene (200 mg), methyprylon (300 mg), and flurazepam (30 mg) 25 days before the sleep study. Her mood at the time of the study was normal. Her sleep patterns during the study are shown in Fig. 1. In the data given below, P indicates placebo.

Night	1	2	3	4	5	6	7	8	9
Dose (mg/kg)	P	P	P	47.09	31.39	15.69	24.54	P	P
Was sleep drug induced?	yes	yes	yes	yes	yes	no	yes	no	no
Sleep Quality	3	4	3	4	4	2	4	2	3

Case II

A 60-year-old woman with a 16-year history of manic-depressive psychosis. At the time of the study she had been off all drugs for more than 5 months and her mood was normal. Her sleep patterns during the study are shown in Fig. 2.

Night	1	2	3	4	5	6	7	8
Dose (mg/kg)	P	P	P	45.45	45.45	45.45	P	P
Was sleep drug induced?	no	no	no	no	yes	yes	no	no
Sleep quality	3	3	3	4	5	5	3	5

Case III

A 60-year-old man with a long-standing history of chronic anxiety and alcoholism. He had been treated with a wide variety of anxiolytics, antidepressants, and hypnotics with only moderate success. At the time of the study he had been off all drugs, including ethanol, for more than 1 month and was mildly anxious.

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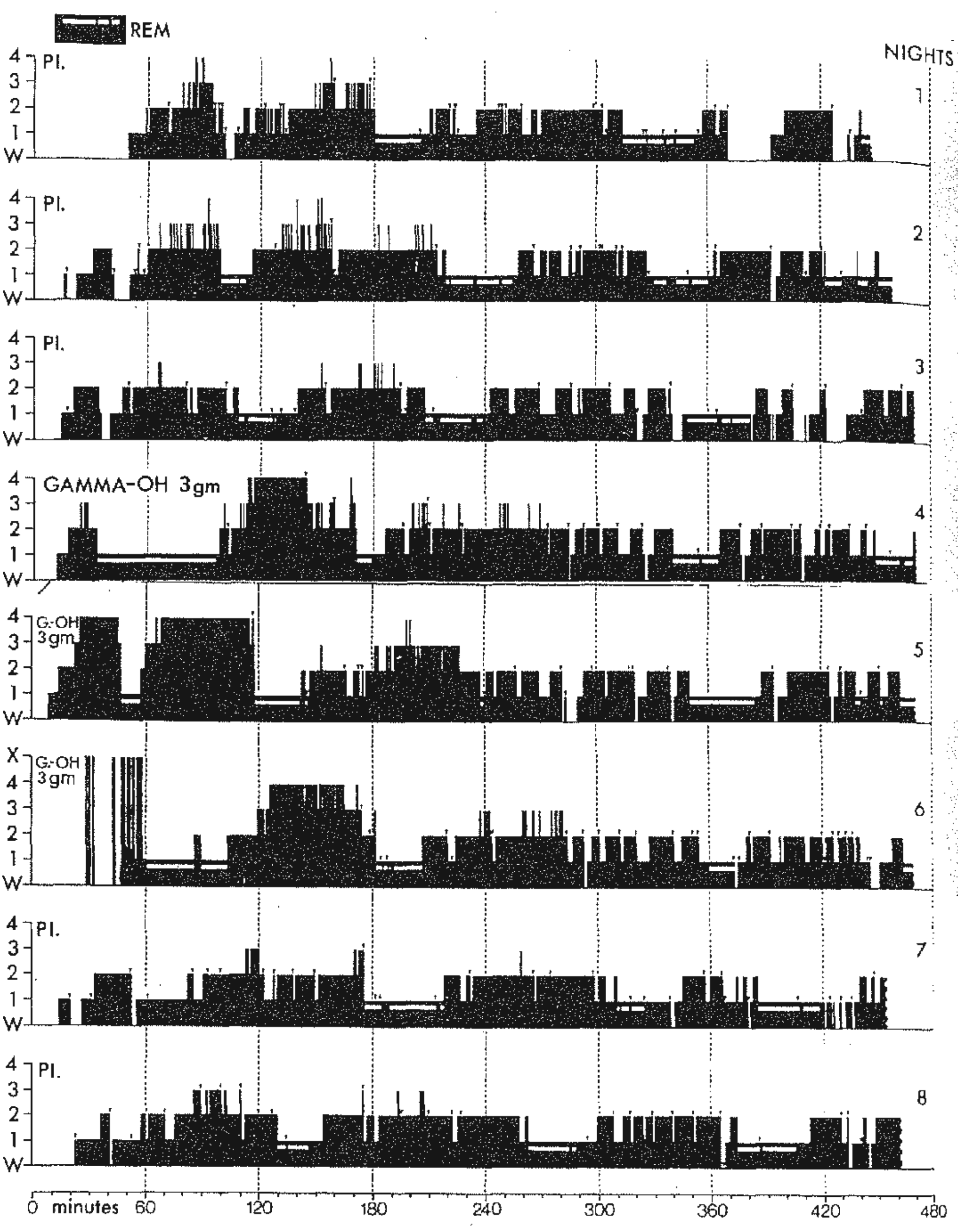

Fig. 2. Case II sleep patterns. See caption to Fig. 1.

Night	1	2	3	4	5	6	7	8
Dose (mg/kg)	P	P	P	50.00	50.00	50.00	P	P
Was sleep drug induced?	no	no	yes	yes	yes	no	yes	yes
Sleep quality	3	3	4	4	5	5	4	4

Case IV

A 50-year-old woman with a 12-year history of recurrent depressions for which she had been treated with ECT and tricyclic antidepressants. At the time of the study she had been off all drugs for 3 weeks and was complaining of depression and fatigue.

Night	1	2	3	4	5	6	7	8
Dose (mg/kg)	P	P	P	52.08	26.04	17.36	P	P
Was sleep drug induced?	no	no	no	yes	yes	yes	yes	yes
Sleep quality	5	4	5	3	3	4	4	4

Case V

A 57-year-old man with a 6-year history of mild bipolar mood swings now effectively controlled by lithium. At the time of the study he had been off all drugs for more than 1 month and his mood was normal.

Night	1	2	3	4	5	6	7	8	9
Dose (mg/kg)	P	P	P	36.23	54.34	36.23	36.23	P	P
Was sleep drug induced?	no	yes	yes	no	yes	no	no	no	yes
Sleep quality	2	4	5	4	3	5	5	4	4

Case VI

A 53-year-old man with a long-standing history of manic and depressive mood swings. These are well controlled by lithium. At the time of the study he had been off all drugs for more than 2 months and his mood was normal.

Night	1	2	3	4	5	6	7	8
Dose (mg/kg)	P	P	P	32.96	32.96	32.96	P	P
Was sleep drug induced?	no	yes	no	yes	yes	no	no	yes
Sleep quality	3	5	4	4	5	4	4	6

Case VII

A 59-year-old man with a long-standing history of chronic anxiety and depression. He has been treated with a wide variety of anxiolytics, antidepressants, and hypnotics without success. At the time of the study he had been off all drugs for more than 3 months and was complaining of anxiety and mild depression.

Night	1	2	3	4	5	6	7	8
Dose (mg/kg)	P	P	P	29.60	19.73	39.41	P	P
Was sleep drug induced	no	yes	yes	yes	no	yes	no	no
Sleep quality	3	2	4	4	4	4	3	4

Case VIII

A 37-year-old man with a 24-year history of narcolepsy. He suffers from attacks of narcolepsy and cataplexy, from sleep paralysis, hypnogogic and hypnopompic hallucinations, and nocturnal dysomnia. At the time of the study he was under adequate control on *d*-amphetamine 10 mg t.i.d.

Night	1	2	3	4	5	6	7	8
Dose (mg/kg)	P	P	P	44.77	22.38	14.92	P	P
Was sleep drug induced	no	no	no	no	yes	no	yes	yes
Sleep quality	2	5	3	4	4	4	4	4

Sleep-induction with GHB was indistinguishable on the whole from the normal process of falling asleep. The patients were unable to guess any better than chance whether or not they had received the drug ($p > 0.05$, ns). With higher doses, patients reported feeling dizzy, light-headed, and somewhat inebriated before falling asleep. Other patients reported feeling very weak before losing consciousness. One patient (Case II) a 60-year-old manic-depressive woman, actually reported being unable to move one night while still awake. Her sleep tracing revealed a progression from wakefulness through stage X to REM sleep (Fig. 2, night 6). Since patients may be conscious during stage X (Yamada *et al.*, 1967), the reported paralysis coupled with the sleep onset REM period suggests a GHB-induced attack similar to hypnagogic sleep paralysis seen with dissociative sleep onset REM periods in cases of compound narcolepsy (Rechtschaffen and Dement, 1967).

The quality of sleep was no different with GHB than it was on the pre-drug night or combined placebo nights ($p > 0.05$, ns). There were no hangovers on awakening.

B. Sleep-EEG Data

Each patient responded to GHB in a somewhat different manner. Nevertheless, an overall response pattern emerged. The earliest electroencephalographic effects were seen about 15 min after the oral administration of GHB. At this time, bursts of high-voltage theta waves appeared. The patients were still conscious, though often drowsy, during this period. The theta bursts frequently became continuous and then merged with NREM sleep. The first REM sleep period usually appeared after a short latency and was often prolonged in duration. REM periods lasting 45 min or longer were not uncommon. REM sleep was shifted to the first third of the night, but the total duration of REM sleep per night was not changed. GHB increased the duration of delta sleep, and typically a period of delta sleep followed a prolonged initial REM period (Figs. 1 and 2).

With higher doses, EEG patterns emerged which were different from those of normal sleep. These were scored as stages X, Y, and Z as described earlier. These stages were devoid of well-formed sleep spindles. It was usually possible to minimize the appearance of these EEG patterns by giving less drug. However, transitional patterns, especially between stages X, 1, 2, and REM did occur. At times, even lengthy periods of normal EEG sleep on drug nights would be interrupted by short intervals or bursts of moderate- to high-voltage theta or delta rhythms. This was most likely to occur at moments of arousal or preceding the shift to a lighter stage of sleep. For example, in a shift from stage 2 to stage 1 or wakefulness, a burst or an epoch or two of moderate to high-voltage theta and delta rhythms might intervene. At these times, the record was scored according to the EEG pattern which dominated 50% or more of the epoch.

The Mann-Whitney U-test was used to evaluate the data. For each sleep parameter, two different comparisons were made. First, night 3, the last pre-drug night, was ranked against the drug nights. Second, night 3 and the last two nights of each study, i.e., the combined placebo nights, were ranked against the drug nights. The first two nights of each study were considered adaptation nights. Since the eight patients represent a clinically heterogenous group, each sleep parameter was ranked separately for each patient. For the sake of comparison with other data in the sleep literature, the mean value and standard deviation of each sleep parameter averaged for all patients is also given (Table I).

1. *Total sleep time*

(a) drug nights *vs.* pre-drug night: $p > 0.05$, ns
(b) drug nights *vs.* combined placebo nights: $p > 0.05$, ns

Table I. Mean Value and Standard Deviation of Each Sleep Parameter

	Pre-drug night	Drug nights	Combined placebo nights
1. Total sleep time	396.08 ± 21.39 min (N = 8)	398.55 ± 34.04 min (N = 26)	384.12 ± 57.24 min (N = 24)
2. Sleep latency (based on 7 patients excluding the narcoleptic)	38.14 ± 25.59 min (N = 7)	28.07 ± 15.69 min (N = 22)	35.39 ± 19.28 min (N = 21)
3. Delta sleep	14.41 ± 17.45 min (N = 8)	34.14 ± 24.28 min (N = 26)	16.46 ± 18.90 min (N = 24)
4. Delta sleep in first third of night	5.66 ± 9.75 min (N = 8)	19.08 ± 17.69 min (N = 26)	5.94 ± 7.85 min (N = 24)
5. REM sleep	89.32 ± 32.14 min (N = 8)	75.58 ± 23.88 min (N = 26)	79.10 ± 24.69 min (N = 24)
6. REM percent in first third of night	23.11 ± 12.47% (N = 8)	37.40 ± 18.51% (N = 26)	26.54 ± 15.82% (N = 24)
7. REM latency (based on 7 patients excluding the narcoleptic)	65.81 ± 40.66 min (N = 7)	32.28 ± 34.36 min (N = 23)	66.79 ± 36.38 min (N = 21)
8. REM density	63.17 ± 8.77% (N = 8)	59.33 ± 12.73% (N = 26)	66.15 ± 7.40 min (N = 24)
9. REM density in first third of night	66.27 ± 12.11% (N = 7)	50.42 ± 20.09% (N = 25)	66.13 ± 12.51% (N = 22)
10. Movement time in first third of night	2.71 ± 2.19 min (N = 8)	1.87 ± 1.32 min (N = 26)	2.87 ± 2.11 min (N = 24)

2. *Sleep Latency* (based on 7 patients, excluding the narcoleptic)

(a) drug nights < pre-drug night: $p < 0.05$
(b) drug nights < combined placebo nights: $p < 0.05$

3. *Delta Sleep*

(a) drug nights > pre-drug night: $p < 0.01$
(b) drug nights > combined placebo nights: $p < 0.01$

4. *Delta Sleep in first third of night*

(a) drug nights > pre-drug night: $p < 0.01$
(b) drug nights > combined placebo nights: $p < 0.01$

5. *REM Sleep*

(a) drug nights *vs.* pre-drug night: $p > 0.05$, ns
(b) drug nights *vs.* combined placebo nights: $p > 0.05$, ns
(c) pre-drug night *vs.* first post-drug night: $p > 0.05$, ns
(d) pre-drug night *vs.* post-drug nights: $p > 0.05$, ns

6. *REM Percent in first third of night*

(a) drug nights > pre-drug night: $p < 0.01$
(b) drug nights > combined placebo nights: $p < 0.02$

7. *REM Sleep latency* (based on 7 patients, excluding the narcoleptic)

(a) drug nights < pre-drug night: $p < 0.01$
(b) drug nights < combined placebo nights: $p < 0.01$

8. *REM Density*

(a) drug nights *vs.* pre-drug night: $p > 0.05$, ns
(b) drug nights *vs.* combined placebo nights: $p > 0.05$, ns

9. *REM Density in first third of night*

(a) drug nights < pre-drug night: $p < 0.05$
(b) drug nights < combined placebo nights: $p < 0.05$

10. *Movement time in first third of night*

(a) drug nights *vs.* pre-drug night: $p > 0.05$, ns
(b) drug nights *vs.* combined placebo nights: $p > 0.05$, ns

DISCUSSION

The pharmacological properties of GHB, including its hypnotic and anesthetic actions, were first studied by Laborit and his collaborators (Laborit, 1964). Earlier, Sampson, Dahl, and White had demonstrated the soporific action of other short chain fatty acids (Sampson and Dahl, 1955; White and Sampson, 1956). With the advent of EEG sleep studies, Jouvet *et al.* (1961) and later Matsuzaki *et al.* (1964) found that short chain fatty acids such as butyrate, isovalerate, caproate, and GHB and its lactone induced both NREM and REM sleep in the cat and that prolonged periods of REM sleep often appeared after a short latency. Interest in GHB heightened when it was isolated from the mammalian nervous system and its derivation from γ-aminobutyric acid (GABA) was

experimentally demonstrated (Roth and Giarman, 1969). However, the normal rate of formation of GHB in the nervous system is not known. The liver, which can also synthesize GHB, has been considered as an alternate source (Roth, 1970).

The behavioral and electroencephalographic effects in GHB in humans have been described by a number of workers (Schneider *et al.*, 1963; Metcalf *et al.*, 1966; Ohye *et al.*, 1966; Yamada *et al.*, 1967). The paradoxical presence of theta and delta rhythms in waking subjects has been a consistent finding. In doses of 60-70 mg/kg, GHB produces coma lasting 1 to 2-hr (Vickers, 1969). No specific electroencephalographic changes mark the transition from wakefulness to coma and the EEG shows continuous irregular medium and high-voltage theta and delta rhythms at this time (Metcalf *et al.*, 1966). Lower doses produce a reversible somnolent state (Vickers, 1969).

In our hands, this somnolent state was readily reversed by such external stimuli as the call to wake up and such internal stimuli as a full bladder. The EEG showed the typical electrical patterns of NREM and REM sleep, and in distinction to the EEG patterns observed with other hypnotics (Kales *et al.*, 1970), delta sleep was prolonged and REM sleep was not suppressed. In fact, on many nights, GHB specifically activated the process of REM sleep. The state induced by GHB, then, closely resembles true sleep as defined by behavioral and electroencephalographic criteria (Dement, 1967).

GHB and REM Sleep

REM sleep rarely appears when GHB is given during the day and even when the drug is given at bedtime to healthy young adults, REM sleep appears only after a normal latency (Yamada *et al.*, 1967). This is in contrast to its effect in our patients in whom REM sleep was usually induced after an abnormally short latency (Figs. 1 and 2). Many of our patients, however, had nights with short REM sleep latencies even in the absence of the drug. An early REM sleep period, however, was a more consistent finding following GHB, and the average REM sleep latency fell from 65.81 min on placebo nights to 32.28 min with GHB.

Our patients all had histories of mental depression, recent drug withdrawal, or narcolepsy, conditions in which abnormally short REM latencies have previously been described (Kupfer and Foster, 1972; Oswald, 1968, 1971; Rechtschaffen and Dement, 1967). Since the first REM sleep period in man does not usually appear until about 90 min after the onset of sleep, the early REM sleep periods in these disorders have been attributed to abnormally low REM sleep thresholds caused by increased REM pressure or to ineffective REM inhibitory mechanisms.

It is noteworthy that clinical conditions with persistent overt early REM sleep periods are characterized by emotional lability, vulnerability to stress, and

disturbances in personality functioning. This is so for schizophrenia (Snyder, 1972), depression, narcolepsy, and withdrawal from centrally active drugs, particularly sedative drugs. The personality disturbances in narcolepsy, notably the high incidence of depression, have been emphasized recently by a number of workers (Broughton and Ghanem, 1975; Roth and Nevsimalova, 1975). In fact, Kupfer (1976) has recently proposed that persistent overt early REM periods are biological markers for primary depressive illness.

We suggest that GHB may be used to probe the REM threshold and that the early induction of a REM sleep period following the administration of GHB at bedtime is indicative of an abnormally low REM sleep threshold. This, in turn, implies a fault in the neurological mechanism controlling REM sleep. We suggest that this fault or defect expresses itself in a vulnerability to stress and that it is one of the abnormalities persisting in depressed patients following clinical recovery which predisposes them to a recurrence of their illness (Mendels and Chernik, 1975). For example, in the case illustrated in Fig. 2, GHB was given to a 60-year-old woman with a long-standing history of manic-depressive illness. At the time of the study she appeared clinically well and had been off all drugs for 5 months. Her REM sleep latency on placebo nights was within normal limits (average sleep latency: 106 mins). GHB markedly reduced the REM sleep latency and on one night even induced a sleep onset REM period. We would have predicted from this that she was not entirely well, and indeed a few months later, continuing off all medications, she became manic.

Narcolepsy and GHB

The induction with GHB of sleep paralysis in conjuction with a sleep-onset REM period was of considerable interest. This phenomenon has been uncommon but we have had a number of reports of sleep paralysis following the administration of GHB. These episodes are comparable to those occurring naturally in compound narcolepsy (Rechtschaffen and Dement, 1967) and encourage speculation that a disorder of GHB metabolism or of a pharmacologically analogous compound exists in narcolepsy.

Jouvet (1969) proposed that acetylcholine and a deaminated catabolite of serotonin trigger the noradrenergic mechanisms of REM sleep. Cholinergic mechanisms have been implicated specifically in the tonic events of REM sleep, the activation of the EEG and the decrease in muscle tone (Jouvet, 1972). GHB both increases the concentration of brain acetylcholine (Giarman and Schmidt, 1963) and shares structural features with it (Feldstein *et al.*, 1970). It is conceivable, then, that GHB acts by increasing acetylcholine levels at critical receptor sites within the nervous system or acts directly on these receptor sites themselves. GHB also shares structural features with the two serotonin catabolites reputed to have soporific properties: 5-hydroxytryptophol and 5-hydroxy

indoleacetaldehyde (Feldstein *et al.*, 1970). The aldehyde, in particular, has been mooted as the active sleep-inducing metabolite (Feldstein *et al.*, 1970; Sabelli and Giardina, 1970). However, GHB's structural similarity to these compounds may be less meaningful since neither actually has been shown to induce REM sleep and their exact role in sleep physiology remains undefined (Rechtschaffen *et al.*, 1968; Feldstein, 1973; Morgane and Stern, 1973).

THERAPEUTIC APPLICATIONS OF GHB

GHB's major clinical disadvantage is its short duration of action. In cases of severe insomnia we have had to repeat the drug two or three times during the night to maintain sleep. Although GHB shortened the sleep latency, the practical significance of this is not clear since our subjects fell asleep after about ½-hr even without the drug. GHB, however, did not suppress REM sleep, and there was no REM sleep rebound after its withdrawal. The absence of a REM rebound on withdrawal likely makes it a less habituating hypnotic than other drugs (Oswald and Priest, 1965). We see it as potentially useful for the large number of patients who have difficulty falling asleep but who once asleep are able to remain so.

GHB may also be useful for certain disorders complicated by specific types of insomnia. For example, we used GHB to treat the insomnia of a small group of narcoleptic patients (Broughton and Mamelak 1975). We were interested in the relationship between their impaired nocturnal sleep (Rechtschaffen *et al.*, 1963) and their daytime symptomatology. We gave the drug in repeated doses during the night. GHB increased the total nocturnal sleep time and the total duration of nocturnal REM sleep. The incidence of daytime attacks of cataplexy declined and daytime functioning improved. This unique therapeutic effect distinguishes GHB from the synthetic hypnotics (Daniels, 1934).

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Narcolepsy: A Family Study

Mortimer Mamelak,[1,2] Victoria J. Caruso,[1]
and Keith Stewart[1]

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We obtained medical and psychological assessments and 48-hr polysomnographic recordings on five sisters, three of whom had narcolepsy. Of the three, two were identical twins. All three narcoleptic sisters cited emotional stress and environmental demands for sustained performance as the major factors which aggravated their symptoms, and corresponding to this, the illness followed a different life course in each of the three. Most striking were the differences between the twin sisters in clinical symptoms and polysomnographic signs. One sister suffered from all the symptoms of narcolepsy and her sleep recording showed the typical sleep onset REM periods of the disease. Her twin suffered only from excessive daytime drowsiness and her sleep recording was normal – at least by the usual criteria. The sleep of all three narcoleptic sisters, however, was significantly more fragmented than that of their normal siblings. Our data suggested that excessive sleep fragmentation was a basic feature of narcolepsy and that it betrayed a constitutional predisposition for sleep to dissociate into its components and to become distributed around the nycthemeron. This process could be aggravated by emotional stress and by environmental demands for sustained vigilance, and this in turn, created the differences in symptoms and signs between individuals with identical genetic predispositions.

It has been well documented that narcolepsy runs in families (Krabbe and Magnussen, 1942; Daly and Yoss, 1959; Yoss and Daly, 1960; Bruhova and Roth, 1972; Bruhova, 1973; Kessler *et al.*, 1974; Kessler, 1976). More recently, investigators have been interested in the relative contributions to the disease of genetic and environmental factors. Narcolepsy has been viewed as one of a

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[1] Sunnybrook Medical Centre, University of Toronto Clinic, Toronto, Ontario.
[2] All correspondence should be directed to Dr. M. Mamelak, Sunnybrook Medical Centre, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5.

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number of clinical disorders such as cleft lip and palate, diabetes, and schizophrenia which do not show classical Mendelian patterns of inheritance but in which a number of factors combine to create a liability to the disease. Once a given threshold of liability is reached the illness becomes clinically manifest (Kessler *et al.*, 1974; Kessler, 1976; Thomson, 1976; Leckman and Gershon, 1977).

In the case of narcolepsy, both genetic and environmental factors are thought to contribute to this liability (Imlah, 1961; Zarcone, 1973; Dement *et al.*, 1976). In fact, two thresholds of liability have been proposed. The first, when reached, results in excessive daytime drowsiness; the second, in narcolepsy and its auxiliary symptoms of cataplexy, sleep paralysis, and nocturnal hallucinations, i.e., in compound narcolepsy (Kessler *et al.*, 1974; Kessler, 1976; Leckman and Gershon, 1977). Although a substantial genetic contribution to this liability has been demonstrated, the few case reports on narcolepsy in identical twins have emphasized the differences between the twins in the extent to which they suffer from the disease (Imlah, 1961; Mitchell and Cummins, 1965). This, in turn, has directed attention back to the other etiological determinants.

A unique opportunity for examining the relative contributions of these multiple determinants recently presented itself to our clinic when a severely ill narcoleptic woman and her mildly somnolent identical twin sister were referred for investigation and treatment. The twins were part of a sibship of 11 children, seven of whom were still living (see Fig. 1). Of the seven, three – the twins and one older sister – suffered from narcolepsy. Their mother, too, apparently had suffered from the disease, but the diagnosis was never confirmed in her lifetime. We were able to obtain medical and psychological assessments and continuous

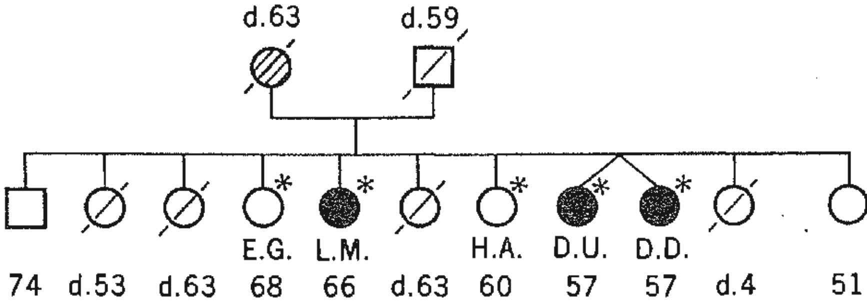

Fig. 1

bicircadian polysomnographic recordings on the identical twins and their narcoleptic sister and on two other sisters, close in age to their narcoleptic siblings, but free of the disease themselves. The clinical data emphasized the importance of life-style and life events in determining the severity of the symptoms in those genetically predisposed to the disease, and the polysomnographic data pointed to sleep fragmentation rather than to sleep onset REM periods as the critical feature distinguishing the sleep of narcoleptics from normals.

METHODS

After complete medical and psychological histories were obtained, all five sisters were admitted to the sleep laboratory for polysomnographic recordings. Continuous 48-hr recordings were obtained on four of the five sisters and a 33-hr recording was obtained on the fifth. During their stay in the sleep laboratory, all the sisters remained in bed for the recording period except for three daily 60-min breaks for meals and for additional breaks to the bathroom as needed. They could read if they so wished but were otherwise encouraged to sleep. Blood group studies and dermatoglyphic analyses were done on the twins.

At the time of the polysomnographic study, only one sister (EG) was using centrally active drugs. She was taking 250 mg of α-methyldopa. t.i.d., propanolol 10 mg t.i.d., protriptyline 5 mg t.i.d., and diazepam 10 mg t.i.d. for hypertension and depression. She had been on this regimen for a number of years and it was inadvisable for us to stop it. D.W. had been using phenmetrazine 12.5 mg q.d. or b.i.d. intermittently for about a year but had stopped it 1 week before starting the study. The other sisters were not on any drugs.

The polysomnographic records were scored according to international criteria using 20-sec epochs (Rechtschaffen and Kales, 1968). The 48-hr recordings were divided into nocturnal and diurnal periods. The nocturnal period was defined as the interval between the onset of sleep for the night to the time of awakening for breakfast (Fig. 2). Sleep during the remainder of the day was scored as part of the diurnal period. In Tables I and II, the total sleep time refers to the sum of stages 1, 2, 3, 4, movement time, and REM. The actual sleep time refers to the sum of Stages 2, 3, 4, and REM. The true sleep efficiency refers to the total sleep time expressed as a percentage of the recording period, be this the nocturnal or diurnal recording period. Similarly, the actual sleep efficiency refers to the actual sleep time as a percentage of these recording periods. To be scored as a separate REM sleep period, a period had to be have begun at least 15 min or more after the end of the previous REM sleep period. The REM latency refers to the time between the onset of Stage 2 of 60 sec or more in duration to the onset of Stage REM of 60 sec or more in duration. The REM

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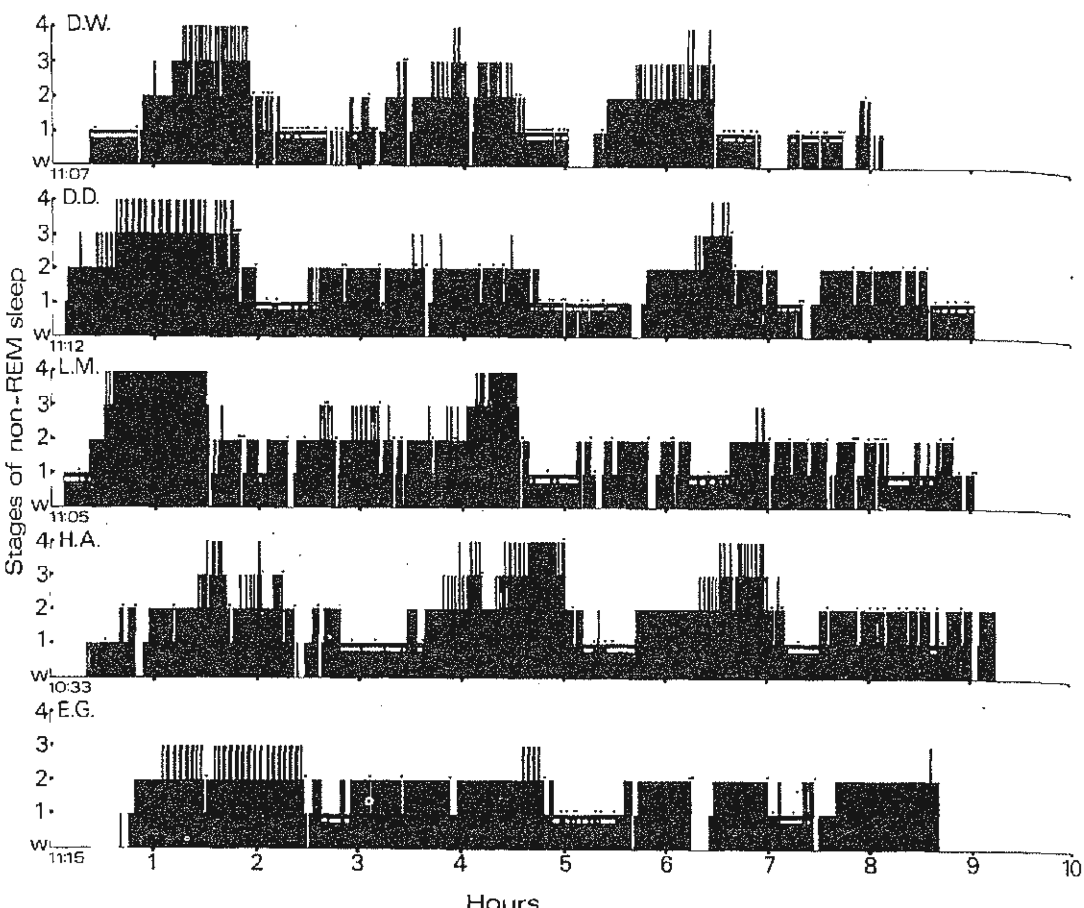

Fig. 2. This figure illustrates the sleep patterns during the first nocturnal sleep period of the continuous 48-hr recording in the five sisters. The vertical axis indicates the stages of NREM sleep, the horizontal axis indicates time in hours. The horizontal white bars at the level of Stage 1 indicate REM sleep and the triangles above each sleep pattern indicate movements. Note that D. W., D. D., and L. M. are narcoleptic and that D. W. and D. D. are identical twins.

density refers to the profusion of rapid eye movements in each REM period and was determined by estimating the relative number of rapid eye movements in each REM period on a scale of 0–8 and the averaging these values over the full REM period (Snyder, 1968). The values obtained for each REM sleep period were then normalized to obtain an average value for REM sleep density during the nocturnal or diurnal recording periods.

The overall degree of sleep fragmentation was assessed by arbitrarily defining any period of sleep preceded by and ending in 1 min or more of either wakefulness, movement time, or Stage 1 as a sleep fragment. The duration of each fragment was expressed both in minutes and as a percentage of the summed duration of all the fragments for the nocturnal or diurnal periods. This sum will hereafter be referred to as the total nocturnal or diurnal fragment time. The fragments of nocturnal or diurnal sleep were divided into three categories:

Table I. Polysomnographic Data for the Two Nocturnal Periods of the 48-Hour Recordings of the Five Sisters[a]

	D.W.		D.D.		L.M.		H.A.		E.G.	
	1	2	1	2	1	2	2	2	1	2
Total nocturnal period (min)	478.00	379.33	533.00	507.66	529.66	575.65	526.66	544.66	472.33	520.00
Stage W (min)	63.66	109.33	34.66	24.33	43.00	31.33	14.66	14.00	13.33	27.33
1 (min)	45.33	30.33	48.33	75.00	50.66	90.66	47.66	39.33	39.00	49.33
2 (min)	146.33	104.66	262.66	269.66	253.00	289.33	269.33	306.66	322.00	314.33
3 (min)	59.33	23.66	47.00	29.33	22.00	21.00	56.66	29.33	32.66	34.00
4 (min)	27.66	3.00	38.00	26.33	71.00	34.33	43.00	41.00	0.00	1.00
REM (min)	113.33	87.00	89.33	70.33	74.33	96.00	84.00	100.00	58.33	80.00
Movement time (min)	22.33	21.33	13.00	12.67	15.66	13.00	11.00	14.33	7.00	14.00
Total sleep time (min)	392.00	248.66	485.33	470.66	480.00	531.33	500.66	516.33	452.00	478.66
Actual sleep time (min)	346.66	218.33	437.00	395.66	420.33	440.66	486.00	477.00	413.00	429.33
True sleep efficiency %	82.00	66.00	91.00	93.00	91.00	92.00	95.00	95.00	96.00	92.00
Actual sleep efficiency %	73.00	58.00	82.00	78.00	79.00	77.00	92.00	88.00	87.00	83.00
No. of REM periods	5	3	5	3	5	6	4	4	3	5
REM sleep latency (min)	0.00	0.00	109.33	106.33	0.00	0.00	134.33	77.66	115.00	96.33
REM density	302.00	230.00	132.71	178.00	112.90	94.80	130.20	134.50	110.00	107.00
True REM sleep efficiency %	76.00	86.00	81.00	76.00	87.00	88.00	95.00	96.00	89.00	91.00
Actual REM sleep efficiency %	76.00	85.00	79.00	65.00	82.00	88.00	86.00	92.00	81.00	89.00

[a] D.W., D.D., and L.M. have narcolepsy. D.W. and D.D. are identical twins. There were no statistical differences between the narcoleptic and nonnarcoleptic family members in any of the above variables at the 0.05 level. We used the t test for two independent samples.

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Table II. Polysomnographic Data for the Two Diurnal Periods of the 48-Hour Recording on the Five Sisters[a]

	D.W.		D.D.		L.M.		H.A.		E.G.	
	1	2	1	2	1	2	1	2	1	2
Total diurnal period (min)	1022.33		935.00	889.33	927.66	799.66	934.33	823.33	961.33	871.33
Stage W (min)	798.00		746.00	656.00	743.66	607.33	650.66	701.00	929.00	763.33
1 (min)	26.00		10.00	34.33	35.33	18.33	74.00	44.00	13.00	27.00
2 (min)	58.66		114.66	64.33	103.66	111.33	159.66	71.66	19.33	77.00
3 (min)	35.33		23.33	31.00	20.66	17.33	11.63	5.00	0.00	3.66
4 (min)	35.33		21.33	74.00	10.66	30.66	2.00	0.00	0.00	0.00
REM (min)	58.66		16.00	25.66	9.33	11.00	32.33	0.00	0.00	0.00
Movement time (min)	10.33		3.66	4.00	4.33	3.66	4.00	1.66	0.00	0.33
Total sleep time (min)	214.00		185.33	229.33	179.66	188.66	279.66	120.66	32.33	107.66
Actual sleep time (min)	188.00		175.33	194.99	144.33	170.33	205.66	76.66	19.33	80.66
True sleep efficiency %	21.00		20.80	26.00	19.00	24.00	30.00	15.00	3.00	12.00
Actual sleep efficiency %	24.00		18.70	22.00	16.00	21.00	22.00	9.00	2.00	9.00
No. REM periods	6		1	2	1	1	2	0	0	0
REM latency (min)[b]	0.00		77.00	72.66	0.00	7.00	73.33	–	–	–
Avg. REM density	202.30		362.50	165.00	189.20	254.80	103.10	–	–	–
True REM sleep efficiency %	92.00		96.00	81.00	100.00	94.00	89.00	–	–	–
Actual REM sleep efficiency %	89.00		96.00	81.00	100.00	94.00	75.00	–	–	–

[a] D.W., D.D., and L.M. have narcolepsy. D.W. and D.D. are identical twins. There were no statistical differences between the narcoleptic and nonnarcoleptic family members in any of the above variables at the 0.05 level. We used the t test for two independent samples.

[b] REM Latency refers only to the first diurnal sleep period during which REM sleep occurred.

Table III. Proportion of Sleep Spent in Fragments[a]

Time periods (min)	First nocturnal period			Second nocturnal period			First day (First and second nocturnal periods)		
	0–30	31–60	61+	0–30	31–60	61+	0–30	31–60	61+
Narcoleptic D.W.	52.19	8.93	38.88	62.39	37.61	–	52.31	22.55	25.13
Narcoleptic D.D.	61.67	17.21	21.12	57.03	25.69	17.29	50.28	34.57	15.14
Narcoleptic L.M.	59.56	25.37	15.07	47.00	53.00	–	60.84	15.81	11.51
Normal H.A.	20.50	6.62	72.88	28.86	24.38	46.66	26.58	23.09	50.32
Normal E.G.	16.77	39.38	43.85	35.95	25.76	38.29	20.46	37.63	41.91

[a]The differences between the narcoleptic and normal family members during the first and second nocturnal periods and during the first day were significant at the 0.001 level for fragments 30 min or less in duraction and at the 0.05 level for fragments 60 min or less in duration. We used the t test for two independent samples.

those up to 30 min, those of 31–60 min and those greater than 61 min, and the percentage of the total nocturnal or diurnal fragment time spent in each of the three categories was estimated (Table III).

Finally, we specifically assessed the degree of REM sleep fragmentation. Both a true and actual REM sleep efficiency were determined. This was done by arbitrarily defining the time from the first epoch of a REM sleep period to the last epoch of that period as the total REM sleep period duration. Because of fragmentation, it consisted of REM sleep epochs and also of interspersed epochs of wakefulness, MT, Stage 1, and Stage 2. The actual REM sleep efficiency refers to the percentage of the total REM sleep period duration consisting only of REM sleep epochs. The true REM sleep efficiency refers to the percentage of the total REM sleep period duration consisting of epochs of Stage 2 plus Stage REM.

RESULTS

The Family

Case Histories

E.G. stated that she had never suffered from narcolepsy or any of its symptoms. She had spinal meningitis at the age of 3 and was left with both her

legs permanently paralyzed. Like many other members of her family, she also suffered from asthma, diabetes, coronary artery disase, and hypertension. She complained of depression and attributed this to the death of her husband a number of years earlier.

L. M. first became aware that she had narcolepsy in her early 20s. Initially her symptoms were mild and consisted only of daytime drowsiness. She identified the break-up of her first marriage in her early 30s as a major stress which aggravated her disease. Cataplexy first appeared at that time. Sleep paralysis, nightmares, hallucinations, and long periods of wakefulness became commonplace at night. Over the years these symptoms gradually abated, and she now again suffers from only mild drowsiness during the day and from the rare cataplectic attack. She recalled, however, that when her second husband died, all her symptoms reappeared with their former intensity. In recent years her life has been comfortable and stress-free. It is not necessary for her to work, and she is able to rest whenever she pleases. She feels well, in spite of coronary artery disease and peripheral vascular disease.

H. A. stated that she had never suffered from narcolepsy. In her late 30s, however, when working long hours as a waitress, she required *d*-amphetamine to stay awake. This difficulty lasted for about 1 year. At present, she complains only of mild postprandial drowsiness but of lesser degree than that in her narcoleptic sisters. She revealed that she has had rare but recurrent episodes of sleep paralysis for most of her adult life. Other than this, she believed her nocturnal sleep was normal. Her personal life was stable. Like many of her siblings, she too suffered from coronary artery disease and hypercholesterolemia.

D. W. was the patient initially referred to us. She recalled excessive daytime drowsiness and sleep attacks in her early 20s. She believed these symptoms started when she gave birth at the age of 21 to her first child and that the symptoms were exacerbated in the ensuing years by the turmoil in her marriage. Cataplexy first appeared during this time. Her illness became progressively worse over the years and, when first seen in our clinic, she was virtually incapacitated. Her nocturnal sleep was markedly fragmented – she slept for about an hour or two and was then awake for an equal time. She complained of sleep paralysis, nocturnal hallucinations, and nightmares and, during the day, of exhaustion, fatigue, sleepiness, and attacks of sleep and cataplexy. She was using small doses of phenmetrazine daily withour much benefit, but was hesitant to increase them for fear of developing tolerance and addiction. She had been on *d*-amphetamine and on tricyclic antidepressants but discontinued these drugs because of their side effects. On psychological assessment, she was clearly depressed and during her stay in hospital required individual and marital psychotherapy. D. W. also suffered from asthma and hypercholesterolemia.

D. D. is the twin of D. W. She first noticed excessive daytime drowsiness in her mid-20s. At that time, she was under a great deal of stress working full time and raising a child on her own. She developed insomnia, sleep paralysis,

nocturnal hallucinations, and daytime attacks of sleep and cataplexy. When she remarried a number of years ago, her condition improved and she attributed this to the emotional and financial security which her marriage brought her. She no longer had to work and could rest when she pleased. At present, she suffers from only mild daytime drowsiness and fatigue. She commented spontaneously on the relationship between stress and her symptoms. She revealed that she was very dependent on her husband, a fireman. His absence from home on nights when he was on call regularly led to the recurrence of nocturnal hallucinations and nightmares. Since her remarriage, much of her earlier mental depression had disappeared. D. D. also has peripheral vascular disease and hypercholesterolemia. She had asthmatic attacks as a child but now suffers from these only when she has an upper respiratory infection.

Twin Testing

On dermatoglyphic analysis alone, the probability that D. D. and D. W. were monozygotic was 0.5864. D. D. and D. W. shared the following blood group antigens: A, Rh, P, Fy (a^+), kk, Le (a^-b^+) MNS, Lu (a^-). On the basis of the blood group antigens alone, the probability that the two sisters were identical twins was 0.9105. Combining the dermatoglyphic and blood group analyses, the probability that D. D. and D. W. were monozygotic twins was 0.988.

Polysomnographic Findings

The polysomnographic data for the two nocturnal and diurnal recording periods are shown in Tables I and II. The sleep fragmentation data for the two nocturnal recording periods and for the first nocturnal and diurnal periods together, are given in Table III. Figure 2 illustrates the sleep architecture of the first nocturnal periods of 48-hr polysomnographic recordings in the five sisters.

DISCUSSION

Marked differences were observed between the identical narcoleptic twin sisters in their clinical symptomatology and polysomnographic patterns. D. W. suffered gravely from all the symptoms of narcolepsy; her polysomnographic recording revealed the gross fragmentation of her nocturnal sleep and showed the characteristic sleep onset REM sleep periods of the disease. D. D. her twin, suffered only from excessive daytime drowsiness and her polysomnographic recording was normal, at least by the usual criteria. Specifically, she showed no sleep onset REM periods at any time during her 48-hr recording (Tables I, II, Fig. 2).

It is noteworthy that both twins suffered gravely from compound narcolepsy in the third and fourth decades of their lives. In D. D.'s case, her symptoms improved with time, but in D. W.'s they did not. In both, the demands of life were greatest in those years as each one had to cope with a troubled marraige and with the stresses of child rearing. For D. D., in addition, there was the demand of full-time work; and for D. W. the emotional and physical exhaustion entailed by recurrent attacks of asthma. It was D. W.'s view that her asthma aggravated the narcolepsy, and the narcolepsy, her asthma. D. D.'s symptoms virtually disappeared when remarriage brought emotional and financial security, but, to this day, nocturnal hallucinations and nightmares recur whenever her husband is away from home at night in the course of his duties as a fireman. D. W. continued to suffer from asthma all her life, and, in addition, she was never able to resolve her personal and marital conflicts. Her symptoms reached the point where they completely debilitated her.

The two nongenetic factors which stand out as the major contributors to the liability to narcolepsy in the twins are emotional stress, and environmental demands for sustained performance or vigilance during the day. Long hours of work, or the chronic demands for attention made by children may serve as examples of the latter. L. M., the other narcoleptic sister, cited very similar factors in the genesis of her symptoms. Now that she (L. M.) was free from emotional stress and from the need to work she suffered from only mild daytime drowsiness. Similarly, H. A., one of the sisters who claimed never to have had narcolepsy, suffered from excessive daytime drowsiness requiring the use of *d*-amphetamine when she had to work long hours as a waitress. At root, these two factors may indicate that narcoleptics fatigue more readily than do normal individuals and, so, require more sleep and rest. Conversely, they may indicate that narcoleptics do not sleep or rest effectively, particularly when they are under stress, and that this causes the excessive sleepiness and fatigue observed during the day.

Some evidence in support of this latter suggestion may be adduced from our polysomnographic data. The only statistically significant difference we could find between the narcoleptic and nonnarcoleptic members of the family was in the degree of sleep fragmentation. Total sleep time, NREM sleep time, sleep onset REM periods, REM time, or any of the other commonly measured sleep parameters did not consistently distinguish the sleep of the narcoleptic sisters from their siblings. But the sleep of these sisters, no matter how mild their clinical symptoms, was significantly more fragmented than the sleep of their nonnarcoleptic siblings.

We recognize that our sample is very small and that the two normal family members are not ideal control subjects. E. G. was on a mixture of drugs whose combined effect on sleep has not been established; some tricyclic antidepressants, for example, increase sleep fragmentation, at least in the early weeks after their administration (Dunleavy *et al.,* 1972). H. A., on the other

hand, may well have had a tendency towards narcolepsy herself as her history suggests. Thus her sleep, too, may have been more fragmented than that of a truly normal subject. The differences in the degree of sleep fragmentation between the narcoleptic and nonnarcoleptic siblings, then, could have been even more pronounced. It is to be noted that the short sleep fragments we measured consisted of NREM sleep or of REM sleep or of a mixture of the two. We specifically examined REM sleep fragmentation with the REM sleep efficiency method because of Montplaisir's data, which indicated that REM sleep is more fragmented in narcoleptics than in normals (Montplaisir, 1976; Montplaisir *et al.,* 1978). With this method, the REM sleep of the narcoleptic sisters appeared somewhat more fragmented than the REM sleep of their normal siblings, but the differences were not statistically significant. We were thus unable to confirm Montplaisir's findings.

We were particularly interested in the overall degree of sleep fragmentation because of the many reports that disturbed sleep was an essential feature of the illness (Daniels, 1934; Rechtschaffen *et al.,* 1963; Zarcone, 1973; Dement *et al.,* 1976) and one often critical for its development (Mitchell and Dement, 1968, Broughton, 1971; Broughton and Ghanem, 1976; Montplaisir, 1976; Montplaisir *et al.,* 1978). Indeed, our study demonstrated that excessive sleep fragmentation was a more reliable sign of narcolepsy than was the sleep onset REM period. However, we now believe that both excessive sleep fragmentation and sleep onset REM periods represent different manifestations of the same process: the tendency for narcoleptic sleep to dissociate.

Normal sleep is composed of finely articulated and closely synchronized subsystems (Dement and Mitler, 1975). Sleep fragmentation leads to their dissociation. In normal subjects, unusual procedures are necessary to achieve this. Thus, sleep onset REM periods and other signs of sleep dissociation such as intermediate sleep appear in healthy subjects when sleep is interrupted in attempts to create 90-min or 3-hr days (Weitzman *et al.,* 1974; Carskadon and Dement, 1975; Carskadon, 1976).

In narcoleptics, the pressures of ordinary living may suffice for this purpose. If, as we suggest, the breakdown of sleep into short segments represents an early sign of dissociation, then it is evident that narcoleptic individuals, like D. D. and L. M., have excessively dissociated sleep even when leading very protected lives. D. D.'s history indicated that she could not sustain even minor emotional stress without an exacerbation of her symptoms. It would have been interesting to examine her sleep patterns at these times. Earlier in her life, when she was under far more stress and was suffering from the full narcoleptic syndrome, including cataplexy, she almost certainly would have shown sleep onset REM sleep periods and other advanced signs of sleep dissociation. Sleep onset REM sleep periods are almost invariably present in patients with cataplexy (Dement *et al.,* 1976). We infer from these case studies that narcoleptics, generally, have a predisposition to sleep dissociation and that stresses which promote mild dissociation in

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normal individuals are often catastrophic for narcoleptics. The vulnerability of narcoleptics to jet lag, shift work, or other forms of sleep disruption (Mitchell and Dement, 1968; Broughton, 1971; Broughton and Ghanem, 1976; Dement *et al.,* 1976) thus becomes easier to understand.

Our study suggests that an interaction between innate and exogenous factors is necessary for the full clinical expression of the illness. This is consistent with a stress diathesis model for narcolepsy. Given a specific genetic predisposition, the symptoms and signs of the illness wax and wane with the sleep-dissociating stresses encountered. D. D., and D. W., the identical twins, provide good examples of this. Because of their very different life circumstances, D. D. suffers at present from only mild daytime drowsiness and her sleep recording appears normal; in contrast, D. W. has all the symptoms of narcolepsy and her sleep recording shows its characteristic features. D. D.'s case also illustrates the reversibility of narcoleptic symptoms and signs with changing circumstances and demonstrates that even when the sleep-dissociating pressures are mild, some indications of narcolepsy are always present if there is sufficient genetic predisposition. Conversely, when there is less innate predisposition, as in H. A.'s case, the sleep-dissociating forces must be very great before symptoms and signs appear.

The interpersonal tensions and environmental demands which appeared to precipitate and exacerbate the illness in our patients are common life stresses which cause fatigue in everyone. Narcoleptics, however, are especially sensitive because they likely are rarely well rested to begin with – their nocturnal sleep so often is fragmented and insufficient (Daniels, 1934; Rechtschaffen *et al.,* 1963; Zarcone, 1973; Montplaisir, 1976; Montplaisir *et al.,* 1978). Should they attempt to compensate for this by sleeping during the day, they further fragment and dissociate their sleep at night (Montplaisir, 1976; Montplaisir *et al.,* 1978). A vicious circle is readily set in motion, and sleep progressively dissociates until its components become distributed around the nycthemeron. Physicians have long implicitly recognized this dilemma and have counseled narcoleptics to sleep as much as they can at night. Often, hypnotics have been prescribed to assist this (Daniels, 1934; Zarcone, 1973).

With this in mind, we treated D. W., the most severely ill of the narcoleptic sisters, with a combination of γ-hydroxybutyrate (GHB) at night and methylphenidate during the day. We chose GHB because unlike the synthetic hypnotics it promoted both NREM and REM sleep and would therefore best synchronize these sleep subsystems with the nocturnal period (Mamelak *et al.,* 1977), and because tolerance had not been reported to develop to its hypnotic effects with chronic use (Vickers, 1964). D. W. takes 2.25 g of GHB at bedtime and 1.50 g upon awakening about 3 hr later. During the day, she uses 10 mg of methylphenidate before breakfast and lunch and then again in midafternoon. With this treatment, her sleep has become limited to the nocturnal period and her nightmares, hallucinations, and attacks of sleep paralysis have all disappeared.

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She is able to remain awake all day and has no sleep attacks or cataplexy. Her only remaining complaint is that she lacks as much energy as she would like to have. She has now been on this regimen for over 18 months and has not developed any adverse effects or tolerance to it (Broughton and Mamelak, 1978, 1979).

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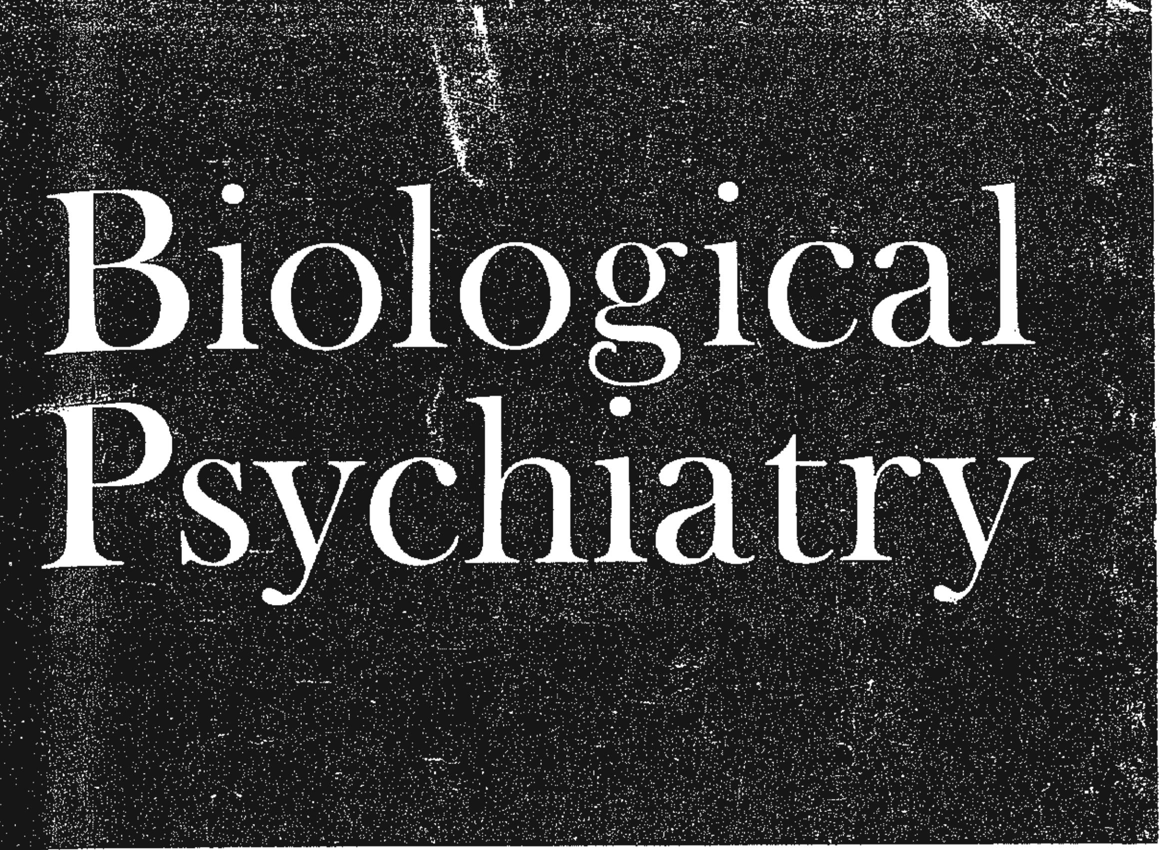

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Short Report

Treatment of Narcolepsy and Sleep Apnea with Gammahydroxybutyrate: A Clinical and Polysomnographic Case Study

Mortimer Mamelak and Peter Webster

Departments of Psychiatry and Medicine, Sunnybrook Medical Centre, University of Toronto Clinic, Toronto, Ontario, Canada

Summary: Gammahydroxybutyrate was administered to a patient who experienced narcolepsy associated with central sleep apnea. The treatment relieved the major symptoms of narcolepsy, and significantly decreased the number of apneic periods. Gammahydroxybutyrate did not cause the prolonged and potentially fatal apneic periods associated with the use of other hypnotic agents.
Key Words: Gammahydroxybutyrate—Narcolepsy—Central sleep apnea.

We have been examining the use of gammahydroxybutyrate (GHB) for the treatment of narcolepsy (Broughton and Mamelak, 1979). In this report, we present data on the use of GHB for the treatment of a case of narcolepsy associated with central sleep apnea.

CASE HISTORY

The development of narcolepsy 6 years before he was first seen in our clinic, forced our patient, a 53-year-old air traffic control instructor to leave his job. By the time he was referred to us, he had gradually increased his medications to the point where he was taking 120 mg of methylphenidate and 250 mg of imipramine daily. In spite of this, he was unable to work, and he remained chronically drowsy and subject to numerous attacks of sleep and cataplexy. He slept poorly at night and suffered from sleep paralysis and frightening nocturnal hallucinations. He told us that he snored and was a restless sleeper, and his wife added that she had often observed him to stop breathing for short periods during his sleep. Until the development of his illness, the patient had been in good health, and we were unable to find any abnormalities on examination. His blood pressure was 120/85 mm Hg,

Accepted for publication November 1980.
Address correspondence and reprint requests to Dr. Mamelak at Sunnybrook Medical Centre, University of Toronto Clinic, 2075 Bayview Avenue, Toronto, Ontario, Canada.

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there were no anatomical obstructions to his airway, and although he weighed 180 lb, at 5′10″, he was only slightly overweight. All laboratory tests and particularly the waking pulmonary function studies were normal. As an air traffic controller he had worked the morning and afternoon shift for 18 years until he became ill. Before that he had worked all three shifts for 13 years as a radio operator. There was no family history of narcolepsy or excessive daytime drowsiness.

Our initial view was that this patient had narcolepsy and sleep apnea but that he had developed tolerance to his medication and would improve if he was withdrawn and restarted on a lower dose. His diagnosis was confirmed in the sleep laboratory, and he was withdrawn from all medications. Seven weeks after withdrawal he was started on chlorimipramine, 25 mg, q.d., and methylphenidate, 10 mg, t.i.d. He immediately felt better and was able to return to work. Three months later, however, his symptoms were as florid as ever, and we decided therefore to try him on GHB, in spite of warnings about the use of hypnotic drugs in patients with sleep apnea (Guilleminault et al., 1976). We discontinued the chlorimipramine and started GHB in a dose of about 30 mg/kg twice each night. The first dose was given at bedtime, 11.00 p.m., and the second about 3 hr later, when the patient spontaneously awoke. We continued to administer the methylphenidate before breakfast and lunch and then again in midafternoon. There was a striking improvement in his nocturnal sleep. The nightmares and hallucinations disappeared, and his sleep became subjectively deeper and more continuous. Over the course of the next few weeks, there was a more gradual decrease in the number of daytime attacks of sleep and cataplexy, and after 3 months these had virtually disappeared. Daytime drowsiness, however, continued to be a problem, particularly late in the afternoon, although it was milder than it had been on the tricyclic antidepressant-methylphenidate combination. From our patient's point of view, however, the significant result of the treatment was that he was again able to work a full day. Now, 2 years after starting GHB, his improvement has been sustained, and there are no signs that tolerance or side effects are developing to his drugs.

METHODS

During the first week of the trial, GHB was administered under careful supervision in the respiratory intensive care unit. Treatment effectiveness was assessed from the patient's capacity to work during the day, as well as from the reports he provided about his symptoms. The Stanford Sleepiness Scale (Hoddes et al., 1973) was not used because of poor compliance with its requirements during the long course of the study, nor was the multiple sleep latency test, because information about this test was just beginning to appear in the literature when we undertook this trial (Dement et al., 1978).

Electroencephalographic (EEG) sleep studies were conducted with a Grass model 76B polygraph. Nasal and thoracic respiration were monitored by means of a thermistor and strain gauge, respectively. Data are presented on the sleep and respiratory patterns on 3 consecutive nights 7 weeks after the complete withdrawal of medication and then again on 3 consecutive nights during the 11th week of treatment with 2.25 g of GHB twice nightly and methylphenidate, 10 mg, t.i.d.

Note that because of circumstances in the patient's life, the total recording times on the 3 nights during the 11th week were about a half-hour less than during the drug-free period. The EEG sleep data were analyzed according to Rechtschaffen and Kales (1968) using 20 sec epochs. The sleep parameters listed in Tables 1 and 2 were defined according to Broughton and Mamelak (1980). Only respiratory arrests of 10 sec or longer in duration were counted as apneic periods. The apnea index, i.e., the number of respiratory arrests per hour of sleep, was calculated for each hour of each sleep stage, as well as for the total sleep time. Student's *t*-test was used for comparing the data obtained on drug-free nights with that obtained on treatment nights.

RESULTS

The recordings demonstrated that nocturnal sleep on base-line nights was highly fragmented; rapid eye movement (REM) sleep occurred at sleep onset on 2 of the 3 nights, and on all base-line nights, there were about 100 apneic periods of the central type, each averaging about 20 sec in duration (Fig. 1, Tables 1 and 2). The periods tended to occur in chains, with cycle times from the beginning of one apneic period to the beginning of the next of between 40 and 60 sec. They occurred predominantly during non-REM (NREM) stages 1 and 2 and during REM sleep and were uncommon during NREM stages 3 and 4 (Fig. 1, Table 3).

After 2 weeks on GHB, the total nocturnal sleep time was increased but was still only just over 6 hr. However, there was an improvement in the cohesion of nocturnal sleep, and particularly of REM sleep, at least as defined by our criteria. GHB significantly decreased the total apnea index and the index during NREM stage 1 sleep and REM sleep (Table 3). Apneic periods were also redistributed and now occurred, for the most part, during the induction period soon after the drug was given and then again as the drug was wearing off, although they could occur with arousals during the main period of action of the drug (Fig. 1). The average duration of these periods, as well as the range of their duration, was the same on GHB as off.

DISCUSSION

This case suggests that GHB can safely and effectively be used in the treatment of narcolepsy associated with central sleep apnea. As in other cases, GHB provided relief from the major symptoms of narcolepsy and at the same time significantly decreased the number of apneic periods without producing any of the prolonged and potentially fatal apneic periods reported with the use of other hypnotic agents (Guilleminault et al., 1976).

The coincidence of narcolepsy and sleep apnea is not uncommon. About 5–10% of all cases of narcolepsy are reported to show this relationship (Dement, 1976). As in our patient, apneic periods in these cases are reported to be predominantly central in type and to occur during NREM stages 1 and 2 and during REM sleep and are uncommon during NREM stages 3 and 4 (Guilleminault et al., 1972). Jung and Kuhlo (1965) and Lugaresi (1972) and his associates have argued that respiratory arrests at sleep onset or during the lighter stages of sleep represent an

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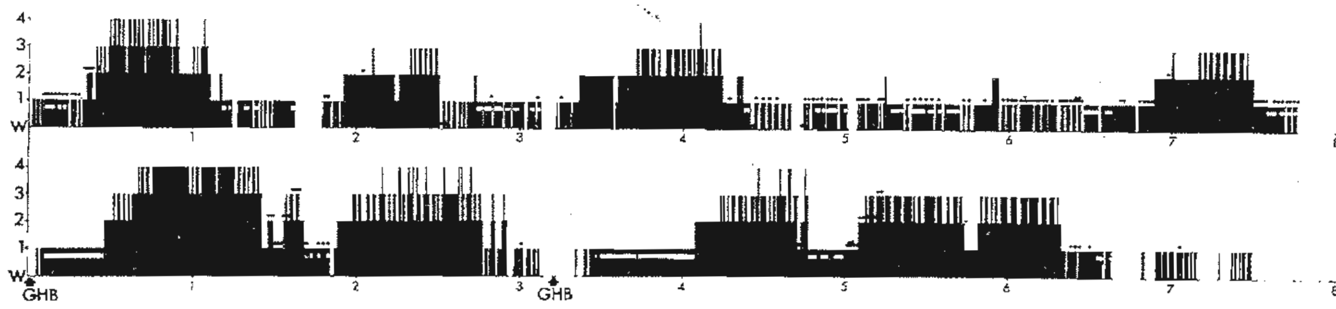

FIG. 1. The sleep pattern 7 weeks after the withdrawal of all drug treatment (top) and the pattern during the 11th week of gammahydroxybutyrate (GHB) treatment. Both nights are the second of the 3 consecutive nights recorded at these times. The vertical axis refers to the stages of NREM sleep (where W represents wake), and the horizontal axis refers to time in hours. The horizontal white bars at the level of stage 1 refer to REM sleep, and the closed circles above each night's sleep pattern indicate the apneic periods.

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TABLE 1. *Sleep parameters during base-line nights*

Parameter	Night 1	Night 2	Night 3	Average	SD
Total recording time (min)	481.00	469.00	482.33	477.44	7.34
Stage (min) Wake	131.33	103.00	122.66	119.00	14.52
1	107.00	117.33	108.66	111.00	5.55
2	100.33	114.66	133.66	116.22	16.72
3	52.66	50.33	40.33	47.77	6.55
4	18.00	8.33	5.33	10.55	6.62
REM	61.66	64.00	63.00	62.89	1.17
Movement time (min)	10.00	11.33	8.66	10.00	1.34
Total sleep time (min)	339.66	354.66	351.00	348.44	7.82
No. apnea episodes	71	88	136	98.33	33.71
Average apnea duration (sec)	18.86	17.98	18.99	18.61	0.55
Range (sec)	10–38	10–28	10–34	—	—
REM latency (min)	0.00	0.00	76.33	25.44	44.07
REM density (%)	26.48	25.63	28.52	26.88	1.49
No. REM sleep periods	6	8	6	6.67	1.15
REM sleep efficiency (%)	55.22	60.19	68.48	61.30	6.70
Shifts out of NREM sleep per 100 min NREM sleep	6.43	6.21	10.59	7.74	2.47
Shifts out of REM sleep per 100 min REM sleep	66.49	51.56	50.79	56.28	8.85
Sleep fragments (%)					
0–30 min	77.70	67.66	53.73	66.36	12.04
31–60 min	22.29	32.34	13.20	22.61	9.57
61+ min	0.00	0.00	33.06	11.02	19.09

TABLE 2. *Sleep parameters after 11 weeks of treatment with gammahydroxybutyrate*

Parameter	Night 1	Night 2	Night 3	Average	SD
Total recording time (min)	433.66	449.00	444.33	442.33[a]	7.86
Stage (min)					
Wake	45.00	72.66	54.33	57.33[a]	14.07
1	83.66	68.00	59.33	70.33[a]	12.33
2	130.33	98.33	109.00	112.55	16.29
3	76.66	86.00	70.33	77.66[a]	7.88
4	46.00	46.66	58.00	50.22[a]	6.75
REM	44.66	69.66	86.33	66.88	20.97
Movement time (min)	7.33	7.66	7.00	7.33[b]	0.33
Total sleep time (min)	381.33	368.66	383.00	377.66[a]	7.84
No. apnea episodes	42	46	58	48.67[b]	8.33
Average apnea duration (min)	18.62	20.59	23.48	20.90	2.44
Range (sec)	10–36	10–34	10–40		
REM latency (min)	63.00	0.00	0.00	21.00	36.37
REM density (%)	12.24	9.71	7.34	9.76[a]	2.45
No. of REM periods	4	5	5	4.67[b]	0.58
REM sleep efficiency (%)	60.00	81.00	92.17	77.72	16.33
Shifts out of NREM sleep per 100 min NREM sleep	7.11	4.33	5.06	5.50	1.44
Shifts out of REM sleep per 100 min REM sleep	31.35	24.40	16.22	23.99[a]	7.57
Sleep fragments (%)					
0–30 min	67.58	12.66	32.69	37.64	27.79
31–60 min	32.41	39.65	47.98	40.01	7.79
61+ min	0.00	47.69	19.33	22.34	23.98

[a] Significantly different from the corresponding base-line value at $p < 0.01$.
[b] Significantly different from the corresponding base-line value at $p < 0.05$.

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TABLE 3. *Apnea index before and during the 11th week of gammahydroxybutyrate (GHB) treatment*

Sleep stage	Apnea index		p value
	Base line	GHB	
1	31.14	19.08	<0.05
2	11.46	8.10	ns
3	0.72	2.04	ns
4	0.00	0.36	ns
REM	14.64	7.14	<0.05
Total sleep time	16.92	8.22	<0.05

exaggeration of the normal physiological tendency to periodic respiration at sleep onset. This periodicity, they suggested, tends to disappear during the deeper stages of sleep due to an undefined stabilization process (Lugaresi et al., 1978*a*). If, as we propose, respiratory stabilization is dependent on the recruitment and integration of a sufficient number of sleep subsystems, this process would be impaired in narcolepsy by the natural tendency of the sleep subsystems to dissociate in this condition (Dement, 1976; Broughtou and Mamelak, 1980). The high incidence of sleep apnea in narcolepsy would thus be the result of this integrative failure. On the other hand, once apnea occurs, it would tend to perpetuate itself, because the arousal terminating each apneic period would interfere with the further recruitment and integration of sleep. If GHB acts by promoting the integration of sleep (Broughton and Mamelak, 1980), then its stabilizing effect on respiration becomes understandable.

Warnings have been issued about the use of respiratory depressants such as opiates, anesthetics, and synthetic hypnotics in patients with sleep apnea (Guilleminault et al., 1976; Lugaresi et al., 1978*b*). GHB, however, is not a respiratory depressant. Although hypnotic doses of GHB are reported to decrease the ventilatory rate, they also increase its amplitude and do not change the overall minute rate of ventilation. And even with high doses of the drug, the respiratory center retains its sensitivity to increases in the partial pressure of CO_2 (Laborit, 1964). Nevertheless, it should be noted that with such anesthetic doses, Cheyne-Stokes breathing occasionally occurs (Laborit, 1964; Steel, 1968), especially when GHB is combined with premedicants. Thus the full effects of GHB on the respiratory mechanisms are not simple and require further definition. Our study, however, suggests that GHB can be used to advantage in the management of patients with narcolepsy-cataplexy and central-type sleep apneas. Further studies are now being undertaken in our laboratory to confirm this finding, as well as to explore the usefulness of GHB in the more common mixed and obstructive types of sleep apnea.

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SLEEP

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EDITORS-IN-CHIEF:

William C. Dement, M.D., Ph.D.
Christian Guilleminault, M.D.
Sleep Research Center TD-114
Stanford University School of Medicine
Stanford, California 94305, U.S.A.

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Gammahydroxybutyrate: An Endogenous Regulator of Energy Metabolism

MORTIMER MAMELAK[1]

Department of Psychiatry, Sunnybrook Medical Centre
University of Toronto, Toronto, Ontario, Canada

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MAMELAK, M. *Gammahydroxybutyrate: An endogenous regulator of energy metabolism.* NEUROSCI BIOBEHAV REV 13(4) 187–198, 1989.—Gammahydroxybutyrate is a naturally occurring metabolite of many mammalian tissues. Although its administration produces a wide range of pharmacological effects, its normal function has never been clearly defined. GHB can induce NREM and REM sleep, anaesthesia, hypothermia, and a trance-like state which has been considered a model for petit mal epilepsy. It markedly increases brain dopamine levels. It has been touted as a central neurotransmitter or neuromodulator, and high affinity brain receptors, as well as central mechanisms for its synthesis, uptake and release have been demonstrated in support of this. But GHB is also found in many peripheral tissues and in some of these in higher concentrations than in the brain. No explanation has been offered for its presence in these tissues. A number of studies indicate that GHB can reduce energy substrate consumption in both brain and peripheral tissues, and that it can protect these tissues from the damaging effects of anoxia or excessive metabolic demand. Indeed there is some evidence to suggest that endogenous GHB levels rise under these circumstances. GHB appears to act through the endogenous opioid system, since in the brain, at least, GHB raises dynorphin levels and its metabolic and pharmacological effects can be blocked by naloxone. These, and other observations detailed in this review, suggest that GHB may function naturally in the induction and maintenance of physiological states, like sleep and hibernation, in which energy utilization is depressed. GHB may also function naturally as an endogenous protective agent when tissue energy supplies are limited.

Gammahydroxybutyrate Energy metabolism Sleep Hibernation Anoxia

WHAT is the natural function of gammahydroxybutyrate (GHB)? This simple pharmacologically powerful 4 carbon molecule is a normal constituent of many mammalian tissues (131), yet its metabolic role has never been clearly defined in any of these tissues (110, 164, 196). Because of its striking effects on behaviour and because of the extraordinary range of these effects, most investigators have limited their research to studies on the central nervous system. The administration of GHB has been shown to cause rapid and dramatic increases in brain dopamine levels (47,145), and the trance-like state induced by the drug has been touted as a model for petit mal-like seizure disorders (169). To explain these phenomena, it has been proposed that GHB acts as a neurotransmitter or neuromodulator, and in support of this, high affinity brain receptor sites, as well as central mechanisms for its synthesis, release and uptake have been demonstrated (110,196). However, to confirm its role as a neurotransmitter, the effects of GHB must be related to the activity of specific neuronal tracts. This, as yet, has not been achieved.

GHB is also found in many peripheral organs, and in some of these, in concentrations far higher than in whole brain (131). For example, in comparison with the 2 nm/g average concentration in rat brain, brown fat has a GHB concentration of 37 nm/g. Resting tissue levels in kidney, heart and skeletal muscle are also considerably higher than in the brain. Receptors for GHB have not been demonstrated in peripheral tissues (174). Nevertheless, the activity of enzymes capable of oxidizing GHB are 10 to 20 times greater in many of these tissues than in the brain (80). The functions and effects of GHB in the periphery have received far less attention.

Clinical and experimental work indicate that GHB can protect both central and peripheral tissues from the damaging effects of hypoxia or excessive metabolic demand (37, 95, 104, 118). Under these conditions GHB appears to depress cell energy requirements. In the nervous system, where this matter, again, has received the most attention, it has been shown that GHB can produce a profound and fully reversible depression of cerebral energy metabolism in tandem with a widespread but equally reversible inhibition of neuronal activity (208). These effects are dose related and can initially be observed with quite low concentrations of the drug. This suggests that GHB could function naturally to regulate cell activity when metabolic energy is in short supply. The existence of endogenous agents with these properties has been invoked to explain the survival of organisms and tissues in anoxic environments, as well as in the induction of biological states, like sleep and hibernation, in which energy utilization is depressed (68–70).

This essay will examine the major foci of GHB research. In the years that followed its introduction into clinical medicine by Laborit in 1960 (90), information was gathered on the sleep-

[1]Requests for reprints should be addressed to M. Mamelak, M.D., Sunnybrook Medical Centre, 2075 Bayview Avenue, Toronto, Ontario, Canada M4N 3M5.

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inducing and anaesthetic properties of GHB, and in relation to this, on its cardiac, respiratory and thermoregulatory effects (88, 129, 198). The trance-like states induced by GHB then raised questions about its epileptogenic properties, and this in turn, to numerous experiments on its interactions with other centrally active agents (164, 172, 192, 196). A substantial effort, which continues today, was made to determine its tissue source, its kinetics and its synthetic and catabolic pathways (110, 175, 194, 196). Other work was devoted to determining the reasons for the remarkable doubling of brain dopamine concentration observed after the administration of GHB (47, 145, 151, 204). It was hoped that this effect might be used to advantage in the treatment of Parkinson's disease, or one of the other disorders of brain dopamine metabolism. In recent years, work on the tissue protective properties of GHB has gained centre stage. This application has provided the unifying insight that all of the disparate physiological changes induced by GHB may well reflect a single fundamental action on cell energy metabolism. The evidence in favour of this point of view will be presented at the conclusion of this review.

BEHAVIOUR

The central nervous system in man has been found to be remarkably sensitive to the depressant effects of GHB. Clinical studies have demonstrated that as little as 10 mg/kg can produce amnesia and lower the critical flicker fusion frequency (59,60). Doses in this range abolish monosynaptic reflexes, produce hypotonia and have been used therapeutically to relieve spasticity in children with cerebral palsy (109, 191, 204). Oral or intravenous doses of 20 mg/kg to 30 mg/kg promote the normal sequence of NREM and REM sleep when given to normal subjects at bedtime (92,209). Oral doses in this range given during the day produce high voltage slow wave activity and occasionally spindling sleep (109). The sensitivity of sleep mechanisms, and REM sleep in particular, to low doses of GHB is better illustrated when this agent is given to patients with narcolepsy or major depressions. In these conditions, GHB regularly induces REM sleep and sleep paralysis (107). Unique amongst centrally active agents, GHB can induce severe and often prolonged attacks of cataplexy (107,138). This reflects the dissociated activation by GHB of the motor inhibitory component of the REM sleep mechanism. The capacity to promote both NREM and REM sleep has been exploited therapeutically to consolidate the night sleep of narcoleptics, and in this way, improve their alertness during the day (22, 23, 108, 154). Since the oral administration of GHB in a dose of 100 mg/kg produces peak serum levels of about 100 μg/ml, the effects on sleep and motor mechanisms observed with oral doses of 30 mg/kg likely occur at serum levels as low as 50 μg/ml or less (71). It would be useful to know the brain tissue levels at different sites at this time. Although sleep mechanisms in animals are also sensitive to activation by GHB, these model systems have not been fully utilized to define the mechanisms involved (25,117).

In man, oral or intravenous doses greater than 50 mg/kg produce anaesthesia (14, 67, 121, 155, 198). The EEG initially shows intermittent periods of rhythmical high voltage slow activity of 2 c/sec–5 c/sec and 100 μV–200 μV in amplitude against a background alpha rhythm. These theta and delta rhythms then become continuous and at an undefined point consciousness is lost. Sleep spindles may be observed. The record then becomes dominated by rhythmic and nonrhythmic delta activity until, with higher doses, progressively longer periods of electrical silence appear punctuated by K-like complexes. As GHB wears off, these patterns recur in reverse order. Unlike the barbiturates, GHB does not induce an initial period of fast activity. The drug is rapidly metabolized and the central effects of an intravenous dose of 60–70 mg/kg run their course in about 2 hours. These doses lead to initial plasma levels of 200 μg/ml to 300 μg/ml (67). A peculiar feature of these GHB-induced states is that full consciousness may be sustained initially in the presence of continuous high voltage slow activity. In contrast, stimulation during coma may evoke alpha activity in unconscious subjects (121).

The behavioural and electroencephalographic effects of increasing doses of GHB in animals have also been carefully documented. Similar effects have been noted in the cat (178,207), rat (112), rabbit (54), dog (161) and monkey (165). In the rat, for example, 600 mg/kg intraperitoneally produces a reversible continuum of EEG changes characterized by intermittent rhythmic high amplitude slow waves initially which then become continuous and somewhat slower. These give way to isoelectric periods punctuated by polyphasic bursts and spikes. During the intermittent and continuous high amplitude slow wave activity, the rat is immobile, crouched with eyes open. Arousal is possible at this time. But the righting reflex is lost during the period of electrical silence. Complete recovery occurs about 2 hours after the drug has been given. It has been estimated that with these doses, peak serum GHB levels of about 500 μg/ml are attained (61,99). Brain GHB concentrations reach 100 μg/g to 150 μg/g. The righting reflex disappears with tissue concentrations above 100 μg/g. This is about 500 times the average normal brain concentration.

As much as 1,000 mg/kg has been given to monkeys intravenously without harmful effects (165). Serum levels as high as 2,300 μg/ml have been reached. In this species, the initial slowing of the EEG is followed by synchronous high voltage slow activity punctuated by spikes. At plasma levels greater than 300 μg/ml, prepubescent monkeys show paroxysmal rhythmic 2 and a half to 3 per second high voltage slow waves associated with spikes. In general, older monkeys are less sensitive to the effects of GHB than younger monkeys. Nevertheless, when plasma levels exceed 500 μg/ml all animals show myoclonic seizures. At this time, the animals appear to be in a trance-like state and are unresponsive to all stimuli.

Some investigators have been impressed with the similarity between the EEG and behavioural effects of GHB in animals and the clinical spectrum of myoclonus and petit mal seizure disorders. In support of this, it has been shown that agents like pentylenetetrazol and systemic penicillin, which are commonly employed to induce models of absence seizure states in animals, significantly prolong the duration of the EEG changes produced by GHB (169). In addition, in the rat and monkey, the antipetit mal drugs, trimethadione, valproate and ethosuccimide have been shown to antagonize the behavioural and EEG changes produced by GHB (55,166). However, doubts about the epileptic nature of the induced states have been raised by the high doses of GHB necessary for their induction. As discussed earlier, in man, for example, sleep patterns are commonly observed on the EEG when low doses of this drug are given (92, 107, 121, 209) and even higher doses which are sufficient to produce anaesthesia and plasma GHB levels in excess of 200 mg/ml do not produce epileptiform activity on the EEG (121). In rats, low intraperitoneal doses of GHB, between 50 mg/kg and 100 mg/kg, also induce wave forms on the electrocorticogram which are similar to those found naturally in this species in slow wave sleep (56). Finally, a recent study with rhesus macaque monkeys, given 150 mg/kg or 250 mg/kg gamma-butyrolactone orally, concluded as well that the induced EEG changes and the associated behavioural state had more in common with sleep than with nonconvulsive epilepsy (130). Indeed, as will be discussed in greater detail later in this review, both sleep and GHB reduce the cerebral metabolic rate, while even absence seizures or nonconvulsive epilepsy increase this rate (40).

Agents such as caffeine (113), d-amphetamine (167) and

naloxone (170) can block the EEG and behavioural effects of GHB, and yet are not effective against petit mal. Thus, the antagonism between GHB and valproate, ethosuccimide and trimethadione could be based on mutual but opposing metabolic effects which are independent of the antiepileptic mechanisms of action of these drugs. It is noteworthy that valproate and ethosuccimide appear to prevent GHB catabolism and that they actually increase brain tissue levels of this substance (172,192). For this reason, it has been argued that they antagonize the actions of GHB by blocking the release of this substance from cerebral tissue (192,193). On the other hand, it has also been demonstrated that GHB and the antiepileptic drugs both produce significant changes in the relative concentration of different opioid peptides in the brain (93). Indeed, naloxone, ethosuccimide, valproate and trimethadione have all been shown to block the seizure activity and behavioural abnormalities induced by the opioid peptide, leucine-encephalin (171). Thus, common but opposing actions on opioid peptide metabolism may account for the antagonism between naloxone, the antipetit mal agents and GHB. It should be mentioned that this antagonism does not take place at the level of the GHB receptor. Neither naloxone nor any of the antipetit mal agents cited above competes with GHB for its receptor (174). The exact relationship, then, between petit mal epilepsy and the trance-like state induced by GHB remains to be determined. It can be stated more confidently, however, that GHB can block seizure activity induced by a variety of agents. Those induced by kainic acid (34,211), strychnine (88), isoniazid (88) and mercaptopropionate (189) may be cited as examples.

HEART, RESPIRATION AND TEMPERATURE

When used as an anaesthetic agent in man, in an intravenous dose of 65 mg/kg, spontaneous ventilation is maintained with little change in rate or volume (199). Cardiac output falls somewhat, reflecting both a slight decrease in stroke volume and heart rate. GHB currently finds its greatest use in a number of continental European countries as an anaesthetic agent during labour and delivery. It does not impair uterine contractions, and it may even help relax the cervix. Most important, it does not appear to depress the fetus (46, 88, 129, 190).

Respiratory depression will occur with very high doses, and again, young animals appear to be more sensitive to GHB than adult animals. Thus, an intraperitoneal dose of 750 mg/kg produces a 40% decrease in the minute ventilation in the adult rat (64), but the same dose subcutaneously results in apnea and cyanosis in rat pups (63). It is noteworthy that in spite of the apnea, this dose is rarely lethal. As has been demonstrated in other circumstances, GHB appears to promote survival under hypoxic conditions (7,104).

Marked dose-dependent hypothermic effects have been recorded with GHB in a number of animal species including the mouse (74), rat (100), dog (161) and monkey (165). A careful study of this phenomenon in the rat has revealed that heat loss is brought about by a decrease in metabolic heat production as well as by an increase in cutaneous circulation. The anaesthetic and dopaminergic changes described earlier are independent of the hypothermic effects of GHB and continue to be observed even when the animal is kept warm (18, 47, 74). The drop in body temperature produced by GHB can be blocked by naloxone (29) as well as by the dopamine receptor antagonist haloperidol (100). This latter finding, together with the observation that other dopamine agonists like apomorphine and dopamine itself, lower body temperature, would suggest that GHB facilitates the release of dopamine. However, there has not been universal agreement about this.

DOPAMINE

A great deal of attention has been devoted to the effects of GHB on brain dopamine metabolism (3, 4, 25, 47–49, 145, 150, 151, 204). This is because GHB and its congeners, gamma-butyrolactone and butanediol, produce a dose-dependent increase in the concentration of dopamine in the striatal and cortical regions innervated by the nigro-striatal and mesocortical dopaminergic neurones while simultaneously, and paradoxically, depressing activity in these neurones. Brain dopamine levels can double within the hour while almost no effect is observed on brain noradrenaline concentrations. These neurotransmitter changes are not observed with any other central nervous system depressant. Agents such as halothane or chloral hydrate actually increase neuronal activity in the nigro-striatal tracts (25). Since the depression of nigro-striatal activity by other means, such as transection of the tracts, or the application of GABA or tetrodotoxin, also raises dopamine levels in the caudate, the two phenomena appear closely related (3, 4, 183).

Nevertheless, in spite of much effort, the nature of this relationship has proved elusive. Elevated brain dopamine levels following GHB have been demonstrated in rats (47), rabbits (49), mice (74), and cats (182). In rats, it has been shown that pretreating the animal with GHB can produce an activation of tyrosine hydroxylase isolated from dopamine rich areas of the brain which can be measured in vitro, and alter the behaviour of this enzyme so that it is no longer sensitive to end product inhibition by dopamine (126,127). Calcium chelators produce the identical change. For this reason, it has been argued that GHB-induced neuronal inhibition blocks neuronal calcium influx and that this in turn alters the kinetic properties of tyrosine hydroxylase to increase the rate of dopamine synthesis. Tyrosine hydroxylase isolated from noradrenaline rich areas of the brain lacks this calcium sensitivity and, thus, cannot be activated by GHB. This explains GHB's inability to raise brain noradrenaline levels. However, it is not clear how GHB inhibits neuronal activity, nor have the effects of GHB on calcium mobilization actually ever been measured, in nervous tissue, or anywhere else for that matter. And although there is indirect evidence for the activation of tyrosine hydroxylase by GHB in vivo (26, 151, 203) not all investigators have been able to corroborate the activation of tyrosine hydroxylase by GHB in vitro (187,213). Even the observation that GHB modifies tyrosine hydroxylase so that it is no longer sensitive to end product inhibition by dopamine has been challenged. Rather, evidence has been presented that increased storage of dopamine in reserpine sensitive vesicles occurs after treatment with GHB, and that this accounts for the absence of end product inhibition of the enzyme (74, 103, 180). Finally, the cause and effect relationship between neuronal inhibition and elevated dopamine levels has been placed in doubt by the observation that GHB can raise caudate dopamine levels in newborn rat pups before neuronal activity has been established in the nigro-striatal tracts (26).

Inhibition of dopamine catabolism by GHB is unlikely to be a factor in the elevation of brain dopamine. GHB does not inhibit monoamine oxidase or catechol-o-methyl transference, the two enzymes responsible for the breakdown of dopamine (48). Brain dopamine levels could be increased if GHB prevented the release of this neurotransmitter. A fall in brain dihydroxyphenylacetic acid (DOPAC) levels immediately after GHB administration in the rat has been cited as evidence for this (145, 149, 204). However, the initial fall in DOPAC levels has not been observed in all species (74), and in the cat, direct measurement of dopamine release with the push-pull cannula technique has shown that GHB actually produces a graded dose-dependent increase in the rate of dopamine release (25).

More work is evidently needed to fully comprehend the sequence of events leading to the rise in brain dopamine concentration. Whatever the mechanism, all investigators agree that the

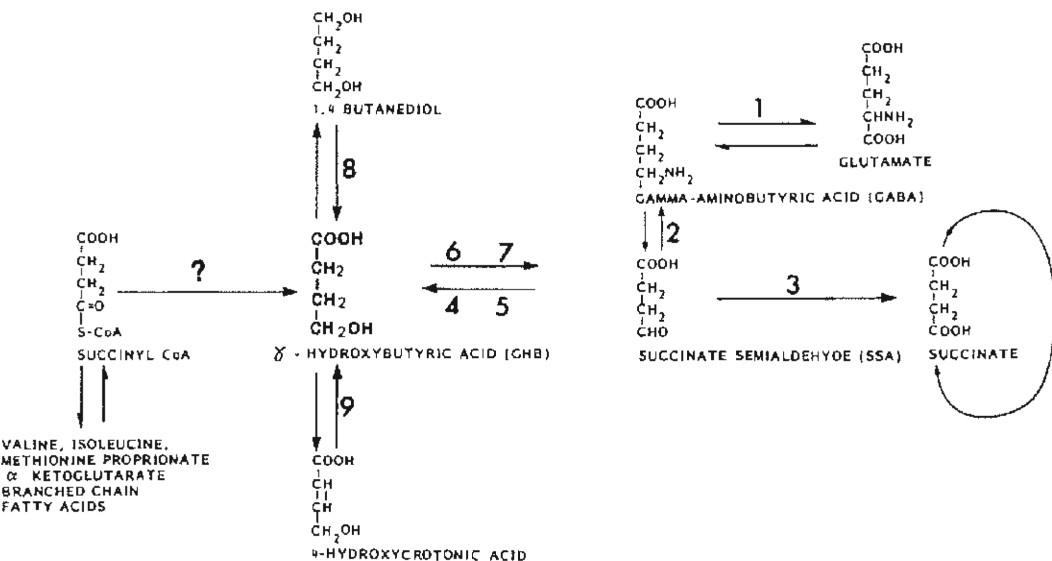

FIG. 1. (1) Glutamic acid decarboxylase, (2) gamma aminobutyric acid transaminase, (3) succinic semialdehyde dehydrogenase, (4) ALR 2, (5) specific succinic semialdehyde reductase, (6) ALR 1, (7) mitochondrial pyridine nucleotide independent oxido-reductase, (8) alcohol dehydrogenase and acetaldehyde dehydrogenase, (9) beta oxidation pathway. See text for further details.

increase is unrelated to GHB's sedative and anaesthetic actions. Sedation and the loss of the righting reflex can be observed with GHB even in animals in which the rise in brain dopamine has been prevented by pretreatment with alpha methyl paratyrosine (48) or by the destruction of dopaminergic neurones (17). EEG slowing can be induced by doses of GHB which are insufficient to alter brain dopamine levels (168), although this last observation has been cast in doubt by a recent report which indicates that intraperitoneal doses of GHB as low as 50 mg/kg can raise these levels (122).

ACETYLCHOLINE, SEROTONIN AND NORADRENALINE

Many anaesthetic agents increase brain acetylcholine levels (159,204). This again has been related to the concomitant depression of cholinergic neuronal activity produced by these agents. In keeping with this, it has been shown that transection of cholinergic tracts in the brain also raises acetylcholine levels in their terminals (158). Gamma-butyrolactone, too, can elevate brain acetylcholine levels, although high doses are necessary (91). The relationship between this gamma-butyrolactone effect and activity in cholinergic neurones has never been directly examined, although given the findings with other central depressants and with tract transection, it has been inferred that the accumulation of acetylcholine is related to neuronal depression. Systemically administered gamma-butyrolactone has been shown to be rapidly converted to GHB by lactonases present in serum and liver but not in brain (147). GHB, however, does not increase brain acetylcholine levels even when given in high doses (91). This finding indicates that the two agents may not have identical effects under all conditions. In high doses sufficient gamma-butyrolactone may enter the nervous system and escape hydrolysis. Under these circumstances its unique actions may become apparent.

GHB has been reported to either increase the turnover rate of brain serotonin (65,179) or to elevate its levels in certain regions (200). It has also been shown to depress neuronal activity in the dorsal raphe to some extent (204). However, it has been argued that these elevations are not related to inhibition of serotonergic neuronal activity because surgical procedures which impair impulse flow in these neurones do not elevate serotonin levels in the terminals (200). GHB does not depress noradrenergic neurones (204) and as mentioned earlier, it has no effect on brain noradrenaline levels. In contrast, GHB profoundly depletes the heart and brown fat of noradrenaline (12).

SYNTHESIS AND ORIGIN OF GHB

The structural similarity of GHB to gamma-amino butyric acid, GABA, would suggest that the two compounds have common physiological properties. Indeed, GHB was first used clinically in the hope that it would serve as a precursor for GABA and as a GABA-mimetic agent, since in distinction to GABA, GHB crosses the blood-brain barrier (88). However, this has not turned out to be the case. Although one study demonstrated that GHB increased brain GABA levels (33), other studies have failed to confirm this observation (50, 114, 124) and indeed, have favoured the view that exogenously administered GHB must first be oxidized to succinate before GABA can subsequently be formed from citric acid cycle intermediates (32, 35, 36, 125). On the other hand, it has been shown that GABA can be converted almost directly to GHB (58,148) and the pathway between these two compounds has been carefully examined (110,196) (Fig. 1). It has been shown that GABA is catabolized to succinic semialdehyde (SSA) and oxidized to succinate (24). A small fraction of SSA is then reduced to GHB (24, 58, 152, 153). The conversion of GABA to SSA and succinate is catalyzed in mitochondria by GABA-transaminase and an NAD-dependent succinic semialdehyde dehydrogenase. The conversion of SSA to succinate is irreversible, and it has been argued that the conversion of SSA back to GABA is also functionally irreversible in vivo because the

concentration of SSA in the brain is many orders of magnitude lower than its Km for the reverse transamination reaction (24). Nevertheless, studies with brain tissue homogenates (124,195), cerebellar slices (195) and purified enzyme preparations containing GHB dehydrogenase and GABA transaminase (197) have shown that GHB can be directly transformed into GABA without first being oxidized to succinate as the in vivo studies would suggest. In vitro, then, the conversion of GABA to SSA does appear to be reversible.

The small fraction of SSA which escapes oxidation to succinate is reduced to GHB by two NADPH-dependent cytoplasmic aldehyde reductases designated ALR-2 and SSA reductase (28, 152, 153). The latter is specific for SSA. It has been estimated that between 0.08% to 0.16% of whole brain GABA is converted to GHB each minute (24, 58, 152, 153). The specific SSA reductase has been found in most of the neuronal cells examined which contain glutamate decarboxylase, the enzyme responsible for the synthesis of GABA (205,206). Peripheral tissues, however, contain very low GABA concentrations. For example, in the liver and kidney GABA levels are less than 1% those in the brain (38). Yet the GHB levels in some peripheral tissues may be more than 10 times higher than in brain (131). Is there another tissue source for GHB (175)? Some investigators have proposed that 1,4 butanediol and 4-hydroxycrotonoate are alternative precursors for GHB in both the periphery and brain (8,194). A possibility never before considered is that GHB may be derived from succinyl-CoA in a reaction analogous to the reduction of hydroxymethylglutaryl-CoA (76). The concentration of GHB would then be regulated by the activity of a unique reducing enzyme. Evidence for this pathway is now being sought by our group.

CATABOLISM OF GHB

The steady synthesis of GHB must be balanced by its catabolism. The kinetic properties of the two aldehyde reductases SSA and ALR-2 indicate that this route is functionally irreversible (24, 152, 153). The reduction of SSA is strongly favoured over the oxidation of GHB. There is some evidence in the brain as well as in the periphery for the catabolism of GHB via beta oxidation. The beta oxidation product, 4 hydroxycrotonoic acid, has been isolated from the brain (194) and kidney (132) and beta oxidation products have been detected in the urine (44, 96, 201). These products have also been recovered from the urine of patients with gammahydroxybutyricaciduria, a rare inborn error of metabolism which has been attributed to a deficiency of succinic-semialdehyde dehydrogenase (51, 52, 75). Significantly, a distinct NADPH-dependent aldehyde reductase (ALR-1), different from those involved in GHB formation, has been isolated from brain, liver, kidney and brown fat. This enzyme promotes the coupled oxidation of GHB to SSA to the reduction of glucuronate to gulonate, and this seems to be the favoured route for GHB catabolism (78–80, 82). Thus, SSA is both a product and precursor of GHB. The activity of ALR-1 is 10 to 20 times greater in peripheral tissues than in brain (80). ALR-1 can be inhibited by acetylsalicylic acid and by alpha-keto analogues of branched chain or aromatic amino acids (82). Like ALR-2, it is sensitive to inhibition by barbiturates and valproate (24,80) and this may be the reason for the elevated brain levels of GHB mentioned earlier following valproic acid administration (172). More recently, evidence for a mitochondrial pyridine nucleotide independent GHB-oxidizing enzyme has been found (81). In brain, over 40% of GHB is oxidized through this route. Even a higher proportion of GHB is oxidized by this enzyme in liver. The metabolic pathways of GHB have been described here in some detail because the formation of this metabolite through a reductive process, and its breakdown through oxidation, suggests that it would accumulate under hypoxic conditions. The significance of this will be elaborated upon later in this review.

RELATIONSHIP BETWEEN GABA AND GHB

In spite of the close structural and metabolic relationship between GABA and GHB, cellular processes clearly distinguish the two. Although the published data are not entirely consistent, it is for the most part true that neither competes with the other for its receptors (11, 41, 72, 101), or for its uptake (10, 62, 135) and release mechanisms (135). However, GABA and GHB do have one important property in common: both depress neuronal activity throughout the neuraxis (85,134). Indeed, they appear to potentiate each other in this regard. Iontophoretic application of GABA and GHB to nigral and neocortical cells shows that both produce neuronal depression, although GABA is more powerful in this regard than GHB. Some investigators report that the actions of GHB can be blocked by the GABA antagonist bicuculline (73,85) while others find that the opposite is true (134,135). Both GHB and GABA increase chloride conductance (73) and recent evidence suggests that GHB, like GABA, acts at the chloride channel (177). The systemic administration of a low dose of GHB, 300 mg/kg intraperitoneally, produces widespread inhibition of neocortical activity (134). Although nigral cells are less sensitive than neocortical cells to the inhibitory effects of GHB, it nevertheless has been demonstrated that intravenous doses of GHB of 150 mg/kg inhibit nigrostriatal activity by 50% (146). If dopamine accumulation in the forebrain can be taken as a sign of neuronal inhibition, then there is evidence that this can be achieved with doses of GHB as low as 50 mg/kg given intraperitoneally (122). Even lower doses in the range of 5 mg/kg infused directly into the cerebral ventricles can depress behaviour and alter the function of dopaminergic neurones (141). Because of the sensitivity of the brain to these low doses, it has been argued that endogenous GHB could play a role in the regulation of neuronal activity. Only small shifts in its rate of production would be necessary for its tissue concentration to rise to levels necessary for such functional effects (146).

EFFECTS OF GHB ON ENERGY METABOLISM

The actions of GHB on brain carbohydrate and energy metabolism mirror this inhibitory effect on neuronal activity. Doses of GHB or of its precursor gamma-butyrolactone, which hardly alter behaviour, and produce only minimal slowing of the EEG, have profound effects on tissue carbohydrate intermediates and tissue energy metabolism (45, 57, 84, 98, 104, 105, 185, 186). In the rat, for example, intravenous doses of GHB ranging from 250 mg/kg to 2,000 mg/kg, or of gamma-butyrolactone ranging from 150 mg/kg to 1,200 mg/kg significantly increase brain concentrations of glycogen and glucose and decrease concentrations of pyruvate, lactate, alpha ketoglutarate and malate (105). Phosphocreatine levels are raised while tissue levels of ATP, ADP and AMP are unchanged. In keeping with this apparent decline in energy substrate consumption, cerebral glucose utilization, as measured with the (C14) deoxyglucose technique, undergoes a significant and dose-related decline starting with intravenous doses of gamma-butyrolactone as low as 75 mg/kg (208). A homogenous inhibition of glucose uptake by grey and white matter is observed. Although no area of the brain is more sensitive than any other, inhibition of glucose utilization in grey matter is far greater than in white matter. An intravenous dose of 600 mg/kg of gamma-butyrolactone, for example, reduces glucose utilization in grey matter by 68% compared with 44% in white matter. Similar results have been reported with GHB (87). It is remarkable that in spite of these extraordinary degrees of metabolic depression full tissue

recovery can take place. Other central nervous system depressants such as the barbiturates also produce comparable effects on intermediary energy metabolism (45,185) but doses of barbiturates which would be necessary to inhibit cerebral glucose utilization as much as that observed with gamma-butyrolactone and GHB would likely be lethal (208). Indeed, all investigators who have compared the actions of the barbiturates with GHB or butyrolactone on tissue carbohydrate and high energy phosphate levels have commented that GHB depresses energy utilization without inducing the deep anaesthesia necessary for effects of similar magnitude with the barbiturates (45, 98, 104).

Studies on the metabolic effects of GHB in cerebral tissue slices have revealed other important differences between this agent and the barbiturates. Although both inhibit glycolysis, GHB augments oxygen consumption, activates glucose 6 phosphate dehydrogenase and increases flux through the pentosephosphate shunt (84,186). These effects have never been demonstrated with the barbiturates. The GHB-induced shift of carbohydrate metabolism to the pentose pathway is limited to the brain and requires intact cells for its demonstration. It has not been observed in diaphragm or kidney. The stimulation of oxygen uptake observed with GHB in cerebral slices requires glucose. GHB does not stimulate respiration in these slices in the absence of substrate. In liver, even in the presence of glucose, stimulation of oxygen uptake by GHB cannot be demonstrated. Studies on the effects of GHB on metabolism in peripheral tissue are sparse and more information is needed.

TISSUE PROTECTION

The sparing effect of GHB or GBL on cerebral energy intermediates is again evident when this tissue is exposed to anoxia. In one study, for example, intravenous doses of gamma-butyrolactone as low as 150 mg/kg spared energy utilization under anoxic conditions by 25 percent (105). This sparing effect could be virtually doubled by raising the dose of gamma-butyrolactone. In another study, 500 mg/kg of intravenous GHB protected rats against the lethal effects of 30 minutes of hypoxia (104). Under these conditions none of the GHB-treated rats died in comparison with 45 percent of the untreated control rats. Even lower doses of GHB, 200 mg/kg, significantly reduced the subcellular response of the brain to hypoxia in rats exposed to atmospheric pressures comparable to those at 10,000 meters (111). Similarly, 100 mg/kg of intraperitoneal gamma-butyrolactone, a dose sufficient to produce only minimal EEG and behavioural effects, given at 2 hour intervals for 24 hours, significantly reduced forebrain neuronal tissue damage at 72 hours produced by 30 minutes of bilateral vertebral and carotid artery occlusion (95). In the gerbil, GHB in doses of 400 mg/kg or 500 mg/kg was shown to exert a protective effect against cerebral ischemia and a sparing effect on tissue energy substrates whether given immediately before or two to three hours after 15 minutes of bilateral carotid occlusion (181). More so than any protective agent studied, including the barbiturates with which it is so often compared, GHB retards the disappearance of oxygen from anoxic cerebral tissue, again demonstrating the potent energy sparing effects of this agent (42).

Gamma-butyrolactone and GHB can also reduce cerebral edema. In man, intravenous doses of GHB as low as 40 mg/kg to 60 mg/kg have been used successfully for many years to reduce intracranial pressure after head injuries (30, 43, 97, 184). In rats, the development of cerebral edema induced by carbonized microspheres given intravenously can be significantly reduced by gamma-butyrolactone (20). And we have recently demonstrated that gerbils anaesthetized with doses of GHB of 250 mg/kg to 500 mg/kg for 6 hours starting immediately after 10 minutes of bilateral carotid occlusion do not show cerebral edema after 24 hours. This protective effect can be partially overcome by naloxone (34).

In the periphery, too, GHB has been shown to protect against anoxic damage and energy insufficiency. Thus, GHB can reduce the pain of angina pectoris and myocardial infarction (27,210). It limits the damage to ischemic myocardium surrounding infarcted regions (37). When myocardial energy resources are inadequate, for example, with ischemia or excessive catecholamine loads, tissue lipid infiltration, mitochondrial swelling, contraction bands and tissue necrosis are observed (13,188). GHB prevents these changes (21, 106, 120). We have recently demonstrated that GHB prevents myocardial lipid infiltration and mitochondrial deterioration in gerbils treated with 0.1 mg/kg isoproterenol (21). GHB has also been shown to minimize the deterioration of myocardial function which follows massive hemorrhage (15, 16, 118). Sodium and lithium gammahydroxybutyrate have been used to prolong the viability of the preserved kidney for organ transplantation (160). GHB also has been shown to inhibit the release of lactate from skeletal muscle in isolated ischemic limbs (102). Finally, we have found remarkable protective effects with GHB against ischemic damage in the hamster gut which again, as in brain, can be overcome by naloxone (19). These observations, then, are consistent with an energy sparing and tissue protective effect across many organ systems.

In considering the protective effect against anoxia it is important to note that GHB also effectively blocks free radical formation and lipid peroxidation, the two damaging sequelae of tissue reperfusion and reoxygenation (89,119). Its free radical quenching actions likely also account for its effectiveness against radiation damage (31,89).

THE NATURAL FUNCTION OF GAMMAHYDROXYBUTYRATE

Adaptation to hypoxic environments or to energy limiting conditions is a common challenge for many living organisms (70). One can only marvel at the extraordinary behaviour of diving birds and mammals capable of surviving under water for periods in excess of an hour. It is less well known that the blood supply to the liver and kidney in some of these species undergoes reductions of profound magnitude at this time (70). In the seal, for example, renal blood flow and renal oxidative metabolism may virtually shut down. Ischemia of this degree in terrestrial species would be irreversibly damaging to the kidneys. Equally astonishing is the months-long survival of many hibernating species in cold environments and without food energy. For some vertebrates this period can last as long as 7 to 8 months. How is this achieved?

Although many mechanisms come into play, it has been suggested that adaptive species must be capable of regulating the set point of cellular metabolic activity in response to the availability of energy. It has been proposed that certain endogenous metabolites mediate this process. One example, in the brain, may be the raised tissue concentrations of the neuroinhibitory amino acids GABA and taurine during hibernation (2, 123, 139, 140). Another proposal is that fluctuations in the relative concentration of different endogenous opiate peptides determine the set point of metabolic activity during hibernation (115,116). This would explain how naloxone and its congeners, which block the activity of these peptides, promote arousal from hibernation and shorten the duration of this state (86, 116, 133). Naloxone, it may be recalled, also blocks the major actions of GHB, antagonizing its behavioural, hypothermic, dopaminergic and EEG effects as well its inhibitory effect on brain glucose utilization (29,170). In contrast, as experiments on male red cheeked sousliks have shown, low doses of GHB between 100 mg/kg and 300 mg/kg markedly impair arousal from hibernation in this species (137). More important, in rats, this agent has been shown to significantly

alter the relative concentration of the opiate peptides dynorphin and beta endorphin in different regions of the brain (93). GHB's effects, then, may be mediated through the endogenous opioid system.

In all, these observations suggest that GHB may act naturally to promote hibernation and that the trance like state induced by GHB may have more in common with this behaviour than with nonconvulsive epilepsy or some other altered state of consciousness. GHB's hypothermic effects and its high concentration in brown fat are in keeping with this (137). In brown fat, GHB may function to counter the activation produced by circulating catecholamines. This would control the release of heat energy and maintain hypothermia and hibernation. Thus, at both the cellular level and that of the whole animal, GHB appears to regulate energy metabolism.

Many biologists view sleep on a continuum with torpor and hibernation (202). Much like these latter states, sleep is thought to serve the function of energy conservation but to a lesser and more intermittent degree. Could diurnal variations in the brain concentration of GHB play a role in the induction and metabolic function of sleep? It is noteworthy that cerebral glucose utilization dramatically falls by as much as 30 percent during NREM sleep (66,83). There, however, is no clear understanding of how this comes about. GHB, too, reduces cerebral glucose utilization. Indeed, its effectiveness as a hypnotic may have as much to do with its energy conserving properties as with its unique capacities to promote both NREM and REM sleep. A reduction of energy consumption during sleep, in compensation for the high energy output during waking, has been cited as a critical feature of sleep throughout the animal kingdom and one which is central to its restorative functions (212). Although other hypnotics like the benzodiazepines have also been shown to decrease the cerebral metabolic rate (142,143), albeit in higher doses than are used clinically to promote sleep, tolerance may develop to this effect as it does to the other sleep-inducing actions of these agents (77). In contrast, tolerance may not develop to GHB's energy-sparing effects, much as it has not been shown to develop to its soporific actions (154).

Does GHB have a role in man? GHB levels have not been systematically determined in human tissues. But it has been shown that fetal brain, in man as in other species, contains high concentrations of GHB which dwindle rapidly after birth (176). Immature animals also appear more sensitive to the depressant effects of GHB than mature animals (63, 64, 165). High fetal brain GHB levels may be one reason for the greater resistance of the fetal brain to anoxia than the adult brain (1,128). A very similar function has recently been ascribed to the high fetal brain concentrations of the neuroinhibitory amino acid taurine, whose levels also fall rapidly after birth (156). Taurine has been shown to increase cerebral resistance to anoxia, and most important, tissue taurine derived from the breakdown of protein, actually rises in concentration in response to hypoxia. Analogous mechanisms could regulate tissue GHB levels. Thus, hypoxia would favour the reduction of succinic semialdehyde to GHB rather than its oxidation to succinate. An hypoxic environment would also tend to inhibit the oxidative catabolism of GHB. Protein degradation, promoted by hypoxia, would favour the formation of succinyl CoA, our putative precursor of GHB in all tissues.

Unfortunately, the effects of hypoxia on tissue GHB levels have never been examined. Brain GHB concentrations have been shown to rise postmortem (39, 144, 176) and liver and brain GHB concentrations increase significantly when cell redox potentials are raised by ethanol (144). GHB levels also appear to rise when metabolic demand is increased. Raised GHB levels in the cerebrospinal fluid of children with seizures (173) and higher brain tissue GHB levels after mercaptopropionic acid-induced seizures may reflect this (58). The tripling of striatal GHB concentration following the intracranial injection of kainic acid may yet be another example (5). This agent is thought to produce neuronal necrosis by over excitation of cellular elements (157). Indeed, the synaptic release of excitatory neurotransmitter agents by ischemia and the subsequent dissociation between metabolic rate and the supply of energy is now considered the first step in the cascade of events leading to ischemic neuronal cell death (53,162). According to our thesis, however, these are the very conditions which would promote GHB accumulation. The rise in tissue GHB levels would then function to depress cell energy requirements and mitigate the damage. These metabolic mechanisms may explain the elevated striatal levels of GHB found in Huntington's disease (6). The reasons for these high levels have never been clear since striatal concentrations of GABA, the accepted precursor of GHB in the brain, are depressed in this condition (6). However, raised brain GHB levels in Huntington's disease would be expected if the neuronal damage in this condition is caused by excitotoxins as has been proposed (136,157). Finally, if our theory is correct, one would expect that the administration of GHB or its analogues would protect nervous tissue from excitotoxic cellular damage. This has clearly been demonstrated in the rat. The damage induced by the intrastriatal injection of kainic acid is almost completely prevented when these animals are pretreated with gamma-butyrolactone (211) and we have recently duplicated this finding with sodium gammahydroxybutyrate (34). Clearly, more definitive studies are required on the biochemical mechanisms regulating tissue GHB levels.

CONCLUSION

Thus, there is evidence, at least in the nervous system, that GHB reduces the level of cellular activity and concomitantly depresses the utilization of glucose and other energy substrates. Under these conditions, exposed tissues become less sensitive to the damaging effects of anoxia or excessive metabolic demand. Indeed, there is circumstantial evidence that tissue GHB levels rise in response to anoxia or excessive metabolic demand, suggesting a natural tissue protective role for this compound. It remains to be explained how the actions of GHB on energy metabolism are effected, and how cellular homeostasis is maintained in the face of reduced energy consumption. The barbiturates share many of GHB's tissue protective properties but the two agents appear to act through different mechanisms since GHB's metabolic effects, in distinction to those of the barbiturates, can be blocked by naloxone. For this reason, it is of considerable interest that dynorphin, the opiate peptide whose tissue concentration is raised by GHB (93), can also depress neuronal activity in a naloxone reversible manner (94) and can improve the survival of animals following ischemic damage to the nervous system (9). This suggests that GHB may act through the endogenous opioid system (29, 170, 192), as the work on hibernation discussed earlier also alludes. GHB, by altering the concentration of specific opioid peptides, may change ion flux across cell membranes (163) and produce the "channel arrest" considered necessary for energy conservation and cell protection (70). It is likely that the interaction between GHB and the opioid peptides will come under careful scrutiny in future years.

The use of GHB as a tissue protector or as an hypnotic is still in the earliest stages of clinical development. Careful examination of its metabolic interactions may help clarify nature's own restorative and protective mechanisms. As the studies reviewed above suggest, GHB may have numerous therapeutic applications. One which immediately comes to mind is to retard the progress of neurodegenerative disorders which have been attributed to excitotoxins or free radicals. GHB may generally benefit conditions in which energy supplies are insufficient to maintain normal metabolic rates. Organ ischemia, status epilepticus, shock and perhaps

even fetal anoxia are examples. The clinical use of GHB may well reify Hochachka's concept that metabolic arrest be used to extend biological time (70).

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Dapoxetine Has No Pharmacokinetic or Cognitive Interactions With Ethanol in Healthy Male Volunteers

Nishit B. Modi, PhD, Mark Dresser, PhD, Dhaval Desai, MD, Christopher Edgar, MSc, and Keith Wesnes, PhD

Dapoxetine is being investigated for the treatment of premature ejaculation. This study evaluated the potential pharmacokinetic and cognitive interactions of dapoxetine 60 mg with ethanol 0.5 g/kg in a single-center, double-blind, randomized, placebo-controlled crossover study in healthy adult male participants (n = 24). Dapoxetine was rapidly absorbed and eliminated; peak concentrations were noted 1.47 hours after administration and decreased with an alpha half-life of 1.33 hours and a terminal half-life of 15.6 hours. Pharmacokinetic parameters (C_{max}, AUC_{∞}, $t_{1/2}$, and t_{max}) of dapoxetine were not altered with concurrent ethanol consumption. Furthermore, coadministration of dapoxetine did not affect the pharmacokinetics of ethanol or potentiate the cognitive and subjective effects of ethanol.

Keywords: *dapoxetine; ethanol; pharmacokinetics; cognition*

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Although premature ejaculation (PE) is a common form of male sexual dysfunction,[1-3] no approved pharmacologic agent is currently indicated for its treatment. Conventional selective serotonin reuptake inhibitor (SSRI) antidepressants are sometimes prescribed off-label for the treatment of PE.[4] When used in the treatment of depression, these long-acting SSRIs require chronic daily dosing for maximal effectiveness and may require weeks to reach maximum plasma concentrations[5]; they may also require chronic daily dosing to be of most benefit for PE.[4,6,7]

Dapoxetine is a short-acting SSRI developed as an on-demand treatment for PE that is rapidly absorbed following oral administration, with peak plasma concentrations approximately 1 hour after administration.[8] Dapoxetine elimination is rapid and biphasic; its alpha half-life is approximately 1.4 hours, and its terminal half-life is approximately 20 hours.[8] Dapoxetine-N-oxide, the primary circulating phase I metabolite, has weak serotonin receptor binding and transport inhibition in vitro (>250-fold less than dapoxetine) and does not contribute to clinical efficacy. Desmethyldapoxetine has a similar pharmacologic potency to dapoxetine in vitro but accounts for <3% of circulating dapoxetine species.

More than 70% of men in the United States consumed alcohol in 2002.[9] Ethanol has known pharmacodynamic effects on cognitive function, such as impaired reaction time and recall. Peak plasma ethanol concentrations are observed approximately 2 hours after oral administration, and a dose of 0.7 g/kg is associated with a maximnm serum ethanol concentration of 502 μg/mL.[10] This study was designed to examine the potential pharmacokinetic, cognitive, and subjective interactions between dapoxetine and ethanol in healthy volunteers.

From ALZA Corporation, Mountain View, California (Dr Modi, Dr Dresser, Dr Desai) and Cognitive Drug Research Ltd, CDR House, Goring-on-Thames, UK (Mr Edgar, Dr Wesnes). Submitted for publication August 3, 2006; revised version accepted November 5, 2006. Address for correspondence: Nishit B. Modi, PhD, ALZA Corporation, 1900 Charleston Road, Building M11-4A, Mountain View, CA 94043; e-mail: nmodi@alzus.jnj.com.
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METHODS

Participants

Healthy men (ages 18-45 years) within 20% of normal weight for height and body build, with a supine

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blood pressure (BP) of 90 to 140 mm Hg systolic and 50 to 90 mm Hg diastolic, were eligible to participate. Moderate ethanol intake (5-20 drinks/week) was a prereqnisite. Participants were excluded if they had clinically relevant abnormalities, as determined by medical history, physical examination, blood chemistry, complete blood count, urinalysis, and electrocardiogram (ECG), or if they had a positive urine drug screen or alcohol breath test. All were required to nse a medically accepted method of contraception throughout the study period and for 3 months after study completion. Consumption of alcohol, caffeine, or products containing grapefruit was not allowed within 48 hours before administration of study medication, and men who regularly consumed >450 mg of caffeine per day were excluded. Participants with a history of smoking or tobacco use within the past 3 months were also excluded. Participants were excluded if they had used any prescription or nonprescription medications (excluding acetaminophen and multivitamins) within 7 days before study start and throughout the study period.

Study Design

This was a single-center, double-blind, randomized, 4-treatment, 4-period, crossover study in healthy adult males, approved by the Ethics Committee of the participating study center (Charterhouse Clinical Research Unit, Ravenscourt Park Hospital, Ravenscourt Park, London, UK) and conducted in accordance with good clinical practice and the International Conference on Harmonization guidelines and Ethics Committee policies, including the ethical principles that have their origin in the Declaration of Helsinki on biomedical research involving human participants. Before participation, each participant was required to read, sign, and date an Ethics Committee–approved consent form explaining the nature, purpose, and possible risks and benefits of the study, as well as the duration of participation.

Participants were assigned randomly to 1 of 4 treatment sequences and received each of the following 4 treatments: (1) a placebo tablet followed by ethanol 0.5 g/kg in ginger ale ("ethanol"), (2) dapoxetine 60 mg (Johnson & Johnson Pharmaceutical Research & Development, Raritan, NJ) followed by ethanol 0.5 g/kg iu ginger ale ("dapoxetine + ethanol"), (3) dapoxetine 60 mg followed by ginger ale as placebo for ethanol ("dapoxetine"), and (4) a placebo tablet followed by ginger ale ("placebo"). Ethanol or ginger ale was administered 30 minutes after dapoxetine or placebo to provide peak concentrations of each at approximately the same time. For the placebo drinks, ethanol-soaked gauze was placed in a double-walled container used to carry the drinks, which were covered in plastic wrap; doses of ethanol in ginger ale were prepared in similar containers covered in plastic wrap. Participants drank doses of either ethanol or placebo through a straw that protruded through the plastic wrap. A washout period of 5 to 21 days was required between treatments.

Assessments

At the initial screening visit, a medical history was obtained, and a physical examination was performed, including vital signs (heart rate, BP, and respiratory rate), 12-lead ECG, blood chemistry, complete blood count, and urinalysis. At each visit, participants were required to pass a urine drug screen and an alcohol breath test. Vital signs were measured at 0 (predose), 1, 2, 4, 6, 8, and 24 hours after administration of dapoxetine or placebo dapoxetine. At study termination, the physical examination, laboratory assessments, vital signs, and an ECG were repeated. All adverse events (AEs) were recorded and assessed in terms of severity and relationship to study drug and followed until resolution or until the end of the study.

Plasma Analysis for Dapoxetine and Its Metabolites

Blood samples (5 mL) were collected at 0, 0.5, 1, 1.25, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 24, 48, and 72 hours after administration of dapoxetine for the measurement of dapoxetine and its metabolites, desmethyldapoxetine and dapoxetine-N-oxide, using a validated liquid chromatography/tandem mass spectrometry (LC/MS/MS) method, with a minimum quantifiable dapoxetine concentration of 1.00 ng/mL and minimum quantifiable desmethyldapoxetine and dapoxetine-N-oxide concentrations of 0.200 ng/mL, using dapoxetine-d_7, desmethyldapoxetine-d_7, and dapoxetine-N-oxide-d_7 as internal standards. Positive ions were monitored by MS/MS in the multiple-reaction monitoring mode for dapoxetine (m/z transition from 306.5 to 157.2), dapoxetine-d_7 (m/z transition from 313.5 to 164.2), desmethyldapoxetine (m/z transition from 292.3 to 157.2), desmethyldapoxetine-d_7 (m/z transition from 299.3 to 164.2), dapoxetine-N-oxide (m/z transition from 322.3 to 157.2), and dapoxetine-N-oxide-d_7 (m/z transition from 329.3 to 164.2). The calibration curve was linear from 1.00 to 1000 ng/mL for dapoxetine and from 0.200 to 200 ng/mL for the metabolites. This method has been described previously.[11]

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Precision and accuracy were determined by replicate analyses of human plasma quality-control samples spiked with dapoxetine and the metabolites. Precision was measured as the percent coefficient of variation of the quality control samples. The ranges of interassay precision were as follows: for dapoxetine, 6.01% to 16.1%; for desmethyldapoxetine, 4.61% to 16.0%; and for dapoxetine-N-oxide, 3.93% to 18.2%. Accuracy was expressed as the percent difference between the mean value for each pool and the theoretical concentration (% bias). The ranges of interassay accuracy were as follows: for dapoxetine, –2.49% to 3.78%; for desmethyldapoxetine, –8.86% to 9.85%; and for dapoxetine-N-oxide, –1.25% to 3.47%.

Plasma Analysis for Ethanol

Additional blood samples were collected at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, and 12 hours after administration of dapoxetine for ethanol analysis using a validated high-resolution gas chromatography with mass spectrometry (GC/MS) method, with a minimum quantifiable ethanol concentration of 2.74 µg/mL and a calibration curve that was linear from 2.74 to 992.0 µg/mL. Precision and accuracy were calculated as described above; the range of interassay precision for ethanol was 2.4% to 5.1%, and the range of interassay accuracy was –4.0% to 0.9%.

Analysis of Pharmacokinetic Parameters

Maximum plasma concentration (C_{max}), time to C_{max} (t_{max}), apparent half-life ($t_{1/2}$), alpha and terminal $t_{1/2}$, and area under the plasma concentration versus time curve (AUC) were estimated for ethanol, dapoxetine, desmethyldapoxetine, and dapoxetine-N-oxide. For dapoxetine and its metabolites, a 2-compartment model with first-order absorption and elimination was used (WinNonMix software, Version 2.0.1, Pharsight Corporation, Mountain View, Calif).[12]

Cognitive and Subjective Assessments

Cognitive function was measured using a battery of tasks from the Cognitive Drug Research (CDR) computerized cognitive assessment system.[13,14] Prior to the first study day, cognitive function was measured 4 times, twice on each of 2 days, with ≥1 hour between tests, to familiarize participants with the tests and to minimize any learning effects during the study. The CDR tests were conducted before dosing and 0.5, 1.5, 2.5, 4, 6, 8, 12, and 24 hours after administration of study drug. The following tests were performed: Immediate Word Recall (IWR), Simple Reaction Time (SRT), Digit Vigilance Task (DV), Choice Reaction Time (CRT), Visual Tracking Task (Tracking), Spatial Working Memory (SWM), Numeric Working Memory (NWM), Delayed Word Recall (DWR), Word Recognition (WR), Digit Symbol Substitution Test (DSST), and the Bond and Lader Visual Analog Scale (VAS) of Mood and Alertness,[15] a questionnaire of 16 analog scales that derives 3 factors that assess change in self-rated alertness, self-rated calmness, and self-rated contentment. Two composite scores from the CDR tests were derived: (1) power of attention, which combines the reaction time measures from the 3 attention tests (ie, SRT, CRT, and DV), and (2) continuity of attention, which combines the accuracy measures from the 3 attention tests (ie, SRT, CRT, and DV).[14]

Statistical Analysis

Statistical analyses were conducted using SAS (SAS Institute, Inc, Cary, NC). Pharmacokinetic parameters for dapoxetine and ethanol were compared between the dapoxetine alone and dapoxetine + ethanol treatments using a mixed-effect analysis of variance (ANOVA) that included treatment, period, and seqnence as fixed effects and subject within sequence as a random effect.[16,17] Log-transformed dapoxetine AUC_{∞} and C_{max} values for dapoxetine and dapoxetine + ethanol were compared using the least squares estimate of the mean parameters for the ratio of dapoxetine + ethanol to dapoxetine alone; ethanol alone was compared to dapoxetine + ethanol in the same manner.

For cognitive and subjective assessments, a repeated-measures ANOVA was used that included fixed effects of sequence, treatment, period, time, and treatment-by-time interaction, as well as a random effect of subject within seqnence. For each CDR measure, predose (baseline) data for each period were subtracted from those at each postdosing time point to derive the difference from baseline scores, on which the analyses were performed. If the treatment-by-time interaction was significant ($\alpha < 0.01$), a standard ANOVA model for crossover designs, which included fixed effects of sequence, treatment, and period, as well as the random effect of snbject within sequence, was used to compare the pharmacodynamic measure at each time point.

RESULTS

Participant Disposition

Healthy participants (N = 24, mean age 25.5 ± 6.4 years) were enrolled. Four subjects discontinued early; 1 withdrew consent, 2 left for personal reasons, and 1 was discontinued for noncompliance. Statistical analyses were conducted on all available

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Table I Pharmacokinetic Parameters of Dapoxetine, Desmethyldapoxetine, and Dapoxetine-N-Oxide Following Administration of Dapoxetine Alone and With Ethanol

	Dapoxetine[a] Mean (SD)	Dapoxetine + Ethanol[b] Mean (SD)	Log-Transformed D + E/D Ratio[c] % (90% CI)
Dapoxetine			
AUC_∞, ng·h/mL	2670 (1000)	2530 (1200)	96.3 (87.26–106.32)
C_{max}, ng/mL	492 (160)	531 (210)	107.2 (97.62–117.65)
t_{max}, h	1.47 (0.51)	1.35 (0.53)	NR
Alpha $t_{1/2}$, h[d]	1.33 (0.14)	1.59 (0.29)	NR
Terminal $t_{1/2}$, h[d]	15.6 (0.81)	15.3 (1.2)	NR
Desmethyldapoxetine			
AUC_∞, ng·h/mL	497 (270)	470 (450)	90.3 (77.66–105.10)
C_{max}, ng/mL	33.0 (11)	32.0 (15)	96.5 (85.38–108.97)
t_{max}, h	2.29 (0.75)	2.26 (0.74)	NR
$t_{1/2}$, h	15.9 (3.8)	14.7 (2.8)	NR
AUC_∞ ratio, dapoxetine/desmethyldapoxetine	5.91 (1.4)	6.57 (2.2)	NR
Dapoxetine-N-oxide			
AUC_∞, ng·h/mL	2080 (690)	2050 (810)	100.8 (92.32–110.05)
C_{max}, ng/mL	96.6 (21)	93.0 (15)	96.5 (91.85–101.38)
t_{max}, h	2.97 (0.64)	3.08 (0.63)	NR
$t_{1/2}$, h	20.0 (4.6)	20.0 (6.9)	NR
AUC_∞ ratio, dapoxetine/dapoxetine-N-oxide	1.23 (0.24)	1.27 (0.24)	NR

NR, not relevant.
a. Dapoxetine 60 mg + placebo ethanol, n = 20.
b. Dapoxetine 60 mg + 0.5 g/kg ethanol, n = 20.
c. The ratio of log-transformed values for dapoxetine + ethanol (D + E) to dapoxetine alone (D), given as a percentage, with the 90% confidence interval (CI).
d. Estimated using a 2-compartment model with first-order absorption and elimination.

pharmacokinetic and pharmacodynamic data for the 20 participants who completed all 4 treatments.

Pharmacokinetic Analysis

Dapoxetine

The pharmacokinetics of dapoxetine were not affected by coadministration of ethanol 0.5 mg/kg (Figure 1). Dapoxetine was rapidly absorbed, with maximal plasma concentrations 1.47 ± 0.51 hours after administration (Table I). Elimination was rapid and biphasic; the alpha and terminal half-life was 1.33 ± 0.14 and 15.6 ± 0.81 hours, respectively. By 24 hours after administration, plasma dapoxetine concentrations had decreased to 4.5% of C_{max}. The pharmacokinetic parameter values for dapoxetine were not affected by ethanol coadministration, as noted by the similar peak concentrations and AUC values. The 90% confidence intervals (CIs) for the ratio of (dapoxetine + ethanol)/(dapoxetine) for $\ln C_{max}$ and for $\ln AUC_\infty$ were within 80% to 125%, indicating that ethanol did not affect the pharmacokinetics of dapoxetine.

Desmethyldapoxetine and Dapoxetine-N-Oxide

Ethanol did not affect the pharmacokinetics of desmethyldapoxetine or dapoxetine-N-oxide (Table I). For desmethyldapoxetine, the 90% CIs of the (dapoxetine + ethanol)/(dapoxetine) ratio for $\ln C_{max}$ were within 80% to 125%, whereas the 90% CI for the $\ln AUC_\infty$ ratio was slightly outside the 80% to 125% range, but the 2 treatments were not significantly different by ANOVA ($P = .258$, dapoxetine vs dapoxetine + ethanol). For dapoxetine-N-oxide, the 90% CIs of the (dapoxetine + ethanol)/(dapoxetine) ratios for $\ln C_{max}$ and for $\ln AUC_\infty$ were also within the 80% to 125% no-effect boundary.

Coadministration of ethanol did not affect the metabolism of dapoxetine to desmethyldapoxetine or dapoxetine-N-oxide; the AUC_∞ ratio for (dapoxetine)/(desmethyldapoxetine) was similar for dapoxetine and dapoxetine + ethanol (5.91 ± 1.4 and 6.57 ± 2.2, respectively), as was the AUC_∞ ratio for (dapoxetine)/(dapoxetine-N-oxide) (1.23 ± 0.24 and 1.27 ± 0.24, respectively).

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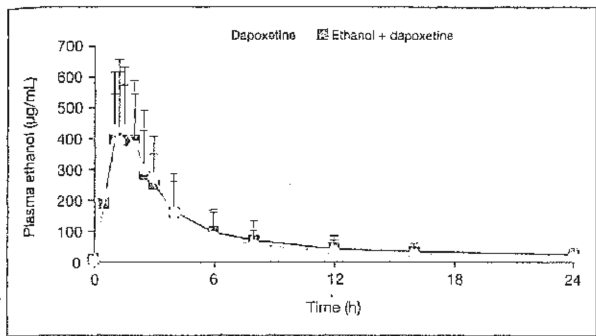

Figure 1. Plasma concentration profile of dapoxetine when coadministered with ethanol. Plasma concentrations of dapoxetine (mean with standard deviation) were measured using liquid chromatography/tandem mass spectrometry (LC/MS/MS) following administration of dapoxetine 60 mg with placebo (n = 20) or ethanol 0.5 mg/kg (n = 20).

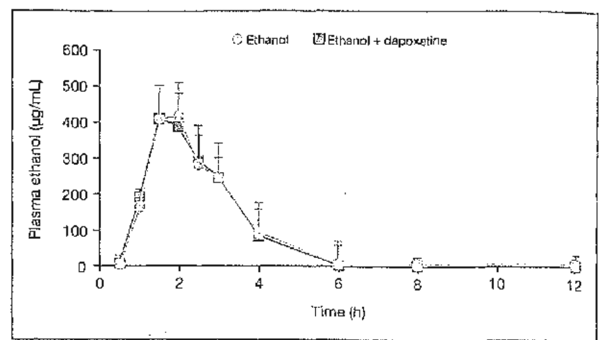

Figure 2. Plasma concentration profile of ethanol when coadministered with dapoxetine. Ethanol concentrations in plasma (mean with standard deviation) were measured using gas chromatography/mass spectrometry (GC/MS) following administration of placebo (n = 20) or dapoxetine 60 mg (n = 20) with 0.5 mg/kg ethanol.

Table II Pharmacokinetic Parameters of Ethanol Administered Alone and With Dapoxetine

	Ethanol[a] Mean (SD)	Dapoxetine + Ethanol[b] Mean (SD)	Log-Transformed D + E/E Ratio[c] % (90% CI)
AUC_∞, µg·h/mL	1020 (330)	970 (220)	100.7 (82.04–123.52)
C_{max}, µg/mL	440 (83)	428 (61)	97.5 (90.46–105.05)
t_{max}, h	1.10 (0.12)	1.07 (0.21)	NR
$t_{1/2}$, h	1.15 (0.75)[d]	1.07 (0.84)[e]	NR

NR, not relevant.
a. Placebo dapoxetine + 0.5 g/kg ethanol, n = 20.
b. Dapoxetine 60 mg + 0.5 g/kg ethanol, n = 20.
c. The ratio of log-transformed values for dapoxetine + ethanol (D + E) to ethanol alone (E), given as a percentage, with the 90% confidence interval (CI).
d. n = 17.
e. n = 15.

Ethanol

Ethanol pharmacokinetics were not affected by coadministration of dapoxetine (Table II); plasma ethanol concentrations over time are shown in Figure 2. The 90% CIs of the (dapoxetine + ethanol)/(ethanol) ratio for $\ln C_{max}$ and for $\ln AUC_\infty$ were within 80% to 125%.

Cognitive and Subjective Assessments

The analysis focused on the approximate time of peak plasma concentrations of both ethanol and dapoxetine (1.5 hours). Area under the effect curve analyses were conducted, and no significant differences between treatments were observed; however, there was a high degree of variability across time points. Peak concentrations of dapoxetine occurred approximately 1.5 hours after administration, whereas peak concentrations of ethanol occurred approximately 1 hour after administration (ethanol was administered 30 minutes after dapoxetine, to match the time to peak concentration for both agents). Ethanol 0.5 g/kg impaired measures of attention and verbal recall and recognition; effects peaked at 1.5 hours and resolved by 4 hours, consistent with the plasma profile of ethanol (Figure 2). Coadministration of dapoxetine did not potentiate the cognitive and subjective effects of ethanol (Figure 3, Table III).

At 1.5 hours, simple reaction time, self-rated contentment, and power of attention and continuity of attention were significantly affected relative to placebo following administration of ethanol alone ($P < .05$, Figure 3A). A trend toward significance was noted for digit vigilance and delayed word recall following treatment with ethanol alone ($P < .1$). Fewer words were recalled correctly during the Delayed Word Recall test, and subjects reported lower levels of contentment and alertness following dapoxetine compared with placebo ($P < .05$). With dapoxetine + ethanol, simple reaction time, response time in digit

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Table III Change From Baseline in Cognitive and Subjective Assessments of Accuracy at Peak Concentrations of Dapoxetine and Ethanol (1.5 Hours After Dapoxetine Administration)

Task	Placebo[a]	Dapoxetine[b]	Ethanol[c]	Dapoxetine + Ethanol[d]
DV, mean % of targets detected	−1.6 (4.3)	−0.6 (5.3)	−5.0 (12)	−3.00 (6.5)
IWR, mean % of words correctly recalled	−2.8 (14)	−7.2 (16)	−9.8 (12)	−11.7 (11)*
DWR, mean % of words correctly recalled	−11.2 (15)	−20.3 (15)*	−18.5 (15)	−21.2 (12)*
Tracking, mean distance from target, mm	0.14 (2.1)	0.53 (1.7)	0.59 (1.8)	1.5 (2.8)
DSST, mean number correct	2.6 (5.2)	1.5 (4.8)	2.5 (4.1)	0.5 (6.3)
Continuity of attention, mean score	−1.0 (2.6)	−2.2 (4.1)	−4.2 (5.4)*	−3.9 (4.2)*

DV, digit vigilance; IWR, immediate word recall; DWR, delayed word recall; Tracking, visual tracking task; DSST, digit symbol substitution test.
a. Placebo dapoxetine + placebo ethanol, n = 20.
b. Dapoxetine 60 mg + placebo ethanol, n = 20.
c. Placebo dapoxetine + ethanol 0.5 mg/kg, n = 20.
d. Dapoxetine 60 mg + 0.5 g/kg ethanol, n = 20.
*$P < .05$ vs placebo.

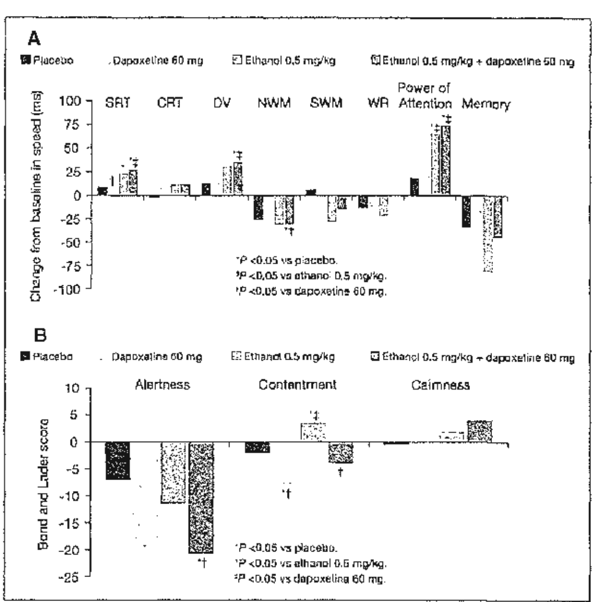

Figure 3. Change from baseline in cognitive and subjective assessments of speed and the Bond and Lader Visual Analog Scale (VAS) at peak concentrations of dapoxetine and ethanol (1.5 hours after dapoxetine administration). Panels A and B show assessments of various pharmacodynamic measures taken at 1.5 hours after administration of dapoxetine, the approximate time of maximum concentrations for both dapoxetine and ethanol. The pharmacodynamic parameters shown are as follows. Panel A: speed measurements for Simple Reaction Time (SRT), Digit Vigilance (DV), Choice Reaction Time (CRT), Spatial Working Memory (SWM), Numeric Working Memory (NWM), Word Recognition (WR), Power of Attention, and Memory.[14] Panel B: Bond and Lader VAS of Mood and Alertness (alertness, calmness, and contentment).[15]

vigilance, and power of attention all increased significantly ($P < .05$) compared to both placebo and dapoxetine alone but not compared to ethanol alone.

At 1.5 hours after administration, dapoxetine alone significantly decreased the percentage of correctly recalled words on the DWR, whereas ethanol alone significantly decreased the score for continuity of attention ($P < .05$ vs placebo for both; Table III). With dapoxetine + ethanol, the percentage of words correctly recalled on both the IWR and DWR was decreased, as was the score for continuity of attention ($P < .05$ vs placebo for all).

Following dapoxetine administration, Bond and Lader scores decreased for alertness ($P < .05$ vs placebo) and contentment ($P < .05$ vs placebo and ethanol alone; Figure 3B); following ethanol administration, Bond and Lader scores for contentment were increased ($P < .05$ vs placebo and dapoxetine alone). Following dapoxetine + ethanol, Bond and Lader scores were decreased for alertness ($P < .05$ vs placebo and ethanol alone) and contentment ($P < .05$ vs ethanol alone).

Adverse Events

Most AEs were of mild or moderate severity. No serious AEs were reported. A total of 28.6% of participants (6/21) reported AEs with ethanol, 75.0% (15/20) reported AEs with dapoxetine + ethanol, 52.2% (12/23) reported AEs with dapoxetine, and 38.1% (8/21) reported AEs with placebo. Somnolence was the most common AE (9.5% with ethanol, 30.0%

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with dapoxetine + ethanol, 13.0% with dapoxetine, and 9.5% with placebo). One participant experienced somnolence rated as severe during the dapoxetine treatment period. Other AEs reported by at least 2 participants on any treatment included headache, nausea, abnormal thinking, alcohol intolerance, asthenia, chills, euphoria, neurosis, and blurred vision. No clinically important changes in laboratory values, vital signs, or ECGs were seen.

DISCUSSION

Dapoxetine is a short-acting SSRI being developed as an on-demand oral formulation for the treatment of PE. Dapoxetine is rapidly absorbed and eliminated, with peak concentrations noted approximately 1.5 hours after oral administration. The alpha half-life of dapoxetine was 1.3 hours; by 24 hours after administration, plasma concentrations of dapoxetine decreased to approximately 5% of peak levels. Because many men consume alcohol,[9] ethanol is likely to be taken concomitantly by men using dapoxetine. Results from this study demonstrated that consnmption of ethanol has no effect on the pharmacokinetics of dapoxetine or its metabolites, desmethyldapoxetine and dapoxetine-N-oxide. Similarly, dapoxetine did not affect the pharmacokinetics of ethanol. Furthermore, pharmacodynamic data (including vital signs and measures of cognitive function) did not support a consistent pattern of synergistic or additive interactions between ethanol and dapoxetine. Definitive conclusions regarding the impact of dapoxetine and ethanol on cognitive function are difficult to derive from the available data because there was a high degree of variability in the pharmacodynamic results from men who received placebos for both dapoxetine and ethanol.

Although SSRI antidepressants are sometimes used in the treatment of PE,[4,6,7] they are not indicated for this application and may require days to weeks to reach the maximum concentrations necessary for effective treatment of depression and/or PE.[5] The pharmacokinetics of SSRI antidepressants are generally not affected by cousumption of moderate amounts of alcohol; fluvoxamine,[18] fluoxetine,[19] and venlafaxine[20] have not shown pharmacokinetic alterations with concurrent alcohol consumption. For example, flnvoxamine 50 mg had no clinically significant effect on the pharmacokinetics of intravenous or oral alcohol (40 mg); however, the mean $AUC_{(0-8)}$ of alcohol increased significantly following multiple doses of fluvoxamine in comparison with placebo.[18] Results from another study demonstrated that coadministration of fluoxetine (30 and 60 mg) with ethanol (45 mL absolute alcohol) did not alter the plasma or blood concentrations of fluoxetine or alcohol compared with either drug alone.[19] In a separate study, administration of alcohol following multiple doses of fluoxetine or amitryptaline (a tricyclic antidepressant) resulted in decreased average alcohol concentrations compared with placebo.[21]

Several cognitive measures were impaired following ethanol 0.5 g/kg in this study, which was broadly consistent with previously characterized effects of ethanol[22]; this dose (~3 drinks) has been used in other drug interaction studies,[20] and dapoxetine 60 mg was the highest dose studied in phase III trials.[23] Peak plasma ethanol concentrations observed here (428-440 µg/mL) were comparable to previously reported maxima of 502 µg/mL and 640 µg/mL following doses of 0.7 g/kg[10] and 40 mg,[18] respectively. Dapoxetine administered alone was associated with declines in delayed word recall, self-rated alertness, and self-rated contentment. Although ethanol + dapoxetine yielded significant impairment on several tests compared with placebo, none were significantly different from those observed with ethanol alone. These results suggest that the addition of dapoxetine did not result in impairment beyond that associated with ethanol.

SSRI antidepressants have not generally been shown to potentiate the cognitive effects of alcohol. Alcohol impaired measures of cognitive function, whereas 50 mg fluvoxamine did not potentiate the effects of alcohol, although 2 measures of cognitive function (speed of responding in the vigilance task and word recognition sensitivity) were possibly affected by fluvoxamine.[18] In another study, fluvoxamine tended to improve recognition but not free recall of words.[24] Similarly, single and multiple doses of flnoxetine did not affect psychomotor activity (stability of stance, motor performance, or manual coordination) or subjective effects of alcohol.[19] In another study, flnoxetine had no effect on auditory reaction time, DSST, or body sway with eyes open or closed but did result in a decrease from baseline in immediate and delayed word recall.[21] Coadministration of fluoxetine with alcohol significantly slowed auditory reaction time, reduced DSST, and increased body sway in both the eyes open and closed conditions and produced further decreases in immediate and delayed word recall.[21]

Most AEs noted in this study were mild or moderate in severity. The overall rate of AEs was high, and 38% of participants reported AEs with placebo. Although this study was not designed to compare the rate of AEs between treatments, coadministration of dapoxetine with ethanol appeared to have an additive rather than a synergistic effect on the total

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number of AEs reported, suggesting that ethanol and dapoxetine induce AEs (such as dizziness, nausea, and diarrhea) through different mechanisms.

CONCLUSIONS

In healthy male volunteers, coadministration of dapoxetine 60 mg with ethanol did not alter the pharmacokinetics of dapoxetine or ethanol or the effects of ethanol on cognitive and subjective states. Although dapoxetine reduced self-ratings of alertness and contentment, neither of these was affected by coadministration of ethanol; however, these effects may merit further investigation.

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the bioassay of samples containing multiple drugs or abnormal levels of normally occurring metabolic products.

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Absorption of Sodium γ-Hydroxybutyrate and Its Prodrug γ-Butyrolactone: Relationship between *In Vitro* Transport and *In Vivo* Absorption

C. ARENA and HO-LEUNG FUNG ˣ

Received August 20, 1979, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Amherst, NY 14260.* Accepted for publication October 11, 1979.

Abstract ☐ A qualitative relationship between *in vitro* transport and *in vivo* absorption of sodium γ-hydroxybutyrate and γ-butyrolactone was demonstrated. As with other short-chain acids, sodium γ-hydroxybutyrate showed capacity-limited transport *in vitro*, consistent with the previous observation that this drug exhibited slower *in vivo* absorption with increasing dose. The prodrug lactone, on the other hand, showed a higher intestinal flux than the acid in the everted gut, and *in vivo* absorption also was more rapid. Capacity-limited transport and absorption of the lactone appeared less evident. Thus, the increased oral hypnotic activity of the lactone over that of the acid most likely is a result of its more favorable intestinal transport characteristics.

Keyphrases ☐ Sodium γ-hydroxybutyrate—relationship between *in vitro* transport and *in vivo* absorption ☐ γ-Butyrolactone—prodrug for sodium γ-hydroxybutyrate, relationship between *in vitro* transport and *in vivo* absorption ☐ Hypnotic agents—sodium γ-hydroxybutyrate and γ-butyrolactone, relationship between *in vitro* transport and *in vivo* absorption

γ-Hydroxybutyrate (I), a metabolite of γ-aminobutyric acid, is found endogenously in the human brain (1). When introduced intravenously, I is a useful anesthetic (2) and is beneficial in Parkinson's disease (3). However, oral administration of this compound results in decreased and variable pharmacological activity (4–6). Recently, oral doses of I totaling 50 mg/kg were shown to be useful in the treatment of narcolepsy and cataplexy in patients, but the duration of sleep induction after each oral dose lasted only for ~2 hr (7).

BACKGROUND

In previous animal studies in these laboratories (8–10), orally administered I was shown to be subject to first-pass metabolism at low doses (≤200 mg/kg) in rats. At higher doses (400–1600 mg/kg), systemic availability approached 100%, presumably due to saturation of first-pass metabolism, but the relative absorption rate appeared to decrease with increasing dose. Thus, although the extent of drug absorption was almost complete, peak plasma I concentrations were relatively insensitive to increases in the oral dose and, in most animals, threshold hypnotic concentrations in plasma were not reached in spite of high oral doses.

The lactone analog of I, γ-butyrolactone (II), is hydrolyzed rapidly and exclusively *in vivo* to I (11, 12) and, therefore, can be classified as a prodrug. Compound II is rapidly and completely absorbed *in vivo* after oral administration over a wide dose range. In contrast to I, the peak drug concentration after oral dosing of II was proportional to the dose, and II was equally effective as a hypnotic whether given orally or intravenously (9).

The reason for the apparent difference in *in vivo* absorption characteristics between I and II has not been delineated. In this paper, *in vitro* experiments that compared the transport properties of these two compounds across the everted rat gut are described.

EXPERIMENTAL

Reagents—Compound I, obtained as the sodium salt[1], and II[1] were used without purification. The buffer and assay reagents[1–3] were all reagent or analytical grade.

Everted Rat Gut Preparation—Male Sprague–Dawley rats, 260–310 g, were sacrificed by decapitation. An intestinal segment, ~12 cm long, was taken from a region 20 cm from the pylorus sphincter; it was everted and mounted according to the technique originally devised by Wilson and Wiseman (13) and modified by Crane and Wilson (14).

Flux Experiment—The everted gut was placed inside a test tube with the mucosal side exposed to 90 ml of a 0.05 *M* physiological tromethamine buffer (pH 7.4) containing the appropriate drug concentration. All flux studies were carried out at 37°. At 5-min intervals up to 25 min, the serosal solution (~1 ml) was removed for the assay and replaced with an equal volume of fresh buffer. Three or four replicate flux experiments were conducted at each initial mucosal concentration.

Spectrophotometric Analysis—The Hestrin (15) assay for short-chain O-acyl derivatives as adopted for I and II by Guidotti and Ballotti (16) was employed. Conversion of I to II was effected by reaction with two parts of concentrated sulfuric acid[2] and subsequent neutralization with 10 parts of 6 *N* NaOH[2].

[1] Eastman Kodak Co., Rochester, NY 14650.
[2] Fisher Scientific Co., Fair Lawn, NJ 07410.
[3] J. T. Baker Chemical Co., Phillipsburg, NJ 08865.

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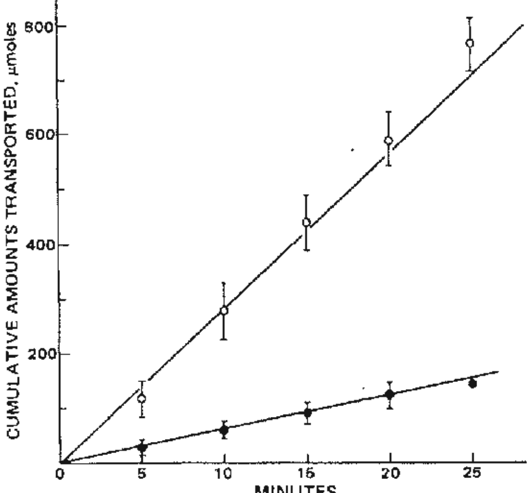

Figure 1—*Mean intestinal transport of I (●, n = 3) and II (○, n = 4) at 0.40* M. *Bars indicate standard deviations. The point shown for I at 25 min represents the mean value of two measurements.*

RESULTS AND DISCUSSION

Transport of I and II through the everted rat gut was examined at various initial mucosal drug concentrations. Intestinal flux was determined for each animal preparation by linear regression of a plot of cumulative amount transported to the serosal side *versus* time. Representative plots showing intestinal transport of I and II at 0.40 *M* are given in Fig. 1. At low mucosal concentrations, linearity of flux was maintained throughout the experiment. However, at high I concentrations, positive deviations (increased flux) occurred at the later time points, suggesting possible tissue damage with prolonged drug exposure. In these instances, initial rates of transport restricted to the linear portion of the curve (usually 0–20 min) were used to calculate flux. In all experiments, the total amounts transported to the serosal side were small (<0.4% for I and <2.5% for II) compared to the total drug available from the mucosal pool. Thus, the initial mucosal concentration remained essentially unchanged throughout each experiment.

Figure 2 shows the relationships between intestinal flux of I and II and their respective mucosal concentrations. Over the concentration range studied, intestinal transport of II was considerably more rapid than that of I. At equimolar mucosal concentrations, the differences in flux between I and II were statistically significant at $p < 0.001$ using the Student t test. Compound II fluxes were ~5, 7, and 10 times higher than I fluxes at 0.40, 0.79, and 1.19 *M*, respectively. In addition, I transport leveled off at concentrations above 0.40 *M*. If nonspecific effects on intestinal permeability could be ruled out, this flux behavior suggested the presence of a capacity-limited transport system for I in the rat intestine. In comparison, concentration-dependent transport of II was less evident.

In these experiments, the ionic strength in the mucosal solution was not constant over the concentration range studied. Although the mucosal solution was prepared with buffer, high I concentrations also could affect the pH slightly because I, as its sodium salt, is mildly basic. The leveling in I flux could, in principle, have been partially contributed to by nonspecific ionic strength and/or pH effects created by increasing mucosal concentrations of the ionic drug. The possibility of this artifact was ruled out by the following experiment.

Flux studies were carried out at 0.08 *M* I under two sets of conditions. In one case, no pH or salt adjustments were made (Condition A: pH 7.4, $\mu = 0.23$ *M*); in the other case, sodium chloride and sodium hydroxide were added so that the pH and ionic conditions were equivalent to those present when flux was studied at 1.19 *M* I (Condition B: pH 8.1, $\mu = 1.34$ *M*). If ionic strength and pH affected flux significantly, then the observed fluxes under Conditions A and B would be different, with the flux of B similar to that observed at 1.19 *M* I. In fact, the flux of I was identical whether or not additional salt or alkalinizing agents were added.

In duplicate determinations, the fluxes obtained under Condition A were 2.2 and 2.3 μM/min; those under Condition B both were 2.4 μM/min. Thus, minor differences in pH and ionic strength contributed by changes

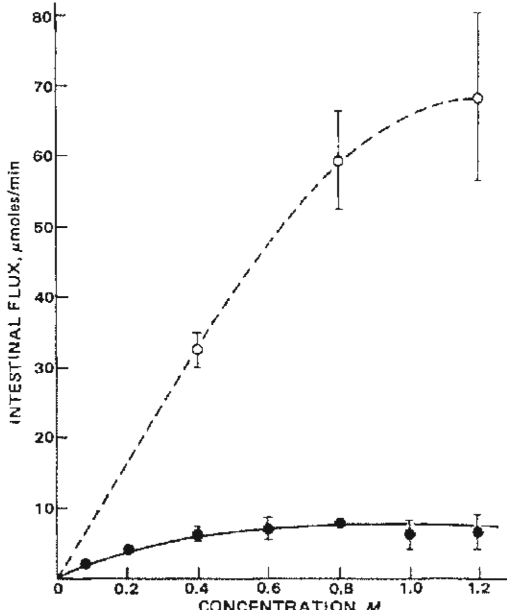

Figure 2—*Concentration effect on the intestinal fluxes of I (●) and II (○). Bars indicate standard deviations. When bars are absent, the standard deviations were too small to be shown.*

in the mucosal I concentration did not affect flux significantly. Since II is nonionic, ionic strength and pH effects produced by increasing II concentrations were presumed to be negligible. Bender *et al.* (17) found the second-order alkaline hydrolytic constant of II to be ~0.2 liter/mole-sec at 25°. At pH 7.4, the hydrolysis half-life would be about 1000 days. Thus, conversion of II to I in the buffered mucosal solution was insignificant during the experiment.

The *in vitro* transport characteristics of I and II are consistent with their *in vivo* absorption properties reported previously (8–10). Compound I, which showed capacity-limited transport *in vitro*, also exhibited relatively slower *in vivo* absorption rates with increasing oral dose (10). Other short-chain acids, such as acetic and butyric acids, also have been shown to be transported *via* an active system (18, 19). Therefore, a capacity-limited absorption mechanism might be a reason for the decreased and variable activity of I when given in high oral doses to humans (4–7). The prodrug lactone II, on the other hand, showed a much higher intestinal flux than I in the everted gut and was almost instantaneously absorbed when orally administered (9). Capacity-limited transport of II, if existent, appeared to occur at much higher drug concentrations.

The present study demonstrated a qualitative relationship between *in vitro* transport and *in vivo* absorption of the two compounds studied. Thus, the increased oral activity of the lactone over that of its open-chain hydroxy acid is most likely a result of its more favorable intestinal transport characteristics. The usefulness of II has not been investigated in humans.

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Temporal Variations in Trough Serum Theophylline Concentrations at Steady State

L. J. LESKO *x, D. BROUSSEAU ‡, A. T. CANADA *, and G. EASTWOOD *

Received June 21, 1979, from the **Drug Concentration Laboratory, University of Massachusetts Medical Center, Worcester, MA 01605*, and ‡*Fisons Corporation, Bedford, MA 01730.* Accepted for publication October 11, 1979.

Abstract □ Temporal variations in serum theophylline concentrations were observed in 14 healthy volunteers receiving multiple doses of theophylline. After repeated oral doses (6.9–18.2 mg/kg/day) of theophylline as either a nonalcoholic aminophylline solution or a controlled-release capsule, trough theophylline levels at steady state were significantly higher ($p < 0.05$) in the morning than in the afternoon or evening. With the solution, the mean ($\pm SE$) trough serum level at 7 am was 11.1 ± 0.9 μg/ml, and at 1 pm it was 9.6 ± 0.8 μg/ml. With the capsule, the mean ($\pm SE$) trough serum level at 8 am was 13.8 ± 0.9 μg/ml, and at 8 pm it was 10.7 ± 0.9 μg/ml. Temporal variations in serum theophylline concentrations have not been reported previously and may be important in therapeutic monitoring.

Keyphrases □ Theophylline—trough serum concentrations at steady state, temporal variations □ Bronchodilators—theophylline, trough serum concentrations at steady state, temporal variations □ Pharmacokinetics—theophylline, trough serum concentrations at steady state, temporal variations

Temporal variation in the absorption and disposition of drugs is an area of pharmacokinetics about which relatively little is known. In the few studies performed, the findings have not been consistent. For example, Shirley and Vesell (1) reported that temporal variations in the disposition of acetaminophen and phenacetin occur. However, Vesell *et al.* (2) observed no temporal variations in the pharmacokinetics of antipyrine (2), and Nakano and Hollister (3) reported no time-related changes in the disposition of nortriptyline. The causes of temporal variations in drug pharmacokinetics may be varied. Circadian rhythm apparently influences the distribution of potassium between body compartments (4), while changes in body posture alter the absorption of cephradine (5) and erythromycin (6) from the GI tract.

One mechanism suggested to account for the temporal variations in the disposition of phenacetin and acetaminophen was the occurrence of diurnal changes in the amount and activity of hepatic microsomal oxidases (1). Theophylline is a drug whose disposition also is determined by microsomal oxidases, so it seemed possible that temporal variations in theophylline disposition may occur. Since this aspect of theophylline kinetics had not been reported previously, one objective of this study was to determine if temporal variations exist.

EXPERIMENTAL

Subjects—The seven male and seven female volunteers were 21–40 years old, and their average weight was 67.5 kg. All volunteers were nonsmokers and were in good physical health with no history of alcoholism or cardiovascular disease.

Drug Administration and Blood Sampling—The volunteers randomly received either a nonalcoholic aminophylline solution or a controlled-release theophylline capsule. The oral theophylline dose was individualized for each volunteer, based on single-dose kinetics, to produce peak serum theophylline concentrations no larger than 18 μg/ml after repeated dosing. The daily doses ranged from 6.9 to 18.2 mg/kg. The solution was administered at 7 am, 1 pm, 7 pm, and 1 am, and the capsule was given at 8 am and 8 pm. Dosing was continued for 6 days prior to each study day. The study days were separated by 1 week during which the volunteers took the alternate formulation.

On each study day, 1 ml of serum was obtained immediately before the morning dose of each dosage form and 6 or 12 hr after administration of the solution or capsule, respectively.

Theophylline Assay—Serum theophylline determinations were made by high-pressure liquid chromatography using a method described previously (7).

Data Analysis—A paired t test was used to analyze within-subject differences between the am and pm trough theophylline concentrations observed for each dosage form.

RESULTS AND DISCUSSION

The am and pm trough serum theophylline concentrations determined for each dosage form are listed in Table I. The percentage changes in trough level are noted for each volunteer. The mean ($\pm SE$) serum theophylline concentration at 7 am for the solution was 11.1 ± 0.9 μg/ml, while at 1 pm the serum theophylline concentration was 9.6 ± 0.8 μg/ml, representing a change of 13%. For the capsule, the mean ($\pm SE$) serum theophylline concentration at 8 am was 13.8 ± 0.9 μg/ml, and at 8 pm it was 10.7 ± 0.9 μg/ml, reflecting a decrease of 24%. The differences between the am and pm serum theophylline concentrations were significant ($p < 0.05$) for each dosage form.

Based on these results, there appear to be temporal variations in theophylline pharmacokinetics. Higher trough levels at 7 or 8 am compared to those at 1 or 8 pm may be related to a shorter plasma half-life at the latter times. Indeed, Shirley and Vesell (1) reported that plasma half-lives of phenacetin and acetaminophen were ~15% shorter at 2 pm than at 6 am. Another possible cause of higher am trough levels may be

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Diclofenac plasma protein binding: PK-PD modelling in cardiac patients submitted to cardiopulmonary bypass

J.O. Auler Jr.[1], E.B. Espada[1], E. Crivelli[1], T.B.G. Quintavalle[1], A. Kurata[1], N.A.G. Stolf[1], A.M. Issy[1], O.E. D. Paschoa[2], M. Danhof[2], D.D. Breimer[2], D.A.F. Chamone[1] and S.R.C.J. Santos[1]

[1]Laboratório de Pesquisa, Instituto do Coração, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo, 05403-000 São Paulo, SP, Brasil
[2]Center for Drug Research, 2300 RA Leiden, Amsterdam, Holland

Correspondence
S.R.C.J. Santos
Laboratório de Pesquisa
Instituto do Coração
Hospital das Clínicas, FMUSP
Av. Dr. Enéas C. Aguiar, 44
05403-000 São Paulo, SP
Brasil

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Abstract

Twenty-four surgical patients of both sexes without cardiac, hepatic, renal or endocrine dysfunctions were divided into two groups: 10 cardiac surgical patients submitted to myocardial revascularization and cardiopulmonary bypass (CPB), 3 females and 7 males aged 65 ± 11 years, 74 ± 16 kg body weight, 166 ± 9 cm height and 1.80 ± 0.21 m^2 body surface area (BSA), and control, 14 surgical patients not submitted to CPB, 11 female and 3 males aged 41 ± 14 years, 66 ± 14 kg body weight, 159 ± 9 cm height and 1.65 ± 0.16 m^2 BSA (mean ± SD). Sodium diclofenac (1 mg/kg, *im* Voltaren 75® twice a day) was administered to patients in the Recovery Unit 48 h after surgery. Venous blood samples were collected during a period of 0-12 h and analgesia was measured by the visual analogue scale (VAS) during the same period. Plasma diclofenac levels were measured by high performance liquid chromatography. A two-compartment open model was applied to obtain the plasma decay curve and to estimate kinetic parameters. Plasma diclofenac protein binding decreased whereas free plasma diclofenac levels were increased five-fold in CPB patients. Data obtained for analgesia reported as the maximum effect (E_{MAX}) were: 25% VAS (CPB) *vs* 10% VAS (control), $P<0.05$, median measured by the visual analogue scale where 100% is equivalent to the highest level of pain. To correlate the effect versus plasma diclofenac levels, the E_{MAX} sigmoid model was applied. A prolongation of the mean residence time for maximum effect ($MRTE_{MAX}$) was observed without any change in lag-time in CPB in spite of the reduced analgesia reported for these patients, during the time-dose interval. In conclusion, the extent of plasma diclofenac protein binding was influenced by CPB with clinically relevant kinetic-dynamic consequences.

Key words
- Diclofenac
- Plasma protein binding
- Cardiopulmonary bypass
- Kinetic-dynamic parameters
- Analgesia
- Visual analogue scale

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) widely prescribed as sodium or potassium salt for chronic pain (1). Diclofenac is prescribed for the relief of chronic or acute pain, by oral (*po*) or intramuscular (*im*) administration, respectively. After cardiac, open heart or abdominal surgery, diclofenac administered *im* during the postoperative period is absorbed rapidly. As this drug shows a high percentage of plasma protein binding, its elimination through the hepatic metabolism (hydroxylation followed by glucuronidation, phases 1 and 2 of drug metabolism) and urinary excretion are mainly dependent on the plasma concentration of the free drug. A low percentage of the drug is glucuronated directly and excreted in the bile (2,3).

In cardiac surgery, loss of blood, high mobilization of liquids and cardiopulmonary bypass (CPB) with hemodynamic consequences such as hypothermia and hemodilution occur (4). During the postoperative period of cardiac surgery with cardiopulmonary bypass, there is a 30% reduction of hepatic blood flow accompanied by changes in the metabolism of diclofenac (4,5). The aim of the present study was to measure the pharmacokinetics and pharmacodynamics of diclofenac on the basis of plasma protein binding, during the postoperative period in surgical patients submitted or not to cardiopulmonary bypass.

Twenty-four surgical patients of both sexes were investigated in a protocol controlled study which was approved by the Local Ethics Committee of the Hospital. Patients selected for this study were divided into two groups: Group I - 10 patients submitted to cardiac surgery with CPB, 65 ± 11 years, 74 ± 16 kg, 166 ± 9 cm height and 1.80 ± 0.21 m^2 body surface area (BSA), mean ± SD. Group II - 14 patients, 41 ± 14 years, 66 ± 14 kg, 159 ± 9 cm height and 1.65 ± 0.16 m^2 BSA, submitted to cholecystectomy, incisional hernioplasty, gastroplasty and gastrectomy without cardiopulmonary bypass (control). The patients evaluated here showed normal renal, liver, and endocrine functions as well as biotransformation of drugs and absence of heart failure. Biotransformation of drugs in general was evaluated by the antipyrine test (6). The conventional antipyrine test associated with oxidative and conjugation capacities using 3-hydroxyantipyrine (HMA) + 4-hydroxyantipyrine (4HA) + norantipyrine (NORA) or 3-hydroxyantipyrine as free or glucuronide forms, respectively, was applied to measure the activity of cytochrome P_{450} subfamilies I, II, and III in the patients studied. Patients with gastric disturbances, allergy or evolution to renal failure were excluded from the study. All patients gave informed writren consent to participate in the study.

The study protocol consisted of two phases. Phase I - selection by clinical and laboratory routine evaluation of all patients during the preoperative period. Phase II - sodium diclofenac, 1 mg/kg, *im*, Voltaren 75® twice daily was administered to patients in the Recovery Unit 48 h after surgery. Venous blood samples were collected during the time-dose interval (0-12 h). Analgesia was measured by the visual analogue scale (VAS) and reported as percentage of pain intensity during the same period, ranging from zero (none) to 100% (maximum pain that each patient could tolerate) (7). Plasma diclofenac levels (as bounded or free drug) were determined by a micromethod after an ultrafiltration procedure followed by HPLC as described previously by the same authors for total or free drug, respectively (8-10).

For the pharmacokinetic evaluation, a two-compartment open model was applied to obtain the decay curve for plasma diclofenac, logC *vs* time. The kinetic parameters listed in Table 1, i.e., systemic bioavailability: area under the curve (AUC_T), drug elimination: total body clearance (Cl_T), drug distribution: volume of distribution (Vd_{AREA}), half-life and rate constants, were estimated (11).

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Diclofenac plasma protein binding was determined for both groups of patients after an ultrafiltration technique followed by the clean-up of only 200 µl of plasma samples using a single extraction with organic solvent in an acidic medium and HPLC was monitored by absorbance at 254 nm (9). The sigmoidal maximun effect (E_{MAX}) model was applied to correlate plasma diclofenac data and analgesia measured by the visual analogue scale for each patient (11-13).

The STATGRAPHICS software was applied to the data obtained in the present study. Chi-square, Spearman, Wilcoxon, Mann-Whitney and rank sum tests for paired and unpaired data were used for statistical analysis. Data are reported as median and $P<0.05$ was considered to be statistically significant (14).

Systemic bioavailability of diclofenac based on AUC_T, rate of bioavailability (RBA), maximum or peak plasma concentration (C_{MAX}) and time to reach the peak plasma concentration (T_{MAX}) showed similar results for both groups. A rapid absorption was obtained for both groups based on higher plasma concentrations at 0.5 h of dose administration. A good level of systemic bioavailability was reached for both groups of patients (Table 1 and Figure 1).

A large interindividual variation in drug distribution was shown for both groups, which was measured by alpha (α), a hybrid rate constant, and the distribution rate microconstants K_{12}, K_{21}, i.e., drug distribution from central to peripheral compartments or blood-tissue drug distribution and tissue-blood drug distribution, respectively. Only the parameter Vd_{AREA}, i.e., drug distribution in the extravascular fluid compartment, showed a significant difference between groups (50% reduction: 0.51 l/kg (CPB) *vs* 0.98 l/kg (control)) (Table 1).

The present results indicate changes in diclofenac distribution not only as a consequence of plasma protein drug binding reduced in patients submitted to cardiopulmonary bypass but also due to changes in plasma volume or in fluid compartments. Therefore, volume of distribution and percentage of protein drug binding were the best parameters to indicate changes in drug distribution with consequences of relevance considering analgesia measured by E_{MAX}, time to reach E_{MAX} (TE_{MAX}) and mean residence time for maximum effect ($MRTE_{MAX}$).

Drug elimination was measured by the following parameters: elimination rate constant (ß), elimination half-life ($t_{(1/2)ß}$) and Cl_T. The data obtained indicate that elimination rate constant was increased by 30% with a consequent reduction of elimination half-life in CPB.

Total body clearance remained unchanged

Table 1 - Effect of cardiopulmonary bypass (CPB) on systemic bioavailability, distribution and elimination of diclofenac.

Data are reported as medians for 10 CPB patients who received 1 mg/kg diclofenac, *im*. C_{MAX}: Peak plasma concentration; T_{MAX}: time to reach C_{MAX}; AUC_T: area under the curve related to systemic availability of drug; RBA: rate of bioavailability; α and ß: hybrid distribution and elimination rate constants; K_{12} and K_{21}: distribution microconstants; binding: percentage of plasma drug bound to protein; Vd_{AREA}: distribution volume; $t_{(1/2)ß}$: elimination half-life; Cl_T: plasma clearance; *$P<0.05$ compared to control (Wilcoxon test, Mann-Whitney test and rank sum test).

Parameters	CPB N = 10	Control N = 14
Systemic bioavailability		
C_{MAX} (ng/ml)	2167	1811
T_{MAX} (h)	0.50	0.50
AUC_T (µg h^{-1} ml^{-1})	4.44	4.04
RBA (h^{-1})	4.62	5.20
Distribution		
α (h^{-1})	2.15	1.98
K_{12} (h^{-1})	0.49	0.70
K_{21} (h^{-1})	0.65	0.62
Binding (%)	94*	99
Vd_{AREA} (l/kg)	0.51*	0.98
Elimination		
ß (h^{-1})	0.39*	0.30
$t_{(1/2)ß}$ (h)	1.80*	2.33
Cl_T (ml min^{-1} kg^{-1})	3.50	5.03

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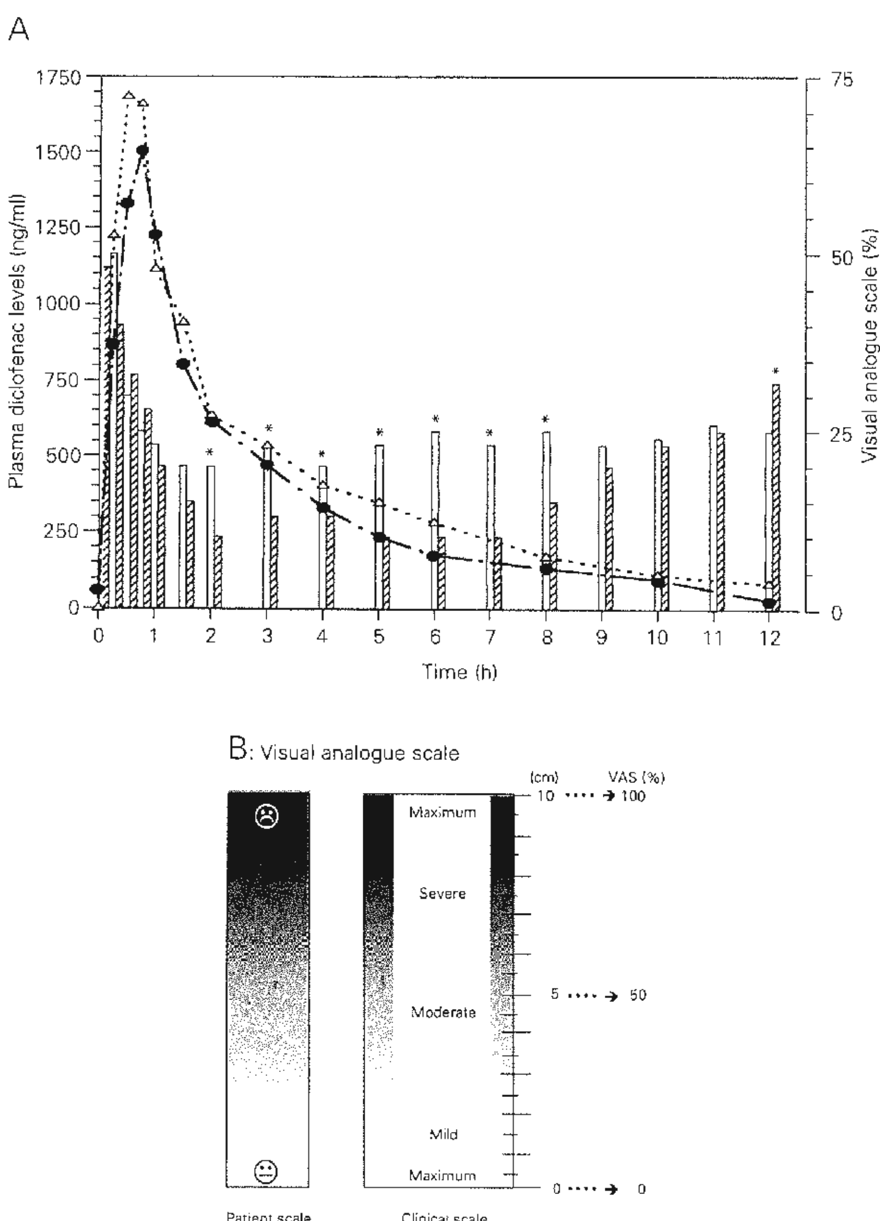

Figure 1 - Effect of cardiopulmonary bypass (CPB) on plasma diclofenac levels and analgesia. Patients received diclofenac (1 mg/kg, *im*) after surgery. *A*, Left ordinate: Δ, plasma diclofenac levels of the CPB group (N = 10); ●, plasma diclofenac levels of the control surgical group (N = 14). Right ordinate: analgesia measured with the visual analogue scale (see *panel B* for the explanation). □, Pain level of the CPB group; ▨, pain level of the control surgical group. *P<0.05 compared to control for analgesia (Wilcoxon test). All data are reported as median values.

for both groups of patients in spite of a 30% reduction in hepatic blood flow as reported previously (4,5). The present data showed wide interindividual variation for both groups when expressed as median: 3.50 CPB *vs* 5.03 ml min^{-1} kg^{-1} control, P = 0.13 (Table 1), or as range of variation IC95%: 2.91-4.43 ml min^{-1} kg^{-1} (CPB) and 3.32-6.03 ml min^{-1} kg^{-1} (control) (P>0.05).

Patients from the Recovery Unit investigated in the study protocol showed different reductions in pain after drug administration, measured by the visual analogue scale (7), for which VAS = 0% means no pain, and VAS = 100% means the highest level of pain that each patient could tolerate. Pain was measured in two different situations: rest and cough during the 0-12-h interval. Considering the cough situation, pain was reduced from 55% to 30% VAS for CPB and from 50% to 35% VAS for control, 0.5 h after drug administration (Figure 1).

The maximum effect was reached at 2 h after drug administration. E_{MAX}:TE_{MAX} measured in patients after cardiopulmonary bypass was 25% VAS *vs* 10% VAS (control), indicating a lower reduction in pain intensity obtained for CPB patients when compared to control.

Analgesia remained unchanged at about 25% VAS between 0.75 and 12 h after drug administration for CPB, and at about 10% VAS for control, during the 2-7-h interval ($MRTE_{MAX}$), i.e., the period of time that the maximum effect remains constant after drug administration. Then, pain recorded at 8, 10 and 12 h after drug administration increased by 15%, 23% and 32% VAS, respectively, for the control, while pain remained unchanged (25% VAS) for CPB during the same period.

Finally, after drug administration, a lower reduction in pain was obtained for patients after CPB when compared to the control, 55% to 25% VAS *vs* 50% to 10% VAS, P<0.05, respectively. A lower analgesia without any change in lag-time was obtained for CPB when compared to control, probably as a consequence of the reduction in volume of distribution and the increase in free plasma diclofenac levels after cardiopulmonary bypass.

Since diclofenac is extensively metabolized by the liver, followed by urinary excretion of several hydroxylated metabolites, the present data suggest a close relationship between elimination rate constant and diclofenac plasma protein binding. Therefore, the estimated kinetic parameters could explain the direct correlation of analgesia with volume of distribution, and the inverse correlation of analgesia with the diclofenac elimination rate constant. Finally, the kinetic evidence reported here could explain the differences in analgesia between the two groups and the higher pain intensity in CPB patients during the period of 0-12 h after drug administration. In conclusion, the extent of diclofenac plasma protein binding was influenced by the cardiopulmonary bypass procedure with kinetic-dynamic consequences of clinical relevance.

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Presynaptic *Gamma*-Hydroxybutyric Acid (GHB) and *Gamma*-Aminobutyric Acid$_B$ (GABA$_B$) Receptor-Mediated Release of GABA and glutamate (GLU) in Rat Thalamic Ventrobasal Nucleus (VB): A Possible Mechanism for the Generation of Absence-Like Seizures Induced by GHB[1]

PRADEEP K. BANERJEE and O. CARTER SNEAD III

Department of Neurology, University of Southern California School of Medicine and Childrens Hospital of Los Angeles, Los Angeles, California

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ABSTRACT

The ventrobasal nucleus of thalamus (VB) is considered to be intimately involved in the genesis of experimental absence-like seizures. Bilateral microinfusion of γ-hydroxybutyric acid (GHB) into VB or systemic administration of γ-butyrolactone, the prodrug of GHB, induces generalized absence-like seizures in rats. In the present study, the basal and K^+-evoked extracellular output of endogenous γ-aminobutyric acid (GABA) and glutamate (GLU) in behaving rat VB nucleus was characterized 1) during unilateral GHB perfusion into VB and 2) during the course of generalized absence-like seizures induced by GHB. Although the basal extracellular release of GABA was inhibited by GHB (250–1500 μM) in a concentration-dependent manner, basal GLU levels remained unaltered. However, K^+-evoked release of both GABA and GLU was significantly attenuated by GHB. During GHB-induced absence-like seizures, a similar decrease in basal GABA or K^+-evoked GABA and GLU levels was observed. These effects of GHB were partially reversed by the specific GHB receptor antagonist NCS 382. (−)-Baclofen (10–50 μM) also produced a concentration-dependent decrease in basal and K^+-evoked levels of GABA and GLU in this thalamic nucleus. The effects of either (−)-baclofen or GHB on the release of GABA and GLU were selectively antagonized by the GABA$_B$ receptor antagonists phaclofen (0.75–2 mM) and CGP 35348 (50–200 μM), respectively. These results suggest that by selectively modulating the basal and K^+-evoked release of GABA and GLU, GHB induces, in the thalamic ventrobasal relay nucleus, an optimal "excitatory" environment conducive to the generation of absence seizures. Moreover, the data raise the possibility that a presynaptic GHB/GABA$_B$ receptor complex occurs in VB.

Generalized absence seizures occur primarily in children and are manifested as rhythmic, bilaterally synchronous 3-Hz SWD associated with sudden unresponsiveness, eye blinking and myoclonic jerks (Mirsky *et al.*, 1986; Berkovic *et al.*, 1987). GHB is a naturally occurring metabolite of GABA (Gold and Roth, 1977) and has the ability to induce absence-like seizures in rats (Godschalk *et al.*, 1976, 1977) that bear electrical, behavioral and pharmacological resemblance to generalized absence seizures in humans (Snead, 1992a). The GHB model of generalized absence seizures in rats has been well characterized in terms of having a distinct ontogeny (Snead, 1994) and a wide pattern of origination in the thalamus and cortex (Banerjee *et al.*, 1993).

The development of generalized spike-wave discharges requires the presence of both a functional cortex and a functional thalamus (Gloor *et al.*, 1990). It has been shown that in completely thalamectomized animals, experimental SWD fail to develop in the cortex (Gloor *et al.*, 1990). Among the various "specific" and "nonspecific" nuclei of dorsal thalamus, experimental SWD evolve most readily, and in synchrony with the neocortical discharges, from the "specific" VB (Avoli and Gloor, 1982; Vergnes *et al.*, 1987; Liu *et al.*, 1992). In the GHB model, bilaterally synchronous SWD evolve most readily from the VB after systemic administration of GHB (Banerjee *et al.*, 1993). Moreover, bilateral microinfusion of GHB into rat VB has been shown to induce generalized absence-like seizures (Snead, 1991). In addition, similar GHB infusions into VB exacerbate absence seizures in genetic (Liu *et al.*, 1991) and other pharmacological models of absence seizures (Snead, 1988). Also, bilateral lesions made in VB have been shown to attenuate significantly the evolution of GHB-induced SWD from both thalamus and cortex

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ABBREVIATIONS: GHB, γ-hydroxybutyric acid; GABA, γ-aminobutyric acid; GBL, γ-butyrolactone; GLU, glutamate; SWD, spike-wave discharges; VB, ventrobasal nucleus of thalamus; HPLC-EC, high-performance liquid chromatography-electrochemical detection; TTX, tetrodotoxin.

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(Banerjee and Snead, 1994). These findings strongly suggest that the VB plays a key role in the generation of absence-like seizures induced by GHB.

GABA is the major inhibitory neurotransmitter in the brain. Though alteration in inhibitory neurotransmission within mutually interconnected thalamic and cortical neurons is thought to be critical to the genesis of absence seizures because these seizures occur more frequently during drowsiness and slow-wave sleep (McCarley *et al.*, 1983; Gloor *et al.*, 1990; Kellaway *et al.*, 1990), the role of endogenous GABA in the pathogenesis of this disorder is still obscure.

The discovery that $GABA_B$ receptor-mediated inhibitory postsynaptic potentials in thalamus activate low-threshold rebound calcium spikes that lead to burst firing and oscillatory behavior in thalamic neurons gave rise to the hypothesis that postsynaptic $GABA_B$ receptor-mediated mechanisms might operate in the pathogenesis of generalized absence seizures (Crunelli and Leresche, 1991). This hypothesis was given further credence by data showing that experimental absence seizures are aggravated by the specific $GABA_B$ receptor agonist (−)-baclofen and are suppressed by specific $GABA_B$ receptor antagonists (Liu *et al.*, 1992; Snead, 1992b). These experiments, taken in conjunction with *in vitro* data demonstrating the role of $GABA_B$-mediated IPSPs in regulating thalamocortical oscillatory behavior *via* low-threshold calcium currents (Crunelli and Leresche, 1991) strongly suggested that postsynaptic $GABA_B$-mediated mechanisms might be involved in the pathogenesis of absence seizures. However, little attention has been paid to the potential role of presynaptic $GABA_B$ receptors, which modulate neurotransmitter release in many areas of the brain (Bonanno and Raiteri, 1993a), in the generation of absence seizures.

In the present study, the extracellular release of both GABA and GLU, the major excitatory neurotransmitter in the brain, was characterized in the VB of behaving, freely moving rats. We also examined the possible involvement of the presynaptic $GABA_B$ receptors of VB in the genesis of GHB-induced absence seizures.

Materials and Methods

Drugs. The specific $GABA_B$ receptor antagonist CGP 35348 was a gift of Dr. R. Bernasconi (Ciba Geigy, Basel). The specific GHB receptor antagonist NCS 382 was a gift of Dr. J. J. Bourguignon (Centre de Neurochimie, Strasbourg). All other drugs were purchased from Sigma Chemical Co. (St. Louis, MO). All analytical reagents were obtained from standard commercial sources and were of the highest available purity.

Surgery and EEG recordings. Adult male Sprague Dawley rats (200–300 g) were used in all experiments. Animals were maintained on a 12-h light/dark cycle and given free access to food and water. Under halothane anesthesia, monopolar EEG recording electrodes were surgically implanted on the surface of the frontoparietal cortex with their tips placed bilaterally at frontal and parietal cortices. Guide cannulas were stereotaxically placed on the surface of dura at the following coordinates for VB: AP, −3.30 mm (from bregma); ML, 2.60 mm according to Paxinos and Watson (1986). Bipolar recording electrodes were constructed by twisting two stainless steel insulated wires (diameter 0.15 mm) and were anchored with the dialysis probes in such a way that the tips of the bipolar electrodes (which were deinsulated before anchoring) and the exposed area of the dialysis probe were approximately 0.5 mm apart.

Seven days after surgery, on the day of the dialysis experiment, the dura was punctured using a thin needle, and the dialysis probe–bipolar recording electrode assembly was inserted through the guide cannula into the VB of awake, freely moving rats. The vertical positioning of probes relative to the intended site of dialysis (5.80 mm from the surface of dura) was predetermined by gluing the probe holder (ESA, Bedford, MA) at a measured distance along the length of the probe. This resulted in accurate placement of the exposed tip of the probe in the desired area of VB. After completion of the experiment, the probe placement was also histologically verified (see below). EEG recordings from the cortical surface and from the site of dialysis were made simultaneously throughout the period during which dialysis was performed.

Microdialysis and quantification of GABA and GLU in dialysate fractions. All dialysis experiments were done in awake, freely moving animals using "loop-type" probes 1.5 mm in exposed tip length (or active dialysis area) prepared from Spectra/Por regenerated cellulose hollow fibers (I.D., 150 μm; molecular weight cutoff, 6000) as described by Dailey *et al.* (1992). Probes were activated by perfusing with 20% ethanol for 1 h followed by potassium Ringer phosphate (KRP) buffer for 2 h at the flow rate of 2 μl/min. The constituents of KRP buffer were (in mM) NaCl 120, KCl 2.4, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 0.9, Na_2HPO_4 1.4 and ascorbate 0.3, pH 7.4.

In vitro recovery of GABA and GLU through probes: Each probe was placed in a standard solution of a mixture of 5 μM GABA and GLU (maintained at 37°C with continuous stirring) and perfused with KRP buffer. After 1 h, three 10-min perfusates were collected and analyzed for GABA and GLU content using an HPLC-EC detecting system (see below). The percentages *in vitro* recovery of GABA and GLU were calculated by dividing the concentration of amino acids present in a 20-μl fraction of standard solution by the concentration of amino acids present in a 20-μl perfusate fraction and multiplying by 100. Probes were washed with KRP buffer overnight before use.

Dialysis protocol: All perfusions were made at a flow rate of 2 μl/min. After a 2-h stabilization period and starting from 3 h, dialysate fractions were collected every 10 min, and a known amount of internal standard, homoserine, acidified in 0.12N HCl, was added. Dialysate samples were either processed for HPLC separation or stored at −80°C until assayed. All drugs were added to the perfusion medium at the 4th hour. All drug perfusions lasted 60 min except that of KCl, which was perfused during the last 20 min of the 4th hour. During high concentration of GHB perfusion (1.5 mM), because of the low GABA levels resulting from the GHB perfusion at this concentration, dialysate fractions were collected every 15 min instead of at the standard 10-min intervals in order to achieve an optimal detection of the GABA signal during the HPLC assay. However, the effect of 1.5 mM GHB on the K^+-evoked release of GABA/GLU was estimated in standard 10-min dialysate samples. Ca^{++}-dependency of the transmitter release was studied by perfusing KRP buffer containing high Mg^{++} concentrations (12.5 mM) and no Ca^{++} ions. Mg^{++} acts as a Ca^{++} antagonist and prevents the influx of Ca^{++} through Ca^{++} channels (Bustos *et al.*, 1992). The Cl^- concentration was balanced during 0 Ca^{++}/high-Mg^{++} or high-K^+ perfusions by withholding appropriate concentrations of NaCl from the buffer. All other drug inclusions into the perfusion medium were made without altering the ionic constituents of the buffer.

(−)-Baclofen, a specific $GABA_B$ agonist, has been shown to decrease the release of GABA and GLU in various brain regions (Harrison, 1990; Calabresi *et al.*, 1991; Thompson and Gahwiler, 1992; Bonanno and Raiteri, 1993b). Therefore, we chose (−)-baclofen to characterize the $GABA_B$ receptor-mediated release of GABA and GLU in the VB. We studied the effect of GHB or (−)-baclofen on the K^+-evoked release of GABA and GLU by including KCl in the last 20 min of the 60-min GHB or (−)-baclofen perfusion on the 4th hour. During antagonist studies, $GABA_B$ antagonists in combination with either GHB or (−)-baclofen were perfused for 60 min on the 4th hour.

Unilateral perfusion of GHB into VB produces no significant EEG or behavioral changes typical of absence seizures, an effect seen only with the bilateral intrathalamic administration of GHB (Snead,

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1991). Therefore, in order to study the effects of GHB-induced generalized spike-wave discharges on the release patterns of GABA and GLU in the VB, we gave GBL, the prodrug of GHB (Roth *et al.*, 1966), i.p. in a dose of 100 mg/kg at the start of the 4th hour of dialysis and measured the extracellular release of GABA and GLU in the VB. The dose of GBL has been standardized as the threshold dose to induce generalized SWD in the GHB model of absence seizures (Snead, 1988). GBL is converted to GHB by a circulating lactonase (Roth and Giarman, 1969) and produces a more rapid onset and predictable time course of SWD than does GHB (Bearden *et al.*, 1980; Snead, 1991). GBL itself is inactive, and the generation of SWD in GBL-treated animals is due solely to the presence of converted GHB in the brain (Snead, 1991).

In another set of experiments, at the peak of GHB-induced absence seizures, *i.e.*, 40 min after the administration of GBL, a 20-min K^+ stimulation was applied to the VB through the dialysis probe.

During the course of GHB-induced SWD, the release of GABA and that of GLU were measured in the VB either in the absence or the presence of specific $GABA_B$ antagonists perfused into the VB. This strategy allowed us to evaluate the effects of $GABA_B$ antagonists on the release patterns of GABA and GLU in VB during the course of GHB-induced SWD without affecting the generalized pattern of SWD.

NCS 382, a specific GHB receptor antagonist (Maitre *et al.*, 1990), has been shown to attenuate GHB-induced SWD significantly (Maitre *et al.*, 1990). NCS 382 (500 mg/kg) was given i.p. 1 h before the administration of GBL (*i.e.*, at the start of third hour of dialysis), and the extracellular levels of GABA and GLU were measured in the VB. We examined the effect of NCS 382 on GHB-mediated changes in the K^+-evoked release of GABA and GLU in VB by giving a 20-min KCl stimulation 40 min after the administration of GBL.

Quantification of GABA and GLU: GABA and GLU were separated from the dialysate fractions using the HPLC method of Donzanti and Yamamoto (1988). Briefly, the samples were precolumn derivatized for exactly 2 min with a mixture of *o*-phthalaldehyde and β-mercaptoethanol before being injected into a 100-μl sample loop (Rheodyne, CA). This derivatized mixture was separated isocratically using a C_{18} (HR-80, ESA, MA) reverse-phase column and was detected on a coulometric electrochemical cell with electrode potentials set at −400 mV (electrode 1) and +600 mV (electrode 2). The mobile phase (0.1M Na_2HPO_4, 50mg/l Na_2EDTA and 25% methanol, pH 6.75) was pumped into the system at a flow rate of 1.2 ml/min. The typical retention times of GLU and GABA are 2.7 and 25.6 min., respectively.

Histologic verification of probe placement. After completion of each dialysis experiment, animals were sacrificed and their brains chilled in isopentane (−40°C) for 60 s. Coronal brain sections 20 μ thick were thaw-mounted on gelatin-coated slides and stained with cresyl violet. The placement of the dialysis probes was verified under light microscopy.

Data analysis. All data were expressed as arithmatic mean ± S.E.M. Only those animals exhibiting correct placement of dialysis probes (as assessed by histology), were incorporated in the data analysis. The number of animals in each group of experiments varied from 4 to 12 (depending on the histologic verification of probe placement). The concentrations of GABA or GLU in the dialysate samples were measured and expressed as moles of amino acids present per microliter of dialysate. K^+-stimulated release experiments were performed in a different set of animals. Paired drug-naive controls were used for each treatment group. Drug- or seizure-induced changes in basal or K^+-evoked levels of GABA and GLU were determined by comparing these levels with the basal or K^+-evoked levels of GABA and GLU in drug- or seizure-free animals. The concentrations of both GABA and GLU were estimated relative to the detector's response to the internal standard, homoserine.

Simultaneous comparisons of multiple-group means were performed by ANOVA followed by either Dunnett's or Tukey's post-hoc test to discern differences between groups. Two-group means were compared using a two-tailed, independent Student's *t* test.

Results

Characterization of the extracellular release of GABA and GLU in VB. *In vitro* recovery of GABA and GLU through the probes varied from 10% to 15%. Immediately after insertion of the probe, the base-line GABA and GLU levels rose significantly; however, this was followed by a steady decline in the levels over time. Within 1.5 h, these levels reached a steady state in which the transmitter levels fluctuated no more than 5% to 6%. Table 1A gives the basal levels of GABA and GLU in drug-naive animals during the 4th hour of dialysis. Figure 1 shows the placement of the dialysis probe within the VB. Along the probe tract, an increase in vascularization and gliosis was observed (see fig. 1B), but no tissue damage was seen at the site of dialysis.

The basal extracellular levels of GABA and GLU were found to be approximately 30% to 40% dependent on the concentration of Ca^{++} (1.2 mM) in the perfusion medium (table 1B). K^+ stimulation (KCl, 30–100 mM, for 20 min) enhanced the extracellular levels of GABA (190%–850%) and GLU (180%–500%) in a concentration-dependent fashion (table 2). The K^+-evoked release of GABA was approximately 50% dependent on the presence of Ca^{++} in the perfusion buffer, whereas the evoked overflow of GLU was more than 70% Ca^{++}-dependent (table 1B).

We studied the action potential-dependency of GABA and GLU release by perfusing the sodium channel blocker TTX (0.5 μM for 1 h, n = 4) into VB. Neither the basal nor the K^+-evoked release of GABA or GLU was significantly altered by TTX perfusion (data not shown).

Effect of GHB perfusion on the extracellular release of GABA and GLU in VB. Perfusion of GHB (250–1500 μM, n = 5–7 for each concentration) into VB inhibited the basal release of GABA in a concentration-dependent fashion (fig. 2). The effect of GHB was significant ($P < .05$) at all time points, except at 2.5 and 3 h, when compared with the basal levels of GABA in GHB-naive animals. The basal release of GLU was not significantly altered during the perfusion of any of these concentrations of GHB. However, the K^+-evoked release of both GABA and GLU was significantly decreased

TABLE 1A

Basal extracellular levels of GABA and GLU in the VB of behaving rats measured during the 4th hour of dialysis

Values are expressed as moles of transmitter amino acids present per microliter of dialysate. KRP buffer was perfused in the VB in the presence of physiological concentrations of Ca^{++} and Mg^{++} (1.2 mM each). Six consecutive 10-min dialysate fractions were collected during the 4th hour of dialysis and analyzed for GLU and GABA content. The amino acids were precolumn derivatized and separated isocratically using a reverse-phase C_{18} column (see "Materials and Methods" for details). There was no significant difference in the dialysate levels of GLU or GABA during the course of the 4th hour of dialysis.

Transmitter	n	10 min	20 min	30 min	40 min	50 min	60 min
GABA fmole/μl	12	40.92 ± 3.07	42.31 ± 4.12	42.07 ± 5.56	41.44 ± 4.13	42.83 ± 2.96	41.21 ± 5.02
GLU pmole/μl	12	01.23 ± 0.12	01.17 ± 0.11	01.21 ± 0.12	01.19 ± 0.09	01.16 ± 0.12	01.20 ± 0.09

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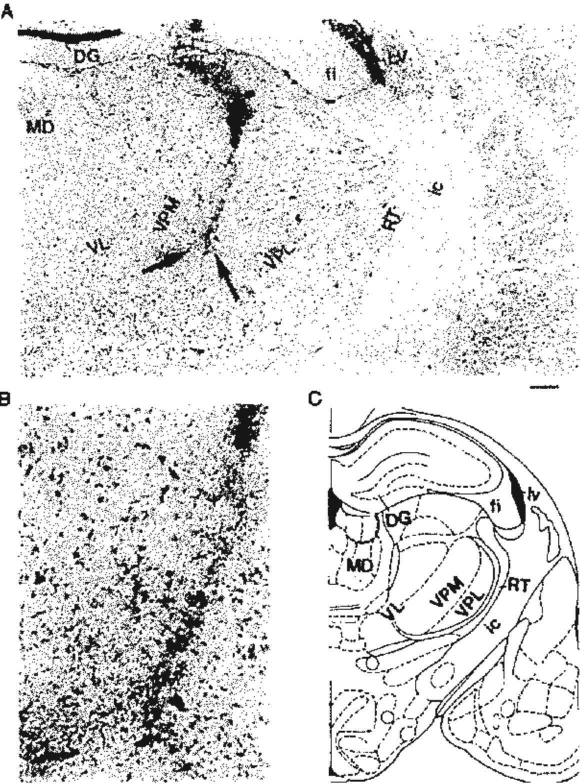

Fig. 1. Photomicrographs (A and B) and schematic drawing (C) of a rat brain coronal section, 3.30 mm posterior from bregma, showing the placement of the dialysis probe in VB. One hour after completion of the dialysis experiment, the rat was sacrificed, and 25-μ serial coronal sections were stained with cresyl violet. Arrows in panel A indicate the presumed site of dialysis within the VB. Panel B shows the probe tract at a higher magnification (× 2.5). Note the increase in vascularization and gliosis along the probe tract; however, there is no tissue damage. VB is a collective term for ventroposterolateral and ventroposteromedial nuclei of thalamus. These two ventral nuclei of thalamus correspond respectively to the lateral and medial subdivisions of the VB. DG, dentate gyrus; fi, fimbria of hippocampus; ic, internal capsule; LV, lateral ventricle; MD, mediodorsal nucleus of thalamus; pc, paracentral nucleus, RT, reticular nucleus; VL, ventrolateral; VPM, ventroposteromedial; VPL, ventroposterolateral nucleus of thalamus. Bars: A, 0.25 mm; B, 0.1 mm and C, 0.60 mm.

by all concentrations of GHB tested when compared with K^+-evoked levels of GABA and GLU in subjects not treated with GHB (table 3).

Extracellular levels of GABA and GLU in VB during the course of GHB-induced generalized spike-wave discharges. After systemic administration of GBL (100 mg/kg, i.p.), the first EEG change, characterized by high-voltage, bilaterally synchronous, 5 to 6-Hz spike-wave discharges, occurred within 5 to 8 min (fig. 3). These electrographic discharges were recorded synchronously from the frontoparietal cortex and the VB or from the dialysis site (fig. 3, panel B). Within 10 min after the onset, these spike-wave discharges became continuous (fig. 3, panel C or D). Two hours after the onset of SWD, sporadic incidents of low-voltage spike-wave bursts were recorded from both thalamus and cortex (fig. 3, panel E). After 3 h, the EEG became normal (fig. 3, panel F).

During the course of GHB-induced SWD, changes in extracellular GABA levels in the VB were similar to those seen after intrathalamic perfusion of GHB (see fig. 4). Basal GLU levels were not altered during seizures. As shown in figure 4, GBL was administered systemically (100 mg/kg, i.p.; $n = 6$) at time 0 (on the abscissa), which represents the start of the 4th hour of dialysis. However, it was not possible to determine what changes in GABA and GLU levels occurred precisely at the time of onset of GHB-induced SWD, *i.e.*, within 5 to 8 min after GBL administration, because in order to detect the GABA peak (during HPLC separation), we would have needed a dialysate sample of at least 20 μl. Because the dialysis was performed at the rate of 2 μl/min, a minimum of 10 min of sampling time was necessary for optimal detector sensitivity to GABA.

In another group of experiments, at the peak of GHB-induced spike-wave activity, *i.e.*, 40 min after the administration of GBL, a 20-min K^+ stimulation (KCl, 30 mM) was given in the VB through the dialysis probe. There was a significant decrease in K^+-evoked overflow of both GABA and GLU in these animals when these levels were compared with K^+-stimulated levels of GABA and GLU in GBL-naive animals (table 3).

Effect of the specific GHB receptor antagonist NSC 382 on basal and K^+-evoked GABA and GLU release in VB during the course of GHB seizures. NCS 382 pretreatment ($n = 5$) significantly attenuated the generation of GHB-induced SWD (data not shown). During this attenuated phase of SWD, the decrease in basal extracellular levels of GABA was markedly less than that observed with GHB alone ($P < .05$ at 1.5 h, fig. 4). NCS 382 pretreatment also partially reversed the inhibitory effects of GHB on K^+-stimulated GABA and GLU levels (table 3).

Effect of the specific $GABA_B$ receptor antagonists CGP 35348 and phaclofen on (−)-baclofen- or GHB-induced changes in basal and K^+-evoked GABA or GLU release in VB. In order to test the hypothesis that the GHB-induced decrease in GABA release in thalamic VB is mediated through presynaptic $GABA_B$ receptors, we studied the effects of two $GABA_B$ receptor antagonists, CGP 35348 (Karlsson *et al.*, 1992) and phaclofen (Bonanno *et al.*, 1988), on GHB-mediated changes in GABA release in VB. Because GHB does not alter the basal levels of GLU, we chose (−)-baclofen, a specific $GABA_B$ agonist that has been shown to decrease the basal release of both GABA and GLU in the cortex (Bonanno and Raiteri, 1993b) and in other areas of the CNS (Harrison, 1990; Calabresi *et al.*, 1991; Thompson and Gahwiler, 1992), to serve as positive control in these experiments.

(−)-Baclofen perfusion into VB (10–50 μM, $n = 5$ for each concentration) produced a concentration-dependent decrease in both basal and K^+-evoked release of GABA and GLU as compared with the basal or K^+-evoked levels of these amino acids in drug-naive animals (table 4).

CGP 35348 (50–200 μM, $n = 5$–6 for each concentration) perfusion into VB dose-dependently antagonized (−)-baclofen's effect on basal and K^+-evoked GLU release (table 5), but had no apparent effect on (−)-baclofen-mediated changes in basal or K^+-evoked GABA release (table 6). Similarly, the GHB-induced decrease in basal or K^+-evoked GABA levels

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TABLE 1B

Ca^{++}-dependency of basal and K^+-stimulated extracellular output of GABA and GLU in the VB of behaving adult rats

Decrease in basal (n = 6) or K^+-stimulated (n = 6) extracellular levels of GABA and GLU (in the absence of Ca^{++} and the presence of high Mg^+ in the perfusion buffer) in two consecutive dialysate fractions collected during the last 20 min of the 4th hour of dialysis. Values are mean ± S.E.M. In control animals, basal (n = 12) and K^+-stimulated (n = 7) levels of GABA and GLU were also measured during the last 20 min of the 4th hour of dialysis but in the presence of physiological concentrations of Ca^{++} and Mg^{++} (1.2 mM each). 0 Ca^{++} 12.5 Mg^{++} was perfused for 60 min on the 4th hour of dialysis (see "Materials and Methods" for details). K^+-stimulated release experiments were performed in a different set of animals, wherein KCl (30 mM) was included in the last 20 min of the 4th hour of dialysis.

Ions	Decrease in Amino Acid Levels			
	GABA (fmole/µl Dialysate)		GLU (pmole/µl dialysate)	
	Basal	K^+-Stimulated	Basal	K^+-Stimulated
Control	40.31 ± 03.82	116.89 ± 10.44	1.19 ± 0.11	3.29 ± 0.62
Ca^{++} (0 mM) + Mg^{++} (12.5 mM)	26.21 ± 01.76*	60.79 ± 04.66**	0.76 ± 0.09*	0.88 ± 0.10**

* P < .05; ** P < .001 as compared with respective control values, *i.e.*, perfusion in the presence of physiological concentrations of Ca^{++} and Mg^{++} (independent Student's t test).

TABLE 2

Effect of K^+-stimulation on the extracellular output of GABA and GLU in rat VB

Increase in K^+-stimulated extracellular levels of GABA and GLU in two consecutive dialysate fractions collected during the last 20 min of the 4th hour of dialysis. The values are mean ± S.E.M. There were seven rats for each concentration of KCl. KCl was included in the last 20 min of the 4th hour of dialysis (see "Materials and Methods" for details). The control represents basal levels of GABA and GLU (in the absence of KCl) during the last 20 min of the 4th hour of dialysis (n = 12). The effect of KCl was significant (P < .001) at all concentrations.

Chemicals	K^+-Stimulated Transmitter Releases	
	GABA (fmole/µl dialysate)	GLU (pmole/µl dialysate)
Control	40.31 ± 03.82	01.19 ± 0.11
KCl, 30 mM	116.89 ± 10.44	03.29 ± 062
KCl, 60 mM	221.71 ± 19.17	04.76 ± 0.73
KCl, 100 mM	358.35 ± 26.71	06.90 ± 0.84

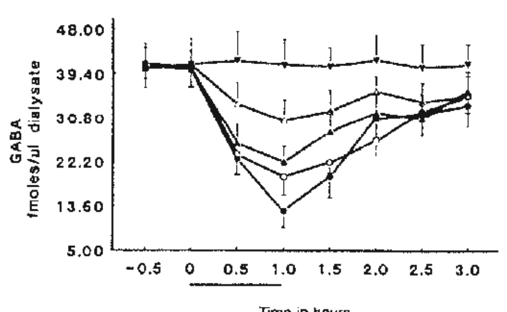

Fig. 2. Dose-dependent decrease in basal extracellular levels of GABA by GHB [in µM: 250 (△), 500 (▲), 1000 (○) and 1500 (●)] perfusion in VB (n = 5–7 for each concentration). Values are mean ± S.E.M. GHB was perfused for 60 min (denoted by scale bar from 0 to 1 h) starting at the 4th hour of dialysis. The point 0 on the abscissa represents the start of the 4th hour. During 1500 µM GHB perfusion, dialysates were collected every 15 min instead of the standard 10-min intervals in order to achieve optimal detection of the GABA signal during the HPLC assay. The effect of GHB (500–1500 µM) was significant (P < .05; Dunnett's test) at all time points, except at 2.5 and 3 h, when compared with the basal GABA levels in GHB-naive animals (▼) (n = 12).

TABLE 3

Effects of GHB and of the specific GHB receptor antagonist NCS 382 on K^+-stimulated extracellular efflux of GABA and GLU

Decrease in K^+-evoked release of GABA and GLU by GHB (perfused into VB) in two consecutive dialysate fractions collected during the last 20 min of the 4th hour of dialysis. Values are mean ± S.E.M. from 5 to 7 rats for each concentration of GHB. GHB was perfused for 60 min starting at the 4th hour of dialysis. In the systemic GHB paradigm, GBL was administered systemically at the start of the 4th hour of dialysis (n = 6). NCS 382 was administered systemically 1 hr before GBL administration (n = 5). KCl (30 mM) was included in the last 20 min of the 4th hour of dialysis. Control values represent K^+-evoked levels of GABA and GLU in the absence of GHB or GBL during the last 20 min of the 4th hour of dialysis (n = 7 or 5).

Drugs	K^+-Stimulated Transmitter Release	
	GABA (fmole/µl dialysate)	GLU (pmole/µl dialysate)
Local:		
Control	116.89 ± 10.44	03.29 ± 0.62
GHB, 250 µM	59.61 ± 03.76*	01.28 ± 0.21
GHB, 500 µM	54.93 ± 04.01*	01.31 ± 0.10*
GHB, 1000 µM	45.59 ± 04.06*	01.12 ± 0.09*
GHB, 1500 µM	40.91 ± 03.92*	00.98 ± 0.09*
Systemic:		
Control	112.79 ± 10.09	03.17 ± 0.59
GBL, 100 mg/kg; i.p.	52.27 ± 04.33*	01.44 ± 0.11*
GBL + NCS 382, 500 mg/kg, i.p.	79.48 ± 03.89•	02.30 ± 0.12•

* P < .001 when compared with non-GHB or GBL values (Dunnett's test). • P < .05 when compared with values for GBL alone (independent Student's t test).

was not significantly altered by CGP 35348 (table 6). However, GHB's effect on K^+-evoked GLU release was partially reversed by CGP 35348 (table 5). On the other hand, phaclofen (0.75–2 mM, n = 5–7 for each concentration) perfusion into VB produced a dose-dependent blockade of both GHB- and (−)-baclofen-induced changes in basal and K^+-evoked GABA levels (table 7). Changes in K^+-evoked or basal GLU levels induced by either GHB or (−)-baclofen were not significantly affected by phaclofen (table 8).

In the systemic GHB paradigm, either CGP 35348 (50–200 µM) or phaclofen (0.75–2 mM) was perfused into VB for 60 min on the 4th hour of dialysis, *i.e.*, during the course of GHB-induced SWD. The effect of phaclofen on GABA release was similar to that observed with local GHB perfusion experiments (partial antagonism of the decrease in basal GABA release during the course of SWD), whereas CGP 35348 was ineffective (data not shown).

In regard to the high concentrations of GHB or of the specific $GABA_B$ receptor antagonists used, it should be noted that in the present experiments, all drugs (except GBL and NCS 382) were perfused into VB through the dialysis probe. Because we found that maximum *in vitro* recovery of GABA and GLU through the probes was about 15%, and because recovery of a substance across the dialysis probe in either direction remains the same (Ungerstedt, 1991), intrathalamic perfusion of small molecules, such as the GHB and $GABA_B$ antagonists used in the present experiments, proba-

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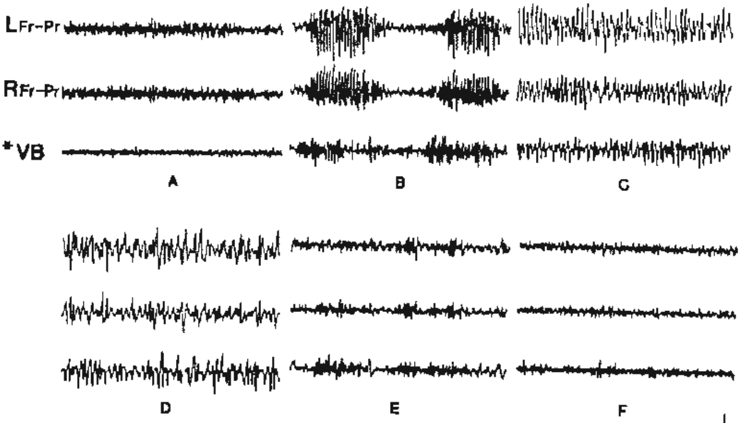

Fig. 3. Simultaneous EEG recordings from a rat frontoparietal cortex and VB or the dialysis site during the course of GHB-induced SWD. EEG recordings were made throughout the period during which dialysis was performed. Panel A shows the background EEG activity during the basal dialysate collection. Within 5 min after systemic administration of GBL (100 mg/kg; i.p.) high-voltage, 5 to 6-Hz spike-wave complexes evolved synchronously from thalamus and cortex (panel B). Panels C and D show continuous spike-wave discharges 30 min and 1 h, respectively, after the onset of seizures. Occassional low-voltage spike-wave bursts were seen 2 h after the onset of seizures (panel E). Panel F shows normalization of EEG 3 h after the onset of seizures. L_{Fr-Pr} and R_{Fr-Pr} denote the left and right frontoparietal leads, respectively. The asterisk denotes the bipolar recording lead. Calibration: 150 μV, 2 s.

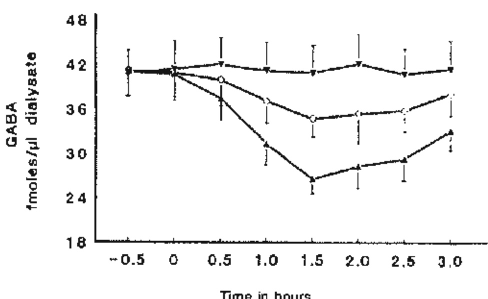

Fig. 4. Decrease in basal extracellular levels of GABA in VB during the course of GHB-induced SWD (▲). Values are mean ± SEM. Generalized SWD were induced by systemic administration of GBL (100 mg/kg, i.p.; n = 6) at time 0 (on the abscissa) which represents the start of the 4th hour of dialysis. GHB-induced suppression of GABA release was significant at all time points, except at 0.5 h ($P < .05$, Dunnett's test), when compared with the basal GABA levels in GHB or GBL-naive animals (▼) (n = 5). In GHB/GBL-naive animals, saline was injected i.p. at the start of the 4th hour of dialysis. The effect of GHB-induced SWD on basal GABA release was partially antagonized by the GHB receptor antagonist NCS 382 (O). NCS 382 was administered systemically 60 min before the administration of GBL. The effect of NCS 382 was significant at 1.5 h ($P < .05$, Dunnett's test; n = 5) when compared with the GABA levels during the course of GHB-induced SWD.

bly results in the delivery of only a fraction of the concentration at the site of dialysis.

Discussion

Efflux of transmitters in the extracellular space occurs from both neuronal and extraneuronal sources (Paulsen and Fonnum, 1989; Anderson and DiMicco, 1992). Hence, during dialysis experiments it is important to identify the source of transmitter in the sampled dialysate fraction. The Ca^{++}- and/or action potential-dependent component of the transmitter present in the extracellular space is generally regarded as being neuronal in origin because transmitter release originating from metabolic or other extraneuronal sources is largely Ca^{++}- and/or nerve impulse-independent (Paulsen and Fonnum, 1989; Timmerman *et al.*, 1992).

Several studies in the past have failed to show a convincing Ca^{++}- and TTX-dependency of basal amino acid levels in the extracellular space and suggest that the major portions of the basal levels of amino acids in the extracellular space may not derive from neuronal processes (Westerink *et al.*, 1987a; Westerink and De Vries, 1989). In the present study, however, the basal release of both GABA and GLU in VB was found to be approximately 40% dependent on the presence of Ca^{++} in the perfusion medium. We observed also no TTX-dependency of either basal or K^+-evoked overflow of these amino acids. Because there is evidence that TTX perfusion into different brain regions in concentrations as low as 0.1 μM significantly inhibits the extracellular release of dopamine, serotonin and acetylcholine (Westerink *et al.*, 1987b; 1988), it is unlikely that the concentration of TTX used in the present experiments (0.5 μM) was suboptimal. Moreover, higher doses of TTX perfusion have been reported to induce strong sedation (Westerink and De Vries, 1989). Although Osborne *et al.* (1990) reported a high TTX-dependent basal extracellular GABA output in anesthetized rat striatum (about 33%) and also indicated that the TTX-dependent amino acid release becomes more pronounced in awake animals (about 55%), those authors used 2.4 mM of Ca^{++} (in the absence of Mg^{++}) in the perfusion buffer rather than the more physiological concentration of Ca^{++} and Mg^{++} (1.2 mM each) that was utilized in the present experiments and by others (Westerink *et al.*, 1988) who also have failed to observe any significant TTX-dependency of amino acid release. It has been shown that with such high concentrations of Ca^{++} perfusion, the basal amino acid output can rise to approximately 200% as compared with the levels measured with 1.2 mM Ca^{++} (Drew and Ungerstedt, 1991). Hence, it is likely that the TTX-dependency of amino acid output observed by Osborne and colleagues actually represents TTX-dependent Ca^{++}-stimulated release, not a basal state of release. Interestingly, in the present experiments the K^+-evoked release of GABA and GLU, which exhibited a good degree of Ca^{++} dependency (about 50% for GABA and more than 70% for GLU), also did not respond to TTX perfusion. Moreover, there is evidence that the increase in extracellular GABA levels in the striatum induced by an neuronal reuptake inhibitor of GABA, (−)-nipecotic acid, is also insensitive to TTX (Westerink and De Vries, 1989). Taking these findings together, it appears that there is not a positive correlation between the stimulated release of amino acid transmitters or the Ca^{++}-dependent component of transmitters in the extracellular

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TABLE 4

Effect of (−)-baclofen perfusion into VB on basal and K⁺-stimulated extracellular efflux of GABA and GLU

Decrease in basal and K^+-evoked extracellular release of GABA and GLU by (−)-baclofen in two consecutive dialysate fractions collected during the last 20 min of the 4th hour of dialysis. Values are mean ± S.E.M. There were five rats for each concentration (−)-baclofen. (−)-Baclofen was perfused for 60 min starting at the 4th hour of dialysis. K^+-stimulated release experiments were performed in a different set of animals (n = 5 for each concentration of (−)-baclofen), wherein KCl (30 mM) was included in the last 20 min of the 60-min (−)-baclofen perfusion. Control values represent basal (n = 12) or K^+-evoked (n = 7) levels of GABA and GLU in the absence of (−)-baclofen during the last 20 min of the 4th hour of dialysis.

Drugs	Extracellular Transmitter Release			
	GABA (fmole/µl dialysate)		GLU (pmole/µl dialysate)	
	Basal	K^+-Stimulated	Basal	K^+-Stimulated
Control	40.31 ± 03.82	116.89 ± 10.44	1.19 ± 0.11	3.29 ± 0.62
(−)-Baclofen				
10 µM	33.45 ± 02.77	72.47 ± 06.71*	1.07 ± 0.08	2.46 ± 0.44*
25 µM	31.44 ± 02.01	67.79 ± 07.01**	0.96 ± 0.08	2.04 ± 0.30**
50 µM	24.20 ± 01.80*	57.27 ± 05.31**	0.82 ± 0.07*	1.54 ± 0.19**

** $P < .001$ and * $P < .05$ when compared with the respective nonbaclofen basal or K^+-evoked values (Dunnett's test).

TABLE 5

Effect of CGP 35348 on (−)-baclofen- and GHB-mediated inhibition of basal and K⁺-evoked extracellular levels of GLU in rat VB

Partial antagonism of (−)-baclofen- or GHB-induced decrease in basal or K^+-evoked extracellular output of GLU by CGP 353348 in two consecutive dialysate fractions collected during the last 20 min of the 4th hour of dialysis. Values are mean ± S.E.M. from 5 to 6 rats for each concentration of CGP 35348. K^+-evoked release experiments were performed in a different set of animals (n = 4–5, where KCl (30 mM) was included in the last 20 min of the 60-min mixed-drug perfusion. Control values represent basal (n = 12) or K^+-evoked (n = 7) levels of GLU in the absence of (−)-baclofen/GHB or CGP 35348 during the last 20 min of the 4th hour of dialysis.

Drugs	Extracellular GLU release (pmole/µl dialysate)	
	Basal	K^+-Stimulated
Control	1.19 ± 0.11	3.29 ± 0.62
(−)-Baclofen 50 µM	0.82 ± 0.07	1.54 ± 0.19
+CGP 35348 50 µM	0.85 ± 0.06	1.84 ± 0.16
+CGP 35348 100 µM	0.92 ± 0.07	2.07 ± 0.20*
+CGP 35348 200 µM	1.03 ± 0.09*	2.31 ± 0.19*
GHB 1000 µM	no effect	1.12 ± 0.09
+CGP 35348 50 µM	—	1.41 ± 0.11
+CGP 35348 100 µM	—	1.67 ± 0.14
+CGP 35348 200 µM	—	1.97 ± 0.20*

* $P < .05$ (Dunnett's test) when compared with either the (−)-baclofen group or the GHB-alone group.

TABLE 6

Effect of CGP 35348 on (−)-baclofen- and GHB-mediated inhibition of basal and K⁺-evoked extracellular levels of GABA in rat VB

Lack of antagonism of (−)-baclofen- or GHB-induced decrease in basal and K^+-evoked extracellular output of GABA by CGP 353348 in two consecutive dialysate fractions collected during the last 20 min of the 4th hour of dialysis. Values are mean ± S.E.M. from 5 to 6 rats for each concentration of CGP 35348. K^+-evoked release experiments were performed in a different set of animals (n = 4–5), wherein KCl (30 mM) was included in the last 20 min of the 60-min mixed-drug perfusion. Control values represent basal (n = 12) or K^+-evoked (n = 7) levels of GABA in the absence of (−)-baclofen/GHB or CGP 35348 during the last 20 min of the 4th hour of dialysis.

Drugs	Extracellular GABA Release (fmole/µl dialysate)	
	Basal	K^+-Stimulated
Control	40.31 ± 03.82	116.89 ± 10.44
(−)-Baclofen 50 µM	24.20 ± 01.80	57.27 ± 05.31
+CGP 35348, 50 µM	26.77 ± 02.03	60.61 ± 06.71
+CGP 35348, 100 µM	30.47 ± 02.71	66.46 ± 07.91
+CGP 35348, 200 µM	29.66 ± 02.06	68.78 ± 08.01
GHB 1000 µM	20.71 ± 03.17	45.59 ± 04.06
+CGP 35348, 50 µM	21.67 ± 03.52	50.61 ± 05.33
+CGP 35348, 100 µM	23.91 ± 04.00	49.17 ± 04.91
+CGP 35348, 200 µM	22.66 ± 03.61	51.67 ± 05.03

space and the TTX-dependency of their release. We did not increase the concentration of Ca^{++} in the perfusion medium, because our aim was to characterize the basal extracellular output of GABA and GLU during the course of generalized absence seizures under normal physiological conditions.

Alternatively, it is also possible that different anatomic areas of the brain may possess different release mechanisms. The possibility that the efflux of GABA and GLU in the VB represents a carrier-mediated transport rather than a classic exocytotic release seems unlikely, because the release of these transmitters exhibited a good degree of Ca^{++}-dependency under stimulated conditions. However, at basal state the release of these transmitters showed only a modest Ca^{++} dependency. Thus it may be concluded that in the present experiments, the release of GABA and GLU in the VB, both in the basal and the stimulated state, originated only in part from the neuronal pool in a Ca^{++}-dependent but TTX-independent fashion. In such a case, one may ask whether the GHB- or (−)-baclofen-mediated decrease in basal GABA or/and GLU release observed in the present experiments represented the neuronal component of the transmitters. Because high-affinity GHB binding sites that have been shown to be responsible for the epileptogenic actions of GHB (Maitre *et al.*, 1990) are present specifically in neuronal structures but not in glial cells (Maitre *et al.*, 1990), the observed effects of GHB on GABA and GLU release after GHB perfusion into VB may be regarded as neuronal in nature. However, a similar explanation may not hold true for the inhibitory effects of (−)-baclofen, because $GABA_B$ receptors are located on both neuronal and non-neuronal structures (Bowery *et al.*, 1987). Hence it is difficult to predict what fraction of the neuronal GABA or GLU was inhibited during (−)-baclofen perfusion into VB. Remember too that a distinct differentiation between neuronal and extraneuronal release of GABA and GLU in the extracellular space in any given CNS nucleus has not been possible *via* the technique of microdialysis.

An imbalance between excitatory and inhibitory neurotransmission within mutually interconnected thalamic and neocortical neurons has been suspected to be critical to the genesis of generalized absence seizures (Gloor *et al.*, 1990). Preservation of GABA-mediated postsynaptic inhibition in the thalamocorticothalamic neuronal network during generalized SWD is a salient feature of absence seizures that

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TABLE 7

Effect of phaclofen on (−)-baclofen- and GHB-mediated inhibition of basal and K^+-stimulated extracellular output of GABA in rat VB

Partial antagonism of (−)-baclofen- or GHB-induced decrease in basal or K^+-evoked extracellular output of GABA by phaclofen in two consecutive dialysate fractions collected during the last 20 min of the 4th hour of dialysis. Values are mean ± S.E.M. from 5 to 7 rats for each concentration of phaclofen. K^+-evoked release experiments were performed in a different set of animals (n = 5), wherein KCl (30 mM) was included in the last 20 min of the 60-min mixed-drug perfusion. Control values represent basal (n = 12) or K^+-evoked (n = 7) levels of GABA in the absence of (−)-baclofen/GHB or phaclofen during the last 20 min of the 4th hour of dialysis.

Drugs	Extracellular GABA Release (fmole/µl dialysate)	
	Basal	K^+-Stimulated
Control	40.31 ± 03.82	116.89 ± 10.44
(−)-Baclofen, 50 µM	24.20 ± 01.80	57.27 ± 05.31
+phaclofen, 750 µM	28.23 ± 01.69	66.49 ± 06.07
+phaclofen, 1000 µM	33.41 ± 02.70	75.31 ± 07.11
+phaclofen, 2000 µM	36.92 ± 02.88*	98.91 ± 06.39*
GHB, 1000 µM	20.71 ± 03.17	45.59 ± 04.06
+phaclofen, 750 µM	28.14 ± 03.50	56.86 ± 04.31
+phaclofen, 1000 µM	32.20 ± 03.61	71.81 ± 05.03*
+phaclofen, 2000 µM	36.51 ± 03.07*	91.43 ± 05.11*

* $P < .05$ (Dunnett's test) when compared with either the (−)-baclofen group or the GHB-alone group.

TABLE 8

Effect of phaclofen on (−)-baclofen- and GHB-mediated inhibition of basal and K^+-stimulated extracellular output of GLU in rat VB

Lack of antagonism of (−)-baclofen- or GHB-induced in basal or K^+-evoked extracellular output of glutamate by phaclofen in two consecutive dialysate fractions collected during the last 20 min of the 4th hour of dialysis. Values are mean ± S.E.M. from 5 to 7 rats for each concentration of phaclofen. K^+-evoked release experiments were performed in a different set of animals (n = 5), wherein KCl (30 mM) was included in the last 20 min of the 60-min mixed-drug perfusion. Control values represent basal (n = 12) or K^+-evoked (n = 7) levels of GLU in the absence of (−)-baclofen/GHB or CGP 35348 during the last 20 min of the 4th hour of dialysis.

Drugs	Extracellular GLU Release (pmol/µl dialysate)	
	Basal	K^+-Stimulated
Control	1.19 ± 0.11	3.29 ± 0.62
(−)-Baclofen, 50 µM	0.82 ± 0.07	1.54 ± 0.19
+phaclofen, 750 µM	0.91 ± 0.09	1.67 ± 0.21
+phaclofen, 1000 µM	0.93 ± 0.10	1.60 ± 0.18
+phaclofen, 2000 µM	0.90 ± 0.09	1.63 ± 0.20
GHB, 1000 µM	no effect	1.21 ± 0.09
+phaclofen, 750 µM	—	1.10 ± 0.10
+phaclofen, 1000 µM	—	1.13 ± 0.11
+phaclofen, 2000 µM	—	1.15 ± 0.20

distinguishes these seizures from generalized convulsive seizures (Gloor and Fariello, 1988). Hence it is logical to expect that during absence seizures, any increase in excitation or decrease in inhibition in the thalamus and/or cortex would be subtle and finely tuned. In one study based on cortical tissue levels of GABA and GLU, an increase in extracellular GLU levels associated with a decrease in GABA levels in the cortex was predicted during the development of penicillin-induced spike-wave discharges (van Gelder *et al.*, 1983). However, no evidence has emerged to support this hypothesis. In the present study, we report that during the course of absence-like seizures induced by GHB, the basal extracellular levels of GABA in the VB remain moderately suppressed. Because we did not observe any significant accompanying alteration in the basal extracellular levels of GLU in the VB during GHB seizures, we suspect that this moderate decrease in GABA levels in the VB during GHB seizures provided, in the thalamus or thalamocortical neuronal network, the increase in excitation (through GLU) necessary to precipitate absence-like seizures.

The observation that GHB specifically inhibits the K^+-evoked release of GLU but not its basal output may be related to the mechanism underlying the generation of absence seizures, whereby a precise control of the increase in excitation in the thalamocortical circuitry is required. We have observed that during the course of GHB-induced SWD, the bilateral administration of N-Methyl-D-Aspartate (NMDA) into different thalamic relay nuclei (including VB) attenuates these paroxysms, converting them to burst suppression (Banerjee and Snead, 1995), whereas lower doses of NMDA have been shown to facilitate spontaneous SWD in rats genetically predisposed to absence-like seizures (Peeters *et al.*, 1990). Furthermore, recent dialysis studies done in the striata of conscious rats suggest that NMDA dose-dependently increases the release of GLU in this brain region (Bustos *et al.*, 1992).

The GHB-induced decrease in the depolarization-induced release of GLU may also dampen excitability to keep the generalized absence seizures from evolving into generalized convulsive seizures. We have observed that pretreatment with GHB in doses sufficient to induce generalized SWD in genetically epilepsy-prone rats (GEPR-9s) significantly suppresses the convulsive episodes upon sound stimulation (Gaza *et al.*, 1994). These *in vivo* data, taken in conjunction with the present microdialysis data, indicate that by moderately suppressing basal GABA release while having no effect on the basal release of GLU, GHB induces a subtle disturbance in the excitation/inhibition equation in the thalamus and/or cortex in favor of excitation.

In the present study, we chose to examine the release characteristics of GABA and GLU in the thalamic VB during the course of GHB-induced SWD for the following reasons: 1) All experimental SWD evolve most readily from this "specific" thalamic relay nucleus, in synchrony with the neocortical discharges (Avoli and Gloor, 1982; Vergnes *et al.*, 1987; Banerjee *et al.*, 1993). 2) In the GHB model, GHB-induced SWD evolve synchronously from VB and from layers I to IV of the frontoparietal cortex (Banerjee *et al.*, 1993). 3) There is a close correspondence between a rapid but reversible, region-specific upregulation of [^{3}H]GHB binding sites during GHB-induced SWD and those thalamic and cortical structures that participate in SWD activity (Banerjee *et al.*, 1993). 4) Bilateral lesions in thalamic relay nuclei (including VB) tend to suppress GHB-induced SWD evolving from both thalamus and cortex (Banerjee and Snead, 1994).

$GABA_B$ receptors are located both pre- and postsynaptically in many mammalian brain regions (Bonanno and Raiteri, 1993a). There is strong evidence in the literature that postsynaptic $GABA_B$ receptor-mediated inhibitory postsynaptic potentials are important to the generation of absence-like rhythms in thalamic and cortical neurons (Crunelli and Leresche, 1991; McCormick, 1992). Alternatively, presynaptic $GABA_B$ receptors located on both GABAergic and glutamatergic nerve terminals (Bonanno *et al.*, 1988; Bonanno and Raiteri, 1992) mediate the inhibition of neurotransmitter release (Bonanno and Raiteri, 1993a).

$GABA_B$ receptors are abundant in the thalamus (Bowery *et*

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al., 1987), but it is not known whether these thalamic $GABA_B$ receptors are located on both the GABAergic and the glutamatergic nerve terminals. If one assumes their presence on both types of terminals in the thalamus, these presynaptic $GABA_B$ receptors would be predicted to exert an important control over excitation/inhibition in thalamocortical pathways by modulating the release of GABA and GLU. Recent electrophysiological and biochemical studies in corticostriatal slices and cortical synaptosomes have provided convincing evidence, based on the specific $GABA_B$ receptor antagonist sensitivity of $GABA_B$ receptors located on GABAergic and glutamatergic nerve terminals, for the heterogeneity of presynaptic $GABA_B$ receptors (Calabresi *et al.*, 1991; Bonanno and Raiteri, 1992, 1993a). For example, in cortical synaptosomes, phaclofen, a baclofen analog, antagonizes the (−)-baclofen-induced inhibition in GABA release without affecting appreciably the release of glutamate, whereas CGP 35348, a more potent $GABA_B$ receptor antagonist than phaclofen, specifically blocks the effect of (−)-baclofen on glutamate release but has little effect on (−)-baclofen-induced changes in the release of GABA (Bonanno and Raiteri, 1992). In the present study, possible presynaptic $GABA_B$ receptor heterogeneity in VB is suggested by the results of (−)-baclofen perfusion, which dose-dependently decreased basal as well as K^+-evoked extracellular output of GABA and GLU. Moreover, the selective antagonism of the (−)-baclofen-mediated decrease in GABA release by phaclofen and that of GLU release by CGP 35348 suggests that the presynaptic $GABA_B$ receptors present in the VB bear pharmacological similarities to those present in the cortex or striatum.

Because GHB exhibited a similar dose-response profile to that of (−)-baclofen in inhibiting the basal release of GABA in VB, one might interpret these data to show that GHB, when administered either locally or systemically in doses that induce generalized absence seizures, modulates the release of GABA in VB through presynaptic $GABA_B$ receptors. This hypothesis is supported by the fact that GHB has been reported specifically to displace bound [^{3}H]baclofen and [^{3}H]CGP 27492, a potent $GABA_B$ receptor ligand, from $GABA_B$ sites in cortical and thalamic homogenates with a low affinity (IC_{50} = approximately 150 μM) (Bernasconi *et al.*, 1992). However, a number of lines of evidence mitigate against the hypothesis that GHB is a simple $GABA_B$ agonist that induces absence seizures by acting directly on the $GABA_B$ receptors.

First, there is no evidence that (−)-baclofen displaces [^{3}H]GHB from high-affinity GHB binding sites in either cortical/thalamic synaptosomal binding studies or autoradiographic studies (Benavides *et al.*, 1982, and our unpublished observation). Moreover, we have been unable to show that GHB displaces [^{3}H]GABA in the [^{3}H]$GABA_B$ autoradiographic studies (our unpublished observation). Second, [^{3}H]GHB and [^{3}H]$GABA_B$ binding sites have different regional anatomic distributions in rat brain (Snead, 1994), and the ontogeny of [^{3}H]GHB binding sites is distinctly different from that of [^{3}H]$GABA_B$ sites. [^{3}H]GHB binding sites in the brain do not appear until the third postnatal week of life, whereas [^{3}H]$GABA_B$ binding is present at birth (Snead, 1994). Interestingly, there is an excellent correlation between the ontogeny of GHB-induced absence seizures and the developmental appearance of [^{3}H]GHB binding sites in the thalamus and cortex (Snead, 1994). Third, it is known that $GABA_B$ receptors are coupled to pertussis toxin (PTX)-sensitive G protein (Bowery *et al.*, 1987). Although there is indirect evidence that GHB receptors are also bound to G protein (Snead, 1992c), [^{3}H]GHB binding behaves very differently in the presence of PTX and nonhydrolyzable GTP analogs than does [^{3}H]$GABA_B$ binding (Snead, 1992c). Fourth, although recent electrophysiological studies in isolated dorsal lateral geniculate (Williams *et al.*, 1993), CA1 pyramidal (Xie and Smart, 1992) and nigrostriatal neurons (Harris *et al.*, 1989) have indicated that GHB induces long-lasting IPSPs and rebound Ca^{++} spikes in a manner similar to (−)-baclofen, the dose-response data in those experiments suggest that the concentration of GHB in brain required to mimic the postsynaptic effects of (−)-baclofen is in the millimolar range. This tissue concentration of GHB may be commensurate with that achieved in brain with the high doses (greater than 500 mg/kg) of GHB associated with burst suppression on the EEG, loss of righting reflex and "anesthesia," but the brain concentrations of GHB associated with doses that induce absence seizures are in the micromolar range (approximately 250 μM) (Snead, 1991). Finally, in the present experiments the action of GHB differed from that of (−)-baclofen in that GHB was able to alter the basal release of GLU in the VB.

These findings together suggest that the GHB and the $GABA_B$ receptor sites are separate from one another in many respects. Even so, both appear to be involved in the pathogenesis of experimental absence seizures. GHB induces absence seizures, $GABA_B$ receptor agonists exacerbate them, and both specific GHB and $GABA_B$ receptor antagonists block the occurrence of SWD in experimental absence models (Maitre *et al.*, 1990; Liu *et al.*, 1992). One hypothesis that reconciles these disparate data is that the GHB sites that are involved in absence seizures are part of a presynaptic GHB/$GABA_B$ receptor complex. In the present study, perfusion of GHB in concentrations of 250 to 1500 μM may have achieved concentrations of GHB at the dialysis site sufficient to stimulate this putative presynaptic GHB/$GABA_B$ receptor complex. Further studies of the relationship between GHB and the $GABA_B$-mediated, G protein-modulated presynaptic function of this receptor complex are under way in our laboratory.

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Send reprint requests to: O. Carter Snead III, M.D., Division of Neurology, Box 82, Childrens' Hospital of Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027.

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Nocturnal γ-Hydroxybutyrate

Effect on Periodic Leg Movements and Sleep Organization of Narcoleptic Patients

Marc-André Bédard, Jacques Montplaisir, Roger Godbout, and O. Lapierre

Centre d'étude du sommeil, Hôpital du Sacré-Coeur, Montréal, Québec, Canada

Summary: Periodic leg movements during sleep (PMS) is a disorder frequently encountered in narcolepsy. In the present study, 12 narcoleptic patients (six with PMS and six without) were recorded in a sleep laboratory for 2 consecutive nights before and after treatment with γ-hydroxybutyrate (GHB) taken at bedtime for 1 month. Treatment resulted in decreased rapid eye movement (REM) sleep latency and increased REM efficiency without change in the total duration of REM sleep. GHB was associated with the appearance of pathological levels of PMS in patients who were unaffected before treatment. These results are discussed in relation to the role of dopamine in the physiopathology of narcolepsy and PMS. **Key Words:** γ-Hydroxybutyrate—Periodic leg movements in sleep—Rapid eye movement sleep—Narcolepsy.

Narcolepsy is a clinical entity characterized by a tetrad of symptoms, namely, excessive daytime sleepiness (EDS) culminating in overwhelming sleep episodes, cataplexy, hypnagogic hallucinations, and sleep paralysis (1). Nocturnal disturbance of sleep, particularly that of rapid eye movement (REM) sleep, is another characteristic component of narcolepsy. For example, narcoleptic patients show sleep-onset REM periods (SOREMPs) during polygraphic recording of sleep (2), and this phenomenon is an essential diagnostic feature. Moreover, REM sleep periods are fragmented, i.e., interrupted by several shifts to other sleep stages or by awakenings (3). This fragmentation has been correlated in time with the onset of cataplexy (4). Finally, isolated components of REM sleep, namely, eye movements and muscle atonia, may occur together with an electroencephalogram (EEG) characteristic of non-REM sleep, a phenomenon known as intermediate sleep (5).

Rechtschaffen et al. (2,6) were the first to suggest that narcolepsy results specifically from a disturbance of REM sleep. Apart from the fact that sleep attacks

Address correspondence and reprint requests to Dr. J. Montplaisir at Centre d'étude du sommeil, Hôpital du Sacré-Coeur, 5400 boul. Gouin ouest, Montréal, Québec, Canada H4J 1C5.

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are often accompanied by SOREMPs, one can indeed view cataplexy, sleep paralysis, and hypnagogic hallucinations as dissociated manifestations of REM sleep. These observations raise the possibility that the symptoms of narcolepsy may result from an inability to maintain REM sleep at night and subsequent increased REM sleep pressure during the day. In addition to REM sleep abnormalities, narcoleptic patients show other disruptions of sleep organization, such as an increased number of awakenings, increased total wake time after sleep onset, and increased time spent in Stage 1 non-REM sleep (2,3).

γ-Hydroxybutyrate (GHB) is a potent suppressor of cataplectic attacks in narcolepsy (7). It is usually taken at bedtime, but because GHB has a short half-life, treatment is normally repeated once during the night. GHB has a strong influence on the sleep organization of narcoleptic patients (8,9). It improves sleep quality; i.e., it reduces Stage 1 non-REM sleep and awakenings and increases Stages 3 and 4 non-REM sleep. GHB also facilitates REM sleep, decreasing its latency and fragmentation and increasing its efficiency. In fact, the therapeutic potency of GHB in narcolepsy is thought to result from a lowered daytime REM sleep pressure following its nocturnal administration (7–9).

Periodic leg movements during sleep (PMS) are found in nearly 50% of patients with narcolepsy (10). Because PMS have been related to decreased dopamine (DA) activity in the CNS (11) and because GHB suppresses the release of DA in the CNS (12), we wanted to document the effects of GHB on PMS and nocturnal sleep organization of narcoleptic patients with and without PMS.

METHOD

Twelve narcoleptic patients, aged 34–55 years, were included in the study. Before entering the protocol, patients were informed of the nature of the study and of their right to withdraw from it at any point. Each patient presented a clear history of EDS and cataplexy, and at least two SOREMPs were recorded during the multiple sleep latency test. Sleep apnea was ruled out by all-night polygraphic monitoring of oronasal airflow and respiratory movements. Human leukocyte antigens (HLAs) were typed in every patient, and all were HLA-DR2 positive.

All previous treatments for cataplexy and EDS were stopped for at least 2 weeks before the initiation of GHB administration, except for two patients who continued to take methylphenidate, 10–20 mg daily, until 48 h before the onset of the study. GHB was administered at bedtime in a single oral dose of 2.25 g.

Polysomnography was performed for 2 consecutive nights before and after 1 month of treatment with GHB. Recording parameters included electrooculogram, central EEG, chin electromyogram (EMG), and anterior tibialis EMG. Sleep stages were scored according to a standard method (13). Sleep latency was defined as the time before the first occurrence of 3 consecutive epochs of Stage 1 non-REM sleep or 1 epoch of any other sleep stage. REM latency was calculated from the sleep onset, and REM periods were defined as subsequent epochs of REM sleep not separated by >15 min of another sleep stage or waking.

Anterior tibialis EMG activity was recorded following a standard procedure (14). Only leg movements lasting 0.5–6.0 s occurring at intervals of between 4 and

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90 s and clustered in groups of at least four movements were counted. A PMS index (number of PMS per hour of sleep) of >5 was used to diagnose pathological PMS.

On the basis of recordings performed before the actual study, patients were selected to form two subgroups of six subjects matched for age, one with PMS (mean age, 47 ± 8 years) and one without PMS (mean age, 42 ± 5 years). All patients of the PMS group had a PMS index of >10. Results of polygraphic recordings obtained in each subgroup before treatment were compared with those obtained in a group of six age-matched normal controls (mean age, 44 ± 7 years) recorded for 3 consecutive nights.

A first-night effect was seen in control subjects; the second and third nights were therefore considered and pooled together when comparing control subjects with the two subgroups of narcoleptic patients before the treatment. Statistical comparisons between the three groups were made by a one-way analysis of variance (Table 1). Significant variables were further analyzed using t tests corrected for multiple comparisons. Comparison of night 1 and 2 in narcoleptic patients did not yield significant differences before or after treatment. This lack of a first-night effect in narcoleptics was reported earlier (15). Therefore, results of both nights were pooled together when comparing baseline and GHB treatment. The effect of GHB was evaluated with paired t tests (Tables 2 and 3) on pooled subgroups of patients as well as on each of the two subgroups separately.

RESULTS

Sleep Patterns in Narcoleptic Patients

Table 1 shows that nocturnal sleep organization is disrupted in narcoleptic patients with or without PMS when compared with a control group. Narcoleptics have shorter sleep latency, increased wake time after sleep onset, longer time

TABLE 1. *Main sleep parameters in control and narcoleptic subjects before treatment*

	Control	Narcoleptics with PMS	Narcoleptics without PMS	ANOVA[a]	t Test[a]		
					AB	*AC*	*BC*
Age (yrs)	43.6 ± 7.1	47.0 ± 8.5	42.0 ± 4.8	—	—	—	—
WASO (min)	5.2 ± 3.9	8.9 ± 5.0	10.6 ± 7.7	0.05	—	0.01	—
Total sleep (min)	425.8 ± 58.3	443.3 ± 69.0	442.8 ± 65.6	—	—	—	—
Sleep latency (min)	10.0 ± 9.0	2.3 ± 1.7	3.2 ± 2.7	0.003	0.001	0.005	—
SWS latency (min)	29.7 ± 15.8	25.4 ± 18.4	32.0 ± 12.7	—	—	—	—
REM latency (min)	85.5 ± 37.2	48.9 ± 88.9	47.4 ± 45.8	—	—	—	—
No. of SOREMPs	0/12 (0%)	7/12 (58%)	5/12 (41%)	—	0.007	0.04	[b]
Stage 1 (%)	10.8 ± 3.4	22.8 ± 8.3	19.4 ± 7.6	0.001	0.0002	0.004	—
Stage 2 (%)	56.2 ± 10.5	48.5 ± 10.1	48.9 ± 6.4	—	—	—	—
SWS (%)	9.3 ± 7.5	9.8 ± 7.9	12.5 ± 8.3	—	—	—	—
Stage REM (%)	23.6 ± 4.4	18.9 ± 8.3	19.1 ± 5.2	—	—	—	—
REM efficiency (%)	82.6 ± 4.9	69.2 ± 13.1	69.8 ± 11.1	0.004	0.003	0.004	—

PMS, periodic leg movements during sleep; ANOVA, analysis of variance; WASO, wake time after sleep onset; SWS, slow wave sleep (Stages 3 + 4); REM, rapid eye movement; SOREMPs, sleep onset REM periods; %, percentage of the night in the stage mentioned; REM efficiency %, min of REM sleep/min of REM period ×100.

Values are means ± SD.

[a] p value; [b]Yate chi-square test.

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TABLE 2. *Effect of γ-hydroxybutyrate on sleep parameters of 12 narcoleptic patients*

	Baseline	γ-Hydroxybutyrate	Paired *t* test[a]
Total sleep time (min)	443.1 ± 65.8	431.8 ± 45.1	—
Sleep latency (min)	2.7 ± 2.3	2.8 ± 3.1	—
SWS latency (min)	29.0 ± 15.5	39.6 ± 19.8	—
REM latency (min)	48.1 ± 69.2	13.2 ± 32.5	0.03
No. of SOREMPs	12/24 (50%)	20/24 (80%)	0.01[b]
WASO (%)	9.7 ± 6.4	10.2 ± 5.1	—
WASO ⅓ (%)	19.8 ± 12.9	11.8 ± 12.2	0.001
Stage 1 (%)	21.1 ± 8.0	20.9 ± 7.3	—
Stage 1 ⅓ (%)	24.5 ± 8.4	20.0 ± 9.7	0.01
SWS (%)	11.1 ± 8.0	15.6 ± 11.5	—
SWS ⅓ (%)	65.7 ± 27.7	65.0 ± 23.3	—
Stage REM (%)	19.0 ± 6.8	18.1 ± 4.4	—
Rem ⅓ (%)	29.2 ± 6.8	39.5 ± 4.4	0.004
REM efficiency	69.5 ± 11.8	75.3 ± 10.6	0.04
REM efficiency ⅓	78.6 ± 16.9	88.5 ± 10.2	0.02
No. of PMS	150.1 ± 170.6	148.1 ± 125.8	—
No. of PMS ⅓	51.5 ± 64.4	64.6 ± 74.1	0.02
PMS index	19.9 ± 21.5	20.6 ± 17.6	—
PMS index ⅓	20.0 ± 24.3	25.7 ± 29.8	0.02

⅓ (%), percentage of the total stage duration retrieved in the first third of the night. For other abbreviations see Table 1.
Values are means ± SD.
[a] p value.
[b] Yate chi-square test.

spent in Stage 1 non-REM sleep, and reduced REM sleep efficiency. REM latency showed a bimodal distribution in narcoleptics because of the presence of both SOREMPs and normal REM latency. This distribution prodnces a large standard deviation that explains the absence of significant difference between controls and narcoleptics. The results of a Yate chi-square test made on SOREMPs confirm the higher incidence of this phenomenon in narcoleptics. No reduction of slow wave

TABLE 3. *Effects of γ-hydroxybutyrate on PMS in two groups of narcoleptic patients*

	Baseline	γ-Hydroxybutyrate	Paired *t* test[a]
Patients without PMS before treatment			
PMS index, total night	3.3 ± 3.5	7.8 ± 6.7	0.06
No. of PMS, total night	22.5 ± 20.5	60.9 ± 51.7	0.04
No. of PMS ⅓	3.5 ± 5.4	16.9 ± 20.4	0.05
No. of PMS ⅔	9.3 ± 11.9	20.0 ± 21.0	—
No. of PMS 3/3	9.7 ± 13.0	24.0 ± 16.3	0.05
Patients with PMS before treatment			
PMS index, total night	36.5 ± 18.8	33.6 ± 15.6	—
No. of PMS, total night	277.8 ± 157.8	242.1 ± 118.6	—
No. of PMS ⅓	99.6 ± 60.0	112.2 ± 82.1	—
No. of PMS ⅔	99.2 ± 74.9	68.8 ± 55.2	—
No. of PMS 3/3	79.0 ± 64.0	61.1 ± 39.4	—

Values are means ± SD. For abbreviations see Tables 1 and 2.
[a] p value.

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sleep (SWS; Stages 3 + 4) was found in either subgroup of patients compared with control subjects. However, there are no significant differences between narcoleptic patients with and without PMS for any of the sleep variables analyzed.

The Effect of GHB on Sleep Organization in Narcolepsy

Table 2 compares the results obtained on nocturnal recordings before and after GHB in narcoleptic patients with and without PMS. When considering the nights as a whole, a single dose of GHB at bedtime was associated with a significant increase in the number of SOREMPs and REM efficiency; these effects were also present in each subgroup of patients. REM latency decreased from 48.1 ± 69.2 to 13.2 ± 32.5 after GHB as a result of increased frequency of SOREMPs.

Stage 1 non-REM sleep and waking decrease in the first third of the night after GHB, whereas REM sleep and REM efficiency in the first third of the night increase. No differences were found between conditions for the second and final thirds of the nights, suggesting an absence of a rebound effect of GHB on sleep organization. Results were comparable in the two subgroups of patients.

Effect of GHB on PMS Activity

Table 2 shows that the number and the index of PMS calculated for all narcoleptic patients for entire nights were not affected by GHB. However, significant increases in the number and index of PMS were observed during the first third of the night.

Additional effects of GHB were revealed when the dependent variables were further broken down by subgroups of patients and by thirds of nights (Table 3). In patients without pathological levels of PMS before treatment, the number of leg movements increased in each third of the night. As a result, the PMS index exceeded the criteria for pathological PMS after GHB in these patients. On the other hand, in patients who were already affected with PMS, GHB was associated with a nonsignificant increase of leg movements in the first third and decrease in the last two-thirds of the nights.

DISCUSSION

Nocturnal Sleep Disruption in Narcolepsy

These results confirm previous reports on nocturnal sleep disturbance in narcolepsy (2,3). In these patients, sleep is interrupted by several awakenings, leading to a decreased sleep efficiency and more Stage 1 non-REM sleep. Although the quantity of REM sleep is normal, its efficiency is low. The present study and those of others (16,17) have shown normal amounts of SWS in narcolepsy, while some have reported a decrease (3). There is no explanation for the lack of consistency on this point.

Some authors have suggested that disturbed sleep in narcolepsy results from the contribution of nonspecific elements such as sleep apneas and PMS (18). The present results and a previous study (19) do not support this hypothesis, since in narcoleptic patients free of sleep apnea and paired for age, there is no difference

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in sleep organization between those with PMS and those without. These results suggest that nocturnal sleep disruption is a primary feature of narcolepsy.

Effects of GHB on PMS

GHB causes a complete cessation of unit activity in the dopaminergic neurons investigated in the nigrostriatal or mesolimbic areas (12). Thus, the increase of PMS seen with GHB in previously unaffected narcoleptic patients is compatible with the hypothesis that PMS may result from a decreased DA transmission in the CNS. This hypothesis is further confirmed by the therapeutic effect of L-Dopa seen in insomniac (11) and narcoleptic (20) patients affected with PMS.

When patients already affected with PMS were considered separately, a nonsignificant decrease of PMS was seen in the last two-thirds of the night. This is compatible with results of another study (21) using a double-dose protocol and showing a decreased number of PMS at the end of the effect of each dose. The decrease seems to occur after the action of GHB and could be attributed to a rebound DA activity, secondary to release of the accumulated neurotransmitter (22), since GHB not only suppresses neuronal firing and DA release but also stimulates DA synthesis (12). However, there is no simple explanation for the difference in the effect of GHB noted between the two subgroups of narcoleptic patients.

GHB and the Triggering Mechanisms of REM Sleep

The facilitation of REM sleep observed in the present study is compatible with results of other studies using repeated administration of GHB in narcoleptic patients (8,9). Short REM latencies after GHB were also shown in patients with affective disorders (23). In normal subjects, however, GHB or its lactone metabolite administered orally (24) or intravenously (25) failed to induce REM sleep except in older normal subjects when GHB was administered orally in the morning (26), i.e., when the circadian propensity for REM sleep is greater. Consequently, it can be postulated that the REM-inducing effect of GHB in humans can be seen only in conditions where REM sleep is already facilitated, i.e., in narcolepsy, depression, and morning recording of older human subjects.

Most REM-inducing substances are known to increase cholinergic transmission in the CNS, and this action is thought to be mediated through cholinergic receptors of "REM-on" cells in the brainstem (27). However, GHB has no cholinomimetic effect and even reduces the firing rate of cholinergic neurons in the rat (28). Furthermore, the REM-inducing property of GHB in cats was not prevented by pretreatment with atropine (29).

It has been postulated that REM sleep may also be controlled by a system located more rostrally, more likely in the hypothalamus (30). We proposed that this rostral system is involved in human narcolepsy, while brainstem cholinergic mechanisms may be impaired in animal narcolepsy. This hypothesis of a rostral REM sleep control would explain the presence of narcoleptic symptoms in patients with lesions located in the vicinity of the hypothalamus (31,32). It may also explain major differences seen between human and animal narcolepsy. The lower

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rate of cataplectic attacks and the closer association with specific emotions noted in the human form would suggest the involvement of a hypothalamic control system. This hypothesis would also account for pharmacological differences. Hyperactivity of cholinergic neurons was found in the brainstem of narcoleptic dogs (33), and cataplexy in these animals is suppressed by muscarinic anticholinergic agents and facilitated by cholinergic agonists (34). In human narcolepsy, no cholinergic hypersensitivity was found (35) and muscarinic anticholinergic drugs did not reduce cataplexy. In addition, GHB was not found to be effective in controlling cataplexy in narcoleptic dogs (36).

The mechanism and the site of action of GHB in human narcolepsy are unknown. There is some evidence that this substance acts as a dopaminergic-inhibiting agent in the hypothalamus. The incertohypothalamic DA system is proposed. This region is GHB sensitive (37) and collaterals originating from this hypothalamic nucleus project to the medullar area associated with the REM sleep–triggering mechanism proposed by Jouvet (30). This theory of a rostral system responsible for human narcolepsy and for the effect of GHB remains highly speculative and will require further experimental confirmation.

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The Treatment of Narcolepsy-Cataplexy with Nocturnal Gamma-Hydroxybutyrate

ROGER BROUGHTON AND MORTIMER MAMELAK

SUMMARY: *Sixteen patients with narcolepsy and cataplexy were treated with gamma-hydroxybutyrate (GHB) given at night and tailored to achieve as continuous a night's sleep as possible. The dosage usually consisted of 1.5-2.25 gm orally at bedtime and then one or two further 1.0-1.5 gm doses with awakenings during the night, and totaled about 50 mg/kg. Apart from one patient who took only the bedtime dose, the subjective quality of night sleep improved in all patients and the number of irresistable daytime attacks of sleep and cataplexy substantially diminished. Some residual daytime drowsiness remained and this usually responded well to low doses of methylphenidate. Improvement has been maintained for up to 20 months without the development of tolerance. Two patients experienced adverse side effects necessitating withdrawal of GHB treatment, but no serious toxic effects have occurred.*

RÉSUMÉ: *Seize malades qui présentaient des épisodes de narcolepsie et de cataplexie ont été traités la nuit avec hydroxybutyrate-gamma. Il était dosé pour donner un sommeil nocturne le plus continuel possible. Le dosage normal était de 1.5-2.25 gm. par voie orale avant le coucher suivi par un ou deux autres dosages de 1.0-1.5 gm. pour les réveils nocturnes. Le dosage total était approximativement de 50 mg/kg. Le sommeil nocturne de tous les malades s'est amélioré, sauf pour un seul qui ne prenait que le dosage avant le coucher, et le nombre d'épisodes de sommeil diurne irrésistible et de cataplexie étaient très diminués. Une somnolence résiduelle et diurne persistait, ce qui habituellement répondait bien au dosage minime de methulphenidate. L'amélioration clinique a été maintenue jusqu'à 20 mois sans l'apparition de tolérance. Deux malades ont eu des effets secondaires qui nécessitaient l'arrêt du traitement, mais aucun effet toxique sérieux n'a eu lieu.*

From the Division of Neurology, Ottawa General Hospital and University of Ottawa, and the Department of Psychiatry, Sunnybrook Medical Centre and University of Toronto, Canada.

Reprints requests to: Dr. Broughton, Department of Medicine (Neurology), Ottawa General Hospital, Ottawa, Canada, K1N 5C8.

INTRODUCTION

The prevalence of narcolepsy has been shown in epidemiological studies to be about 0.1% (Roth, 1962; Dement et al., 1973). Therefore it is more frequent than a number of much better known chronic neurological conditions, such as multiple sclerosis. Moreover, as it generally begins in young adulthood and remains for the patients' lifetime, and as it has marked detrimental effects involving employment, education, recreation, interpersonal relations, driving, accidents in general and other parameters of everyday life (Broughton and Ghanem, 1976), the condition can be truly debilitating. The investigation of narcolepsy by modern polysomnographic techniques has shown that of the classical so-called 'tetrad' of Daly and Yoss (1960), the auxillary symptoms (i.e. those other than sleep attacks) of cataplexy, sleep paralysis, and vivid hypnogogic hallucinations are all based upon abnormal rapid-eye-movement (REM) sleep mechanisms, and that the sleep attacks of patients with narcolepsy-cataplexy begin in REM sleep in 50-100% of attacks (Broughton, 1971; Zarcone, 1973), depending upon the author. These findings have led to the addition of drugs which suppress REM sleep, i.e. tricyclic antidepressants (imipramine, chlorimipramine, and desipramine) or less frequently MAO inhibitors (phenelzine) to traditional stimulant medication, usually methylphenidate. The antidepressants have been largely effective in reducing the auxillary symptoms of cataplexy, sleep paralysis and hypnogogic hallucinations, whereas methylphenidate has been most useful for the sleep attacks and for the more or less continuous daytime drowsiness

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presented by these patients (Zarcone, 1973). Despite these therapeutic improvements over stimulants alone, the treatment of narcolepsy still remains unsatisfactory. In many patients control of symptoms is far from complete. Others show undesirable side effects discussed later.

This situation led us to use a somewhat different therapeutic strategy. Rather than concentrating upon suppressing the daytime symptoms, we decided to attempt to improve their night-time sleep, which is characterized by early or direct entry into REM sleep (Rechtschaffen et al., 1963), much sleep fragmentation with particular inability to sustain periods of REM sleep (Montplaisir, 1976), and by other features, in the hope that daytime pressure for sleep-related symptoms would be reduced. There were at least two reasons for suggesting that disturbed nocturnal sleep might be central to the physiopathogenesis of narcolepsy with cataplexy. First, prolonged periods of sleep deprivation or of irregular sleep precede the onset of major symptoms of the disease in 50-75% of patients (Mitchell and Dement, 1968; Broughton and Ghanem, 1976) with idiopathic narcolepsy. Secondly, narcoleptics are known to be very vulnerable to the effects of shift work, and therefore to alteration in their circadian sleep-wakefulness rhythms. Such disturbances regularly aggravate their symptoms (Broughton, 1971).

We chose the sodium salt of gamma-hydroxybutyrate (GHB) (Laborit, 1964; Muzard and Laborit, 1977; Snead, 1977) in our attempt to "normalize" the nocturnal sleep patterns of patients with narcolepsy and cataplexy. This short chain fatty acid is a normal constituent of the human nervous system (Doherty and Roth, 1976). It possesses definite hypnotic properties. But in distinction to the commonly used synthetic hypnotics, it promotes sleep which more closely approximates that of normal sleep than do other hypnotics, since it does not inhibit either REM or NREM sleep (Jouvet et al., 1961; Matsuzaki et al., 1964; Mamelak et al., 1977; Muzard and Laborit, 1977). GHB also has an additional possible advantage over the synthetic hypnotics in that animal studies had failed to demonstrate the development of tolerance to its hypnotic effects with prolonged use (Vickers, 1969). To date we have treated 16 patients with nocturnal GHB. Preliminary results in our first four patients have already been reported (Broughton and Mamelak, 1976).

PATIENTS AND METHODS

The sixteen patients, 8 men and 8 women, ranged in age from 21-58 years (Mean = 41.8, s.d. 13.6; Table 1). All had histories of diurnal drowsiness, irresistible sleep attacks, and cataplexy. The other main symptoms of the disease were also present in individual patients to varying degrees. In four patients, the symptoms had been particularly debilitating in spite of treatment with the usual combination of methylphenidate and tricyclic antidepressant drugs. The entire protocol and the investigative nature of the study were carefully explained to each patient and consent forms were signed. In all patients, a sleep onset REM period was observed during at least one daytime polysomnographic recording. Before starting treatment with GHB, all previous drug treatment for narcolepsy was discontinued for at least 14 days. A history and physical were performed and the following laboratory tests completed: hemogram, liver survey, renal survey, chest x-ray, EEG and ECG. Each patient was also given a psychological examination and the Minnesota Multiphasic Personality Inventory.

Polysomnographic assessment of sleep-waking patterns was done for at least 48 continuous hours in the baseline state and then at regular intervals while on GHB. In the Ottawa patients (N = 9) recordings were performed without hospitalization using a portable 4-channel apparatus which permitted the monitoring of patients at their habitual activity levels in the normal home or work environment. In the Toronto studies, patients (N = 7) were hospitalized during the recording periods and the usual polysomnographic techniques were employed. None of the patients had histories of loud snoring or of the peculiar gutteral inspiratory snoring which characterizes sleep apnea. Moreover, this symptom was formally excluded by respiratory monitoring (nasal thermistor and abdominal belt transducer) in Toronto studies, where sufficient recording channels made this possible. The Stanford Sleepiness Scale (Hoddes et al., 1973), which is a self-assessed 1 to 7 scale of alertness, was filled in every 30 minutes over at least 3 consecutive days during wakefulness in the pre-GHB baseline period, and during reassessments while on the drug.

Treatment with GHB was started once the initial baseline data was gathered. The treatment schedule was tailored to achieve as continuous a night's sleep as possible. The patient's body weight and his polysomnographic response to GHB were used as guides. Since each sleep inducing oral dose of GHB lasts only two or three hours (Mamelak et al., 1977) — indeed the substance is only detectable in blood that long (Helrich et al., 1964) — and because our aim was to maximize the duration of sleep produced by the drug while minimizing its anaesthetic effects, multiple doses were used. The usual initial dose was 1.5-2.25 gm (10-15 ml) hs, followed by further multiple 1.0-1.5 gm doses during the night with each major reawakening, if at least 2.5 hours had passed since the previous dose. Usually only 2 or 3 doses per night were necessary. Each dose was about 30 mg/kg, but the total quantity of GHB given each night ranged from 3.75 to 6.25 gms, corresponding to approximately 50 mg/kg.

After seven to ten nights on GHB, the 48 hour polysomnographic recording was repeated with the patient continuing to use the drug according to the optimal dose schedule previously established. Major reassessments were again performed after at least one month, six months and 12 months on GHB. On each of these occasions, the clinical effects of the treatment were assessed, the blood and urine studies, chest x-ray and ECG were repeated, and any adverse reactions to the drug noted and investigated.

GHB was obtained from Laboratoire Egic in France, who market this drug in syrup form under the trade

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TABLE 1.

Patients' Symptoms, Previous Treatment and Response to Nocturnal Gamma Hydroxy Butyrate

Patient	Age	Sex	Major Symptoms	Duration of Illness	Previous Medication	Usual GHB Dosage gm/night	Response	Toxicity	Comments
1	21	F	N,SP,HH rare C	6 years	diazepam hs	3.0	+++	none	—
2	22	M	N,C,SP,HH	4 years	diazepam sed^n	3.75	+	none	—
3	23	F	N,C,SP,HH	3 years	none	3.75	+++	none	—
4	25	F	N,C,SP	5 years	benzedrine	2.25	0	none	Took only hs dose
5	32	F	N,C,SP,HH	14 years	dexedrine	5.25	+++	none	Sister of pat. 4
6	38	F	N,C,SP,HH	15 years	dexedrine methylphenidate chlorimipramine	3.75	+++	none	Old gastrectomy
7	40	M	N,C,SP,HH	28 years	dexedrine methylphenidate imipramine chlorimipramine phenelzine	4.50	+++	none	—
8	43	F	N,C,SP,HH	13 years	dexedrine methylphenidate imipramine chlorimipramine phenelzine phenytoin carbamazepine	4.50	++	abdominal pain, muscle weakness	No evidence for epilepsy
9	45	F	N,C,SP	23 years	dexedrine	6.25	+	none	—
10	45	M	N,C,SP,HH	3 years	methylphenidate	4.50	+++	temporary muscle weakness	—
11	52	M	N,C,SP	14 years	desoxyn	3.75	+++	none	Impotence on previous R
12	55	M	N,C	30 years	methylphenidate	3.75	+++	none	—
13	56	M	N,C,SP	31 years	methylphenidate	3.75	+++	dysthesiae left hand	Post-traumatic epilepsy
14	57	M	N,C	43 years	ephedrine	4.50	+++	none	—
15	57	M	N,C,SP,HH	33 years	dexedrine	5.25	+++	none	—
16	57	F	N,C,SP,HH	37 years	dexedrine methylphenidate impramine chlorimipramine	3.75	+++	none	—

0 = no effect; +/- = 0-20% improvement; + = 20-40% improvement
++ = 40-70% improvement; +++ = over 75% reduction of symptoms from baseline
N = irrisistible sleep attacks; C = cataplexy; SP = sleep paralysis; HH = vivid hypnagogic hallucinations

name "GammaOH". We found it best to dilute the syrup in milk or juice, in order to reduce the gastrointestinal upset caused in some patients when the drug was given in undiluted form. Dilution also retarded GHB's rate of absorption somewhat, so that sleep induction was experienced as gradual and more normal.

RESULTS

We wish to report our clinical observations here. The polysomnograpic and Stanford Sleep Scale data and our psychological findings are still being analyzed and will be presented in a future publication. The patient and clinical results are summarized in Table 1.

CLINICAL RESPONSE

The ameliorating effects of GHB on the major daytime symptoms of narcolepsy appeared gradually. By comparison, the subjective quality of night-time sleep improved very rapidly. Over the first 2 to 5 nights, nocturnal sleep became less restless and nightmares, hallucinations, and attacks of sleep paralysis vanished. Some episodes of intense awakenings at about 2-3 hours after taking the initial doses were encountered. These appeared to represent a drug-related rebound phenomenon. Although dreaming continued, it lost its frightening qualities. All patients found it easier to stay awake during the day and noted that after a number of weeks, the irresistible pressure for diurnal sleep and the attacks of cataplexy virtually disappeared. When cataplexy did occur, the attacks were

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usually relatively brief, less intense, and tended to occur late in the day when the individual was very tired. Most patients said that they were much more refreshed after their night sleep and were better able to cope during the daytime. Despite these beneficial effects on the major symptoms of the disease and on the subjective quality of sleep, many patients continued to feel somewhat tired and drowsy during the day. We then added 5 to 10 mg of methylphenidate three times a day to their treatment regimen. It was taken on an empty stomach before breakfast and lunch, and then again in the mid-afternoon. With this addition, the daytime drowsiness and fatique became minimal.

Our patients generally reported that sleep gradually consolidated into a seven to eight hour period. One patient, however, reported that if she slept through the night and failed to take her second dose of GHB, the attacks of narcolepsy and cataplexy recurred on the following day. The single patient (No. 4) who failed to respond at all to GHB treatement, turned out to be taking only the single h.s. dose of the drug. Some patients on their own tried to discontinue GHB treatment and to rely on methylphenidate alone, but they noticed recurrence of their symptoms after a few days.

In patients responding to GHB, the improvement was maintained throughout the trial period. The development of tolerance requiring increasing doses for the same clinical effect on night sleep, sleep attacks or cataplexy has not been encountered. As with traditional forms of treatment, it was found that having patients keep regular hours of retiring and of morning awakening was important for optimal therapeutic effectiveness. At the time of writing, one patient has been on GHB nightly for nearly two years, three others have been on it for over a year, and the remainder have been on it for three months to a year.

SIDE EFFECTS

There have been very few adverse clinical effects with this treatment and no abnormal laboratory findings. Minor side effects of GHB have been seen for the first few days in a number of patients which consisted of a "thick head", ocular discomfort, and other apparent hangover effects, but these were rare after one week. Impotence or reduced libido has never been encountered. We decided to discontinue the drug in two patients. One (patient No. 8) complained of non-specific abdominal pain while using GHB plus muscular weakness in the morning, to the point where she found it difficult to initiate movement. Both of these symptoms disappeared when the drug was stopped. A second patient (No. 13), a male with a post-traumatic narcolepsy and cataplexy, experienced disturbing left arm dysthesiae. He had previously had similar symptoms after the initial head injury. A third patient (No. 10) complained of muscular weakness in the morning, also limited to his left arm. This man had suffered a neck injury a few weeks before starting GHB and his left arm was weak following the event. It had gradually been recovering, but the weakness recurred when he started using the drug. Because his narcolepsy improved so dramatically on GHB, we continued to use the drug in spite of the effect on his arm and the weakness gradually disappeared over a few weeks.

Several patients have also mentioned that GHB caused urinary urgency. On one occasion, enuresis occurred in a patient about an hour after the drug had been given. On the whole, however, urgency has not been a serious problem and our patients report that they void no more frequently during the night on GHB than they did before starting the drug. Another complaint from a number of patients was that GHB produced a dream-like confusional state which could be unpleasant and frightening. This happened when the drug was taken before they were ready for sleep, or when they fought against its sleep promoting actions. This phenomenon is rare if patients cooperate with the drug's hypnotic effects and use it at the minimal dose required for sleep induction and maintenance. No other side-effects were encountered and, in sum, most patients felt they had fewer side-effects and substantially better relief from symptoms on GHB than on any previous medication.

DISCUSSION

The salient finding in this study was the marked clinical improvement produced by nocturnal GHB in patients with narcolepsy-cataplexy. This action was coupled with a paucity of adverse clinical or laboratory findings. When GHB was used at night, and supplemented with small doses of methylphenidate during the day, all the major symptoms of narcolepsy were markedly reduced. The project has involved detailed study of a limited number of patients over substantial periods of time. It is not a double-blind controlled design. But, the therapeutic effects on patients previously uncontrolled by the more traditional drug regimens and the rapid deterioration in those who discontinued the use of the drug on their own for several nights leave little doubt about the compound's effectiveness.

The use of GHB for the treatment of this disease has a number of clear advantages over more conventional therapies. As mentioned, the latter usually use substantial doses of stimulants such as methylphenidate or d-amphetamine, alone, or in combination with tricyclic antidepressants such as imipramine or chlorimipramine. The stimulants, however, cause irritability and anxiety in many patients and more serious side effects in others. One of our patients previously had had a gastrectomy for ulcers attributed to stimulant medication. The antidepressant drugs, on the other hand, may cause dry mouth, sweating, and impotence in males (Zarcone, 1973; Dement et al., 1976). The stimulant-antidepressant combination does not consolidate sleep, and in fact may even further disrupt it. Moreover, tolerance develops in time both to the level of stimulants generally employed and to antidepressants so that after a number of months, many patients complain that their symptoms are again every bit as troublesome as they were to begin with. None of these problems occur with GHB. Nocturnal sleep was restful

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and sustained and patients awoke alert and well rested. There were few side effects and, specifically, no impotence or reduced libido. Tolerance to the drug's actions did not develop, nor did it develop to the relatively small doses of methylphenidate taken during the day, when taken in combination with nocturnal GHB.

Some of the therapeutic and side-effects of GHB may be related to its influence on motor mechanisms. It is known to inhibit muscle tone (Vickers, 1969) and to block the H-reflex response (Uspenskii, 1965; Muzard and Laborit, 1977). In narcoleptics, as well as in normals, the H-reflex response can be abolished by GHB and remains somewhat attenuated for some time after the patient awakens (Mamelak, Sowden and Caruso, unpublished observations). The latter may be due to residual effects of small quantities of unmetabolized drug. This effect may account for the weakness experienced by two of our patients upon arising in the morning. The sustained hypotonia throughout sleep may be as important as any effect on sleep patterns in the subjective feeling of having had a deep refreshing night's sleep. As far as the urinary urgency is concerned, this has been noted by some patients even if they empty their bladders before bedtime, but it has not proved to be a treatment problem. It is intriguing to speculate, however, that the combination of profound sleep and enuresis observed in childhood might be related to a higher brain GHB concentration present in the early years of life.

GHB's mechanisms of action in the treatment of the major symptoms of narcolepsy remains uncertain. It has been known for many years that hypnotic drugs can be helpful for at least some narcoleptic patients (Daniels, 1934; Zarcone, 1973). Recent studies have shown that narcoleptics do not sleep more in the 24-hour period than normal individuals (Hishikawa et al., 1976). Thus, consolidating the fragmented sleep of these patients into a seven or eight hour period by means of hypnotic drugs should theorectically decrease the need for daytime sleep. Perhaps this is how ordinary hypnotics benefit these patients. But, it must be noted that some of our narcoleptic patients slept reasonably soundly at night and that in these patients nocturnal sleep in fact became more fragmented after starting GHB, because they had to wake up for the second dose. If they failed to take it their symptoms recurred. Furthermore, a preliminary review of our polysomnographic data indicates that GHB did not substantially increase the overall duration of sleep in the eight hour night-time period. GHB, then, likely has more specific actions on sleep mechanisms than simply increasing the duration of nocturnal sleep or its gross continuity. As yet, basic neurochemical studies offer few real insights into the drug's mechanism of action, although it has been shown that GHB may be derived from GABA (Roth and Giarman, 1969), and may act as a GABA agonist (Roth et al., 1977) and that it alters dopamine (Roth and Suhr, 1970), serotonin (Spano et al., 1970), and acetylcholine (de la Mora et al., 1970) metabolism. The last three, at least, have been implicated in sleep control mechanisms (Jasper and Koyama, 1969; Jouvet, 1969; Cordeau, 1970; Morgane and Stern, 1972).

Whatever its precise mode of action, this essentially non-toxic constituent of the normal brain does appear to have important clinical therapeutic effects even in otherwise refractory cases of narcolepsy. Moreover, its effectiveness, when given in the night-time period, adds strong support for the postulated importance of the quality of night sleep in the genesis of daytime sleep attacks and cataplexy. It gives promise that GHB itself or similar substances (we have also used gamma-butylactone sucessfully) may lead to substantial improvement in the control of this debilitating neurological disease. The main disadvantage at present is its relatively short duration of action. It is hoped that this might be extended by use of slow release capsules or another approach in order to produce a sustained 7-8 hour overnight effect.

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γ-Hydroxybutyrate Receptor Function Studied by the Modulation of Nitric Oxide Synthase Activity in Rat Frontal Cortex Punches

*Christopher D. Cash, Serge Gobaille, Véronique Kemmel, Christian Andriamampandry and Michel Maitre**

INSTITUT DE CHIMIE BIOLOGIQUE AND ER 2072 CNRS, FACULTÉ DE MÉDECINE, 67085 STRASBOURG, FRANCE

ABSTRACT. Previous results have shown that stimulation of the gamma-hydroxybutyrate (GHB) receptor modulates Ca^{2+} channel permeability in cell cultures. In order to confirm this result, we investigated the consequence of GHB receptor stimulation on nitric oxide synthase (NOS) activity in rat brain cortical punches rich in GHB receptors. The stimulation of these receptors by increasing amounts of GHB induced a progressive decrease in NOS activity. However, for GHB doses above 10 μM, this reduction was progressively lost, either after receptor desensitization or after stimulation of an additional class of GHB receptor having lower affinity. The effect of GHB was reproduced by the GHB receptor agonist NCS-356 and blocked by the GHB receptor antagonist NCS-382. The GHB-induced effect on Ca^{2+} movement was additive to those produced by veratrine, indicating that GHB modulates a specific Ca^{2+} conductance, which explains the modification in NOS activity and the increase in cyclic guanosine monophosphate levels previously reported. BIOCHEM PHARMACOL 58;11: 1815–1819, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. gamma-hydroxybutyrate receptor; GHB; nitric oxide synthase activity; calcium channel; NCS-382

GHB†, which is endogenously present in micromolar quantities in the brain, is derived mainly from the transamination of GABA followed by the reduction of succinic semialdehyde [1]. GHB possesses neuromodulatory functions in brain via high affinity receptors which are characterized by a specific ontogeny, distribution, kinetics, and pharmacology [2]. These receptors, which do not appear to be expressed by glial cells and peripheral tissue, belong to the G-protein-linked receptor family [3]. They are found exclusively in the rostral part of the brain (whole cortex—including frontal cortex—and hippocampus, striatum and olfactory tracts, thalamus, and some dopaminergic nuclei such as A_9, A_{10}, and A_{12}) but also in neuronal cell cultures [4, 5]. Several results have documented that the brain endogenous GHB system exerts a tonic inhibitory control over both the release of dopamine in striatum and frontal cortex and the release of GABA in thalamus and frontal cortex [6, 7]. Other brain regions have not been studied. These effects are most probably mediated by the GHB receptors localized in these brain regions, because most of the neurophysiological and neuropharmacological effects of administered GHB are reduced or blocked by the GHB receptor antagonist NCS-382 (6,7,8,9-tetrahydro-5-[H]-benzocycloheptene-5-01-4-ylidene acetic acid) [8]. When administered peripherally, GHB freely penetrates the brain and interferes with the principal elements of the GHB endogenous system. These interferences, particularly at the level of GHB receptors, result in neuropharmacological effects which are used in therapeutics for sleep regulation in narcoleptic patients [9], for the reduction of withdrawal symptoms in alcohol or heroin addiction [10, 11], and for recreational and psychological benefits [12].

At the cellular level, some molecular events following GHB receptor stimulation have been described. *In vivo* and in brain slices, increases in cGMP and in inositol polyphosphate levels have been observed under the influence of pharmacological doses of GHB [13]. GHB administration generally induces neuronal cell membrane hyperpolarizations [14]. Recently, the stimulation of GHB receptors expressed in differentiated NCB-20 cells appeared to be linked to a reduction in Ca^{2+} channel activities [5]. This effect was blocked by NCS-382 but not by the $GABA_B$ antagonist CGP-55845. Taking into account the GHB effect on Ca^{2+} movements and on cGMP levels, we explored a possible modification of NOS activity induced by micromolar concentrations of GHB in small punches of rat frontal cortex incubated under physiological conditions.

* Corresponding author: Michel Maitre, M.D., Ph.D., Institut Chimie Biologique and ER 2072 CNRS, Faculté de Médecine, 11 Rue Humann, 67085 Strasbourg, France. Tel. 03 88 24 34 93; FAX 03 88 24 35 01; E-mail: maitre@neurochem.u-strasbg.fr

† *Abbreviations:* NOS, nitric oxide synthase; GHB, gamma-hydroxybutyrate; GABA, gamma-aminobutyrate; and cGMP, cyclic guanosine monophosphate.

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MATERIALS AND METHODS

Male Wistar rats (300–350 g; Centre de Neurochimie, Strasbourg, France) were rapidly decapitated after stunning and their brains removed and dissected on a cold glass plate. Coronal sections of 750 μm were cut from the frontal cortex (3.7 mm anterior and 5.2 mm posterior to bregma) according to Paxinos and Watson [15]. Punches were taken from the two slices obtained with the aid of a stainless steel hollow needle with an internal diameter of 1 mm. Punches were immediately transferred into 50 mL of Krebs–Henseleit solution of the following composition: (mM) NaCl 120, KCl 2.0, $CaCl_2$ 2.0, $NaHCO_3$ 26, $MgSO_4$ 1.19, KH_2PO_4 1.18, and glucose 11, which had previously been saturated with 95-5% O_2/CO_2. The solution containing the punches was bubbled with 95-5% O_2/CO_2 at 37° for 60 min. Next, a determined number of punches were transferred into basket-shaped sieves. The baskets were then placed in plastic tubes containing 1 mL Krebs–Henseleit with 20 μM L-arginine, 0.1 μCi U-[^{14}C] L-arginine (11.0 GBq/mmol; 296 mCi/mmol), 1 mM L-citrulline, and 10 μM tetrahydrobiopterin with or without the addition of the various test compounds (veratrine, N^{ω}-nitro-L-arginine, gamma-hydroxybutyrate). The tubes were briefly gassed with 95-5% O_2/CO_2 sealed, and then incubated at 37° for time intervals varying from 20–90 min and as a function of protein (1–4 punches per sieve = 60–240 μg protein). An incubation time of 60 min was usually chosen because of the low amount of citrulline produced. At the end of the incubation period, the sieves were removed and carefully drained into the tubes. Bathing liquid from each tube (0.8 mL) was applied to a 1-mL column of BioRad AG 50W-X8, 200–400 mesh, Na^+ form, and the eluates collected directly into scintillation vials together with two 0.8-mL H_2O rinses. Scintillation fluid (15 mL) was added to each vial, which was then vortexed. After 15 hr, the vials were counted for 10 min to measure the [^{14}C] L-citrulline formed during the course of the NOS reaction.

All measurements were performed by reference to the background formation of [^{14}C] L-citrulline in presence of 1 mM N^{ω}-nitro-L-arginine in the incubation medium. N^{ω}-nitro-L-arginine has been reported to be a strong inhibitor of the calcium/calmodulin-dependent constitutive NOS from brain [16]. Results are percentages of the veratrine-induced stimulation of NOS in the same tissue (taken as 100% reference). Statistical analyses of data (N = 9) were assessed by a one-way Anova followed by a Newmann–Keuls test for dose/effect experiments. Differences between two sets of values were evaluated by the Student's t-test.

RESULTS

Determination of NOS Activity as a Function of Time and Amount of Protein

Veratrine induces brain tissue depolarizations and nitric oxide synthesis through multiple mechanisms, including activation of N-methyl-D-asparate receptors, voltage-sensitive Ca^{2+} channels, and Na^+/Ca^{2+} exchange channels [17]. Under our experimental conditions, the synthesis of nitric oxide induced by 20 μg/mL veratrine was linear for at least 60 min in the presence of two punches of frontal cortex (121 μg protein per tube, Fig. 1). The kinetics were also linear for up to 3 punches in the same tube (i.e. 183 μg protein) incubated for 60 min (Fig. 2). These preliminary studies were used to define the experimental conditions to test the effect of GHB and synthetic analogues on NOS activity.

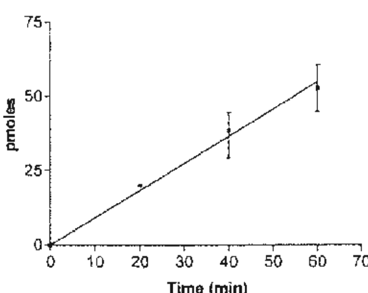

FIG. 1. NOS activity as a function of incubation time. Each sample was incubated in the presence of two punches of the frontal cortex (121 μg protein per tube). Results are expressed in pmol [^{14}C] citrulline formed in the presence of 20 μg/mL veratrine. Backgrounds obtained with 1 mM N^{ω}-nitro-L-arginine were subtracted from all results. Data are given as means ± SEM (N = 9 independent experiments).

GHB Dose–Activity Relationship and NOS Activity

NOS activity was measured in the presence of various concentrations of GHB (2.5 to 100 μM) co-incubated with two punches (121 μg of protein) for 60 min. Results were calculated as percentages of the NOS activity induced by 20 μg/mL veratrine (100% activity = 6 ± 1.2 pmol/min/mg protein) (Fig. 3). Basal levels of [^{14}C] citrulline formation in the presence of 1 mM N^{ω}-nitro-L-arginine were considered as background and always subtracted from the different

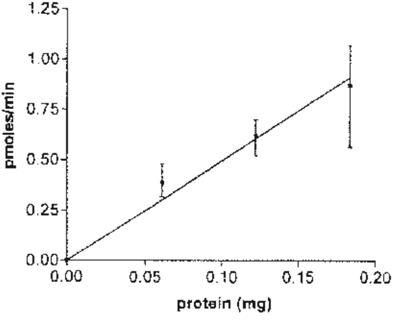

FIG. 2. NOS activity as a function of the amount of tissue. Samples were incubated for 60 min in the presence of either one, two, or three punches (about 60 μg protein per punch). Results (means ± SEM; N = 9) are in pmol [^{14}C] citrulline formed, backgrounds obtained in the presence of 1 mM N^{ω}-nitro-L-arginine always being subtracted.

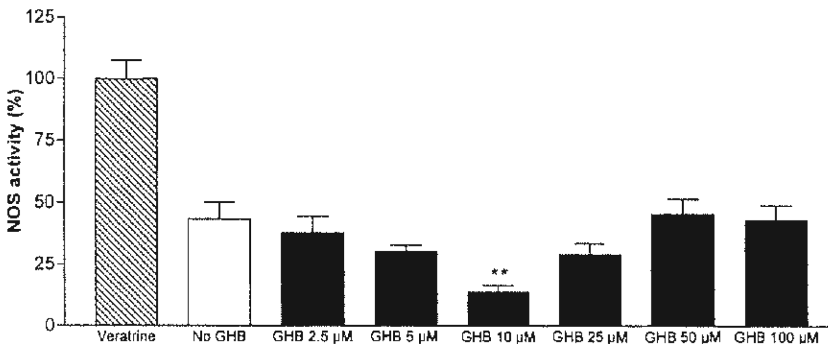

FIG. 3. Effect of GHB on NOS activity. Two punches were incubated for 60 min in the presence of increasing concentrations of GHB (2.5 to 100 μM). Results (means ± SEM, N = 9 independent experiments) are percentages of the control activity obtained with 20 μg/mL veratrine (6 ± 1.2 pmol/min/mg protein). ** = $P < 0.001$ compared to NOS activity measured in the absence of GHB added in the incubation medium.

results. Under these conditions, increasing amounts of GHB from zero to 10 μM induced a progressive reduction in NOS activity (43.2 ± 7% to 13.9 ± 2%). However, when the concentration of GHB in the medium was above 10 μM, a progressive re-increase of NOS activity was seen (28 ± 4% at 25 μM to 45 ± 6% and 43 ± 6% at 50 and 100 μM GHB, respectively). These last two concentrations of GHB induced a NOS activity which was not different from those existing in the punches in the absence of GHB in the incubation medium. Thus, GHB exerts a biphasic effect on NOS activity. Below 10 μM, a reduction was seen. Increasing amounts of GHB above 10 μM induced the progressive disappearance of the GHB effect.

Effects of Synthetic Analogues of GHB

The effects of the GHB receptor agonist NCS-356 (γ-*p*-chlorophenyl-*trans*-hydroxycrotonate) [18] and antagonist NCS-382 [8] were tested under the same conditions in order to characterize the nature of the GHB-induced response pharmacologically. NCS-382 (200 μM) had no effect by itself on NOS activity, but it completely inhibited the decrease in NOS activity induced by a low concentration of GHB (10 μM; 13.9 ± 2.6% in the absence of NCS-382 and 23.9 ± 2.5% in the presence of the antagonist) and also blocked the activating effect of 50 μM GHB (45.5 ± 6.1% in the absence of NCS-382 and 9.5 ± 3.4% in the presence of the antagonist). NCS-356 (50 μM) stimulated NOS, similarly to GHB at the same dosage, and this stimulation was also inhibited by the antagonist NCS-382 (35.3 ± 3.8% to 19.3 ± 3.8%; Fig. 4). Thus, this substance antagonized the GHB-induced inhibition of neuronal NOS observed at low doses of GHB (10 μM), demonstrating that this effect is a specific receptor-mediated effect. At a high dose of GHB (50 μM), it could be suggested that NCS-382 stabilizes the GHB receptor and diminishes its desensitization or, alternatively, that it antagonizes the other class of receptor stimulated only by high doses of GHB.

Additive Effect of Veratrine and GHB on the Stimulation of NOS Activity

When GHB (50 μM) was co-incubated with veratrine (20 μg/mL), a supplementary stimulation of NOS activity was observed compared to those seen with veratrine alone. This additive effect (+27 ± 9%) indicates that GHB modulated the permeability of a calcium channel which was not opened by the action of veratrine. In the presence of the

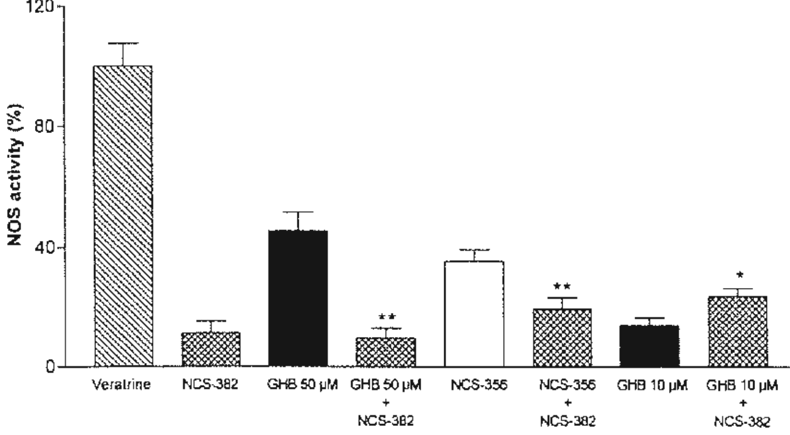

FIG. 4. Effect of synthetic ligands of GHB receptor(s) on NOS activity. Two punches were incubated for 60 min in the presence of: the antagonist NCS-382 (200 μM) alone; the antagonist NCS-382 (200 μM) + GHB (50 μM or 10 μM); the agonist NCS-356 (50 μM) alone; the antagonist NCS-382 (200 μM) + the agonist NCS-356 (50 μM). Results (means ± SEM of 9 independent experiments) are percentages of NOS activity induced by veratrine 20 μg/mL (control activity). * = $P < 0.05$; ** = $P < 0.01$ compared to NOS activity induced by GHB or NCS-356 alone.

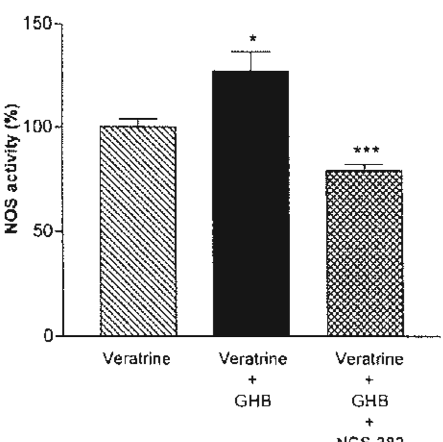

FIG. 5. Effect of GHB on veratrine-induced NOS activity. Two punches were incubated for 60 min in the presence of veratrine alone (20 μg/mL) or of veratrine (20 μg/mL) + GHB 50 μM or of veratrine (20 μg/mL) + GHB (50 μM) + the GHB receptor antagonist NCS-382 (200 μM). Results (means ± SEM of 9 independent experiments) are percentages of control activity (veratrine alone = 100%). * = $P < 0.05$ compared to veratrine alone; *** = $P < 0.0001$ compared to veratrine + GHB.

antagonist NCS-382, the supplementary effect due to GHB disappeared (Fig. 5).

DISCUSSION

GHB is heterogeneously distributed in the rat brain and its maximal concentration is approx. 5–10 μM [19]; however, GHB concentrations increase in the ischemic brain and it is difficult to evaluate the actual GHB levels in the punches of frontal cortex used in this study. Nevertheless, 50–100 μM GHB concentrations are reached in the brain of the animal only after peripheral administration of GHB [20]. Thus, under physiological concentrations of GHB, the present paper describes an inhibition of constitutive NOS by increasing amounts of GHB (up to 10 μM). Neuronal NOS is a Ca^{2+}/calmodulin-dependent enzyme regulated by the steep gradient of Ca^{2+} that occurs in the vicinity of open Ca^{2+} channels [21]. Although constitutive brain NOS is mainly linked to Ca^{2+} influx through the *N*-methyl-D-asparate receptor, it is possible that the GHB receptor also plays a role in this domain. The present study was carried out in order to use neuronal NOS as an indicator of Ca^{2+} movements in the tissue. Interestingly, we confirm the GHB-induced decrease in neuronal Ca^{2+} influx generated by GHB receptor stimulation that has been demonstrated on NCB-20 cells by patch-clamp experiments [5]. These cells are a hybrid between mouse neuroblastoma N18TG2 and Chinese hamster embryonic day-18 brain cells which express many properties characteristic of neurons. When differentiated by dibutyryl cyclic AMP, these cells develop synaptic contacts, and a K^+-evoked, Ca^{2+}-dependent release of GHB takes place. NCB-20 cells also express a homogenous population of high affinity binding sites for [^{3}H] GHB which mediate the GHB-induced modifications in Ca^{2+} conductances, given that this effect was blocked by NCS-382 but not by the $GABA_B$ antagonist CGP 55845. Other electrophysiological results obtained on brain neurons *in vivo* generally support a role for GHB in inducing neuronal cell membrane hyperpolarization [14].

Peripheral administration of GHB in rats and human raises the brain GHB concentration markedly above physiological concentrations. Chronic administration of GHB in rats has been shown to desensitize and down-regulate GHB receptors [22]. Under our experimental conditions (1-hr incubation), concentrations of GHB above 10 μM (25, 50, and 100 μM) induced a progressive re-increase of NOS activity in the punches of frontal cortex. This is probably due to a progressive loss of GHB-induced inhibition of Ca^{2+} influx due to a progressive desensitization of GHB receptors exposed to high GHB concentrations, and NOS activity returns progressively to control values. However, as suggested by the kinetic parameters of GHB binding on rat brain membranes, two populations of GHB receptors exist, one of high affinity (K_{d_1} estimated in the range 30–580 nM) and the other of lower affinity (K_{d_2} in the range of 2 to 16 μM) [2]. High concentrations of GHB in the incubation medium could induce a gradient concentration of GHB in the tissue high enough to stimulate both classes of sites. Under these conditions, the global effect of GHB could be a stimulation of Ca^{2+} influx into the tissue with a stimulation of NOS. Previous results have reported an increase in calcium ion utilization in the substantia nigra, rich in GHB receptors, after local application of GHB at doses above 100 μM [23]. Lower doses have not been tested. In relation to this increase in calcium ion influx and the stimulation of NOS activity at high doses of GHB, an increase in cGMP concentration has been reported in the rat hippocampus *in vivo* following the administration of 400 mg/kg GHB [24]. A possible decrease in cGMP concentrations at low doses of GHB has not been tested and is worthy of investigation.

Thus, several lines of evidence support the notion of a reduction of Ca^{2+} influx in brain tissue after stimulation of GHB receptor, leading to a reduction in NOS activity. This effect could be blocked by the selective GHB receptor antagonist NCS-382. Overstimulation of GHB receptor by high amounts of GHB for 60 min induces the apparent disappearance of this effect. NCS-382 also reduces this second phase either by preventing the desensitization of GHB receptors or by inhibiting the stimulation of Ca^{2+} entry mediated by another class of GHB receptor of lower affinity. The hypothesis that a high dose of GHB could stimulate other kinds of receptors (in particular $GABA_B$ receptors) [25] is unlikely, because the specific synthetic agonist of GHB receptors (NCS-356) reproduces the GHB effect.

It can be concluded that GHB receptor stimulation induces a biphasic effect on calcium ion movements. This could be related to the biphasic effect of GHB on *in vivo* release of dopamine in striatum: low doses induce a decrease

in dopaminergic firing and a reduction of dopamine release, while high doses provoke an extracellular increase of dopamine [26]. As the biphasic modulation of NOS activity via GHB receptor seems unlikely via other mechanisms (e.g. biphasic modulation of NOS phosphorylation) [27], it is proposed that the physiological GHB concentration reduces Ca^{2+} influx and NOS activity. By contrast, the overload of the GHB system by peripheral administration leads to the disappearance of the tonic inhibitory influence of GHB on Ca^{2+} entry, which is followed by an increase in dopamine synthesis and release.

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Drosophila $GABA_B$ receptors are involved in behavioral effects of γ-hydroxybutyric acid (GHB)

Nikola Dimitrijevic, Svetlana Dzitoyeva, Rosalba Satta, Marta Imbesi, Sevim Yildiz, Hari Manev *

The Psychiatric Institute, Department of Psychiatry, University of Illinois at Chicago, 1601 West Taylor Street, MC912, Chicago, Illinois 60612, USA

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Abstract

γ-hydroxybutyric acid (GHB) can be synthesized in the brain but is also a known drug of abuse. Although putative GHB receptors have been cloned, it has been proposed that, similar to the behavior-impairing effects of ethanol, the in vivo effects of pharmacological GHB may involve metabotropic γ-aminobutyric acid (GABA) $GABA_B$ receptors. We developed a fruitfly (*Drosophila melanogater*) model to investigate the role of these receptors in the behavioral effects of exogenous GHB. Injecting GHB into male flies produced a dose-dependent motor impairment (measured with a computer-assisted automated system), which was greater in ethanol-sensitive *cheapdate* mutants than in wild-type flies. These effects of pharmacological concentrations of GHB require the presence and activation of $GABA_B$ receptors. The evidence for this was obtained by pharmacological antagonism of $GABA_B$ receptors with CGP54626 and by RNA interference (RNAi)-induced knockdown of the $GABA_{B(1)}$ receptor subtype. Both procedures inhibited the behavioral effects of GHB. GHB pretreatment diminished the behavioral response to subsequent GHB injections; i.e., it triggered GHB tolerance, but did not produce ethanol tolerance. On the other hand, ethanol pretreatment produced both ethanol and GHB tolerance. It appears that in spite of many similarities between ethanol and GHB, the primary sites of their action may differ and that recently cloned putative GHB receptors may participate in actions of GHB that are not mediated by $GABA_B$ receptors. These receptors do not have a *Drosophila* orthologue. Whether *Drosophila* express a different GHB receptor should be explored.

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1. Introduction

γ-hydroxybutyric acid (GHB) is a naturally occurring metabolite of γ-aminobutyric acid (GABA), found in mammalian tissues including the brain (Roth and Giarman, 1970). Pharmacologically, GHB (sodium oxybate) is considered in the treatment of narcolepsy (Borgen et al., 2004; Tunnicliff and Raess, 2002) and occasionally as an anesthetic (Kleinschmidt et al., 1998). The pharmacological profile of GHB is similar to the profile of ethanol (Poldrugo and Addolorato, 1999). Abuse of GHB, which shares its behavioral effects with a number of classical sedative/hypnotics, is an increasing problem (Nicholson and Balster, 2001; Ricaurte and McCann, 2005). Clinically, there are reports of severe GHB withdrawal symptoms (Craig et al., 2000; Tarahar and Nelson, 2004), and in a rat model, repeated administration of GHB produces both behavioral tolerance and withdrawal (Bania et al., 2003).

It has been proposed that metabotropic GABA receptors, $GABA_B$ receptors, and GHB receptors may mediate the actions of GHB (Andriamampandry et al., 2003; Carter et al., 2003, 2004a,b; Kaupmann et al., 2003). Although a direct binding of GHB to $GABA_B$ receptors has not been conclusively demonstrated (Lingenhoehl et al., 1999; Wu et al., 2004), it appears that to produce its behavioral effects, GHB requires these receptors (Carter et al., 2003, 2004a,b; Kaupmann et al., 2003). In $GABA_{B(1)}$ receptor knockout

* Corresponding author. Tel.: +1 312 413 4558; fax: +1 312 413 4569.
E-mail address: HManev@psych.uic.edu (H. Manev).

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mice, which lack functional $GABA_B$ receptors, GHB application failed to produce either the behavioral or the biochemical responses seen in wild-type mice (Kaupmann et al., 2003). On the other hand, the binding of a putative GHB antagonist, NCS-382, to the specific [^{3}H]GHB-binding sites was unchanged in $GABA_{B(1)}$ receptor knockout mice, suggesting that the behavioral and biochemical effects of GHB are $GABA_B$ receptor-dependent whereas the nature and signaling properties of the specific [^{3}H]GHB-binding sites remain elusive.

Although $GABA_B$ receptor knockout mice are useful for behavioral studies, they are developmentally altered and their use is complicated by inherited pathologies such as seizures (for a review, see Enna and Bowery, 2004). To circumvent these drawbacks, we developed a *Drosophila* model for the adult $GABA_{B(1)}$ receptor knockdown via the injectable RNA interference (RNAi) method (Dzitoyeva et al., 2003). Recent studies demonstrated that *Drosophila* can be successfully used in neuropharmacological research (Bainton et al., 2000; Manev et al., 2003). Fruit flies possess a physiologically active endogenous GABA system (Leal et al., 2004), express $GABA_B$ receptors (Mezler et al., 2001), and when treated with $GABA_B$ receptor ligands display distinct behavioral responses (Dzitoyeva et al., 2003; Dimitrijevic et al., 2004) and developmental abnormalities (Dzitoyeva et al., in press). Previously, GHB was administered to *Drosophila* either via food (Connolly et al., 1971) or by injection (Satta et al., 2003) and in both conditions GHB impaired their locomotor activity. We observed that similar to mammals, *Drosophila* possess the machinery for GHB synthesis and are capable of metabolizing 1,4-butanediol into GHB in vivo (Satta et al., 2003).

In flies (Dzitoyeva et al., 2003), similar to mice (Zaleski et al., 2001), the cAMP-linked $GABA_B$ receptors participate in the behavior-impairing effects of ethanol. Experiments with mutant flies provided evidence that the cAMP signaling system plays a crucial role in the acute response of fruitflies to ethanol vapor (Moore et al., 1998). These authors found that lack of the *amnesiac* gene, which is thought to encode a peptide that increases levels of cAMP, or a mutation in this gene called *cheapdate* increase sensitivity to ethanol. Here, we investigated whether the *cheapdate* mutation influences behavioral effects of GHB. In this work, we hypothesized that $GABA_B$ receptors participate in the behavioral actions of GHB in flies, and that flies can be used as an in vivo model to investigate the behavioral interactions of GHB and ethanol.

2. Materials and methods

2.1. *Drosophila and drug treatments*

Canton-S (wild-type) and *cheapdate* flies were cultured at 25 °C, 50–60% humidity, 12 h/12 h light/dark cycle, on yeast, dark corn syrup, and agar. Studies were performed with 5 to 7-day-old males. For injections (Dzitoyeva et al., 2001, 2003), flies were anesthetized by CO_2 (maximally for 5 min). Using custom-beveled glass pipettes (20×40 μm tip diameter) coupled to a cell injector and a micromanipulator we injected a volume of 0.2 μl/fly by a pulse pressure of 300 kPa under a stereo microscope (Dzitoyeva et al., 2003). Drugs were prepared as 10× stock solutions; 0.2 μl were injected per fly. GHB (Sigma, St. Louis, Missouri, USA), [*S*-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl] amino]-2-hydroxypropyl](cyclohexylmthyl) phosphinic acid hydrochloride (CGP54626; Tocris, Ellisville, Missouri, USA), 5,7,8,9-tetrahydro-5-hydroxy-6H-benzocyclohepten-6-ylideneacetic acid sodium–potassium salt (NCS-382; Sigma, St. Louis, Missouri, USA) and ethanol (Sigma, St. Louis, Missouri, USA) were dissolved/diluted with Ringer solution (NaCl, KCl, $CaCl_2$; 7.5, 0.35, 0.21 g/l; pH 7.6–7.8; sterile-filtered). The stock solutions of CGP54626 and NCS-382 were prepared in dimethylsulfoxide (DMSO; Sigma, St. Louis, Missouri, USA); the DMSO concentration in final solutions was 1%. All control flies were injected with the corresponding vehicle. When using multiple drug treatments, drugs were administered with different pipettes and in the indicated time interval (1–4 h).

2.2. *Behavioral assay*

A *Drosophila* Activity Monitoring System (Trikinetics, Waltham, Massachusetts, USA) coupled to a computer was used to record the locomotor activity of individual flies. In previous studies, we established methods for evaluating the drug-induced changes in locomotor activity, immobility duration, and the number of periods of immobility (Dzitoyeva et al., 2003; Satta et al., 2003; Dimitrijevic et al., 2004). To quantify these parameters, the system was modified, i.e., the space in each individual recording tube was restricted to a length of 8 mm in the center of the photo beam. Flies were placed in the recording tubes within 2 min of injection and the sampling time was set at 1-min intervals. After flies recovered from the injection, they gradually resumed locomotor activity. The *locomotor activity* of flies was calculated as the average number of movements/min recorded and quantified for a 30-min period starting at the time of recovery from the acute immobility induced by vehicle/drug administration. The calculated average 1-min activity of vehicle-treated controls was used to determine the threshold for recovery from injection. Thus, the first 1-min interval after the injection in which a fly reaches this threshold, i.e., the number of "average" moves/min, was used as the time of recovery and to calculate the *duration of immobility*. *Periods of immobility* (i.e., 1-min periods with 0 movements) during the 30-min observation periods following the recovery were also calculated. Typically, 6–10 flies per experimental group were used and experiments were repeated 2–3 times. Statistical analysis was performed by Dunnett's test or Student's *t*-test; $P<0.05$ was taken as significant.

2.3. *Gas chromatography/mass spectrometry (GC/MS) assay of GHB*

This assay was performed as described elsewhere (Satta et al., 2003). Briefly, groups of five flies were homogenized in 200 μl of 100 mM phosphate buffer pH 6; d6-GHB (2,2,3,3,4,4-d6; Cambridge Isotope Laboratories, Andover, Massachusetts, USA) was added (2.5 ng/μl) to each sample as the internal standard. Each sample was applied onto 1 ml of a AG1X8 formate ion exchange column preconditioned with successive additions of 10

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Table 1
The sequences of the sense DNA oligonucleotides corresponding to the $GABA_{B(1)}$ and *Fs(1)Yb* genes used for the in vitro transcription reaction

$GABA_{B(1)}$+T7 promoter (a)	5′-taatacgactcactatattatttgtgtgcatgtgcacaa-3′
$GABA_{B(1)}$+T7 promoter (b)	5′-taatacgactcactatagctgttggcaggatgcagcacg-3′
Fs(1)Yb+T7 promoter	5′-taatacgactcactatatttctgcagtgggaataactt-3′

ml 20% ethanol in 0.5 M formic acid and 10 ml distilled water. GHB was eluted with the successive addition of 4 ml 20% ethanol in 0.5 M formic acid, dried under vacuum, and the residue was dissolved in 200 µl dimethylformamide. After two extractions with 1 ml of hexane, the dimethylformamide layer was dried by evaporation under nitrogen. The samples were derivatized with 100 µl ethyl acetate and 100 µl BSTFA [N,O,-bis(trimethylsilyl)trifluoroacetamide] with 1% trimethylchlorosilane for 10 min at 60 °C, and analyzed on a GC/MS system equipped with a HP-5 column (30 m × 0.25 mm × 0.25 µm). Two µl samples were injected manually. The temperature of the injection port was 250 °C. The oven was initially held at 70 °C for 4 min; thereafter, it was programmed to increase at a rate of 8 °C/min to 100 °C and at a rate of 25 °C/min to 175 °C. The instrument was operated in the selected ion monitoring mode (SIM); the ions 233 and 239 were used for the quantitation of GHB and d6-GHB, respectively. The retention time for GHB and d6-GHB was approximately 9.1 min. The calibration curve ranged from 0.5 to 5 ng/µl GHB; the detection limit for the standard prepared in water was 0.25 ng/µl.

2.4. Synthesis of double-stranded RNA (dsRNA) and RNAi

We targeted two 22-nucleotide long regions; a) 329–350, and b) 1402–1423, of the *Drosophila* $GABA_{B(1)}$ gene. As a control dsRNA, we used a 22nt of the *Fs(1)Yb* gene. 39-mer DNA oligonucleotides with an attached T7 RNA polymerase promoter sequence were synthesized (Integrated DNA Technology, Inc., Coralville, Iowa, USA); these oligonucleotides represented both sense and antisense strands. The sequences of the sense DNA oligonucleotides corresponding to the $GABA_{B(1)}$ and *Fs(1)Yb* genes used for the in vitro transcription reaction are shown in Table 1. Only one match was found in the *Drosophila* genome database pattern search for the $GABA_{B(1)}$ and *Fs(1)Yb* gene, respectively. Equal amounts of oligonucleotides were annealed to form a double-stranded template by heating at 80–85 °C for 5 min and cooling on ice. The in vitro transcription reaction (30 µl volume) for the synthesis of the 22 nt RNA run of transcripts contained 0.1 µg of a

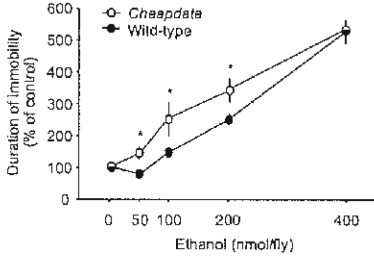

Fig. 1. Increased sensitivity of *cheapdate* flies to injected ethanol. Injections of ethanol caused a dose-dependent prolongation of the immobility observed immediately after injection in both *cheapdate* and wild-type flies. However, the dose response to ethanol-induced prolongation of immobility time in *cheapdate* flies was shifted to the left, indicating their greater sensitivity to ethanol. Results (mean ± S.E.M.; n = 14–16/time point) are expressed as a percentage of corresponding immobility of vehicle-treated controls ["0" ethanol; immobility (min) wild-type = 7.2 ± 1.5; *cheapdate* 6.9 ± 0.5]. *P < 0.05 compared to corresponding wild-type values.

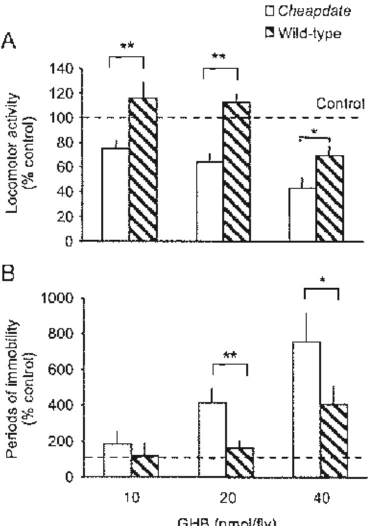

Fig. 2. Increased sensitivity of *cheapdate* flies to behavioral effects of GHB. GHB (doses as indicated) was administered intra-abdominally. Results (mean ± S.E.M.; n = 15–24) are expressed as a percentage of the corresponding vehicle-treated controls [Locomotor activity (movement/min): wild-type = 8.2 ± 2.2; *cheapdate* = 7.7 ± 0.7. Periods of immobility (number of immobile periods in 30 min): wild-type = 3.2 ± 2.0; *cheapdate* = 2.9 ± 1.3.]. Locomotor activity (A) and periods of immobility (B) were measured in a 30-min period starting from the time of recovery (Material and methods). In *cheapdate* flies, locomotor activity was decreased and periods of immobility were increased at lower doses of GHB compared to wild-type flies. A dose of GHB 40 nmol/fly resulted in impaired behavior in both strains of flies (*P < 0.05; **P < 0.001).

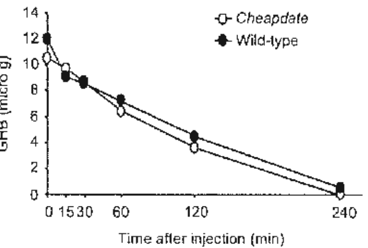

Fig. 3. The elimination rates of injected GHB are comparable in *cheapdate* and wild-type flies. GHB (40 nmol/fly; 5 flies/time point) was injected intra-abdominally, flies were homogenized at different times post-injection (as indicated), and samples were processed for GC/MS assays of GHB. Results are expressed as µg GHB/sample (obtained from 5 flies). Note a similar time-dependent GHB decrease in *cheapdate* and wild-type flies; GHB was barely detectable 4 h post-injection in both strains.

Table 2
Effects of $GABA_B$ receptor antagonist CGP54626 and GHB antagonist NCS-382 on the behavioral effects of GHB

Treatment	Locomotor activity (movements/min)	Periods of immobility (periods/30 min)
Vehicle+Vehicle	8.7±0.9	4.4±1.8
Vehicle+GHB	4.8±0.5*	10.1±1.4*
CGP54626 (0.2 nmol/fly) +GHB	8.0±1.0	4.6±1.2
Vehicle+Vehicle	8.1±1.1	2.5±0.9
Vehicle+GHB	4.6±0.7*	7.7±2.8*
NCS-382 (0.2 nmol/fly) +GHB	4.7±0.8*	8.2±2.7*
NCS-382 (1.0 nmol/fly) +GHB	4.2±0.7*	9.8±1.9*

Wild-type flies were injected with CGP54626 or NCS-382 1 h prior to GHB (40 nmol/fly). Results are expressed as mean±S.E.M.; $*P<0.01$ compared to the corresponding vehicle+vehicle group.

template, 500 μM each CTP, GTP, ATP, and UTP, 1× transcription buffer (Tris–HCl, pH 7.5, 10 mM dithiothreitol, 1% bovine serum albumin), 20 U of RNAse inhibitor, and 50 U T7 RNA polymerase (Gibco BRL, Invitrogen, Carlsbad, California, USA). The reactions were carried out at 37 °C for 1 h. The RNA molecules were annealed together in heat denaturing conditions (65–70 °C for 5 min) and placed on ice. The quality of both RNA and DNA oligonucleotides was confirmed on a 4% NuSive agarose gel (Sigma, St. Louis, Missouri, USA). Flies were injected with 0.2 μl from a stock (100 ng/μl) of $GABA_{B(1)}$ a+b or Fs(1)Yb dsRNAs. They were used for experiments 3 days after the injection; i.e., when a significant reduction of the endogenous $GABA_{B(1)}$ mRNA content was documented in $GABA_{B(1)}$ RNAi flies (Dzitoyeva et al., 2003).

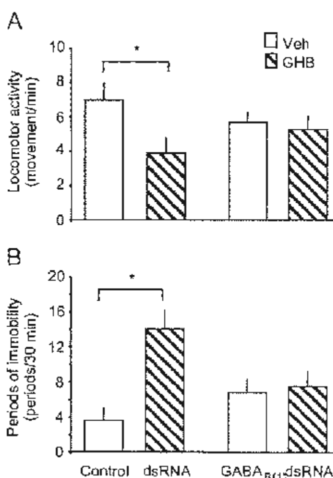

Fig. 4. $GABA_{B(1)}$ receptor RNAi inhibits the behavioral effects of GHB. $GABA_{B(1)}$ dsRNA but not control dsRNA (Table 1) injections into adult wild-type flies prevented the decrease in locomotor activity (A) and the increase of immobility periods (B) induced by GHB (40 nmol/fly). Results are expressed as mean±S.E.M.; $*P<0.01$ compared to the corresponding vehicle-treated group ($n=16$).

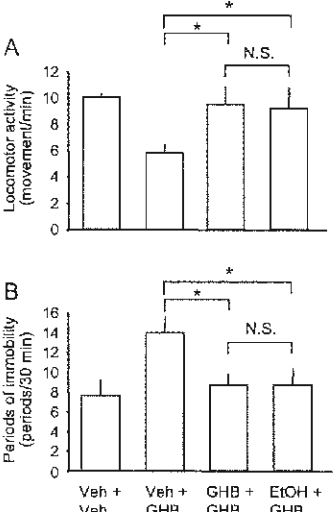

Fig. 5. Previous administration of GHB or ethanol diminishes behavioral responses to subsequent GHB injections. Vehicle, GHB (40 nmol/fly), and ethanol (200 nmol/fly) were injected into wild-type flies 4 h prior to subsequent injections of vehicle or GHB. Thereafter locomotor activity (A) and increase of immobility periods (B) were measured. Results are expressed as mean±S.E.M.; $*P<0.05$ ($n=11$–12).

3. Results

3.1. GHB impairs locomotor activity of flies: cheapdate mutation increases sensitivity to GHB

Both in wild-type and *cheapdate* flies, ethanol injections induced the dose-dependent prolongation of immobility typically observed immediately after the injection. However, the dose–response curve for ethanol-induced prolongation of immobility was

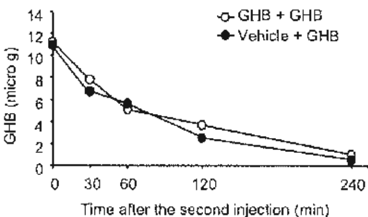

Fig. 6. Previous administration of GHB does not alter the elimination rate of subsequently injected GHB. GHB (40 nmol/fly; 5 flies/time point) or vehicle were injected intra-abdominally. The flies received two injections (GHB +GHB or vehicle+GHB), 4 h apart, and were homogenized at different times after the second injection (as indicated). Samples were processed for a GC/MS assay of GHB. Results are expressed as μg GHB/sample (obtained from 5 flies). Note a similar time-dependent GHB decrease in GHB+GHB and vehicle+GHB groups.

shifted to the left in *cheapdate* vs. wild-type flies (Fig. 1). The effects of GHB injections were qualitatively different from the effects of an ethanol injection. Although GHB slightly prolonged immobility immediately after injection, this action was neither significant nor dose-dependent (not shown). Instead, once GHB-injected flies recovered from the injection their locomotor activity level was lower and interrupted by periods of immobility (Fig. 2). These effects of GHB were observed in both *cheapdate* and wild-type flies, but *cheapdates* were more sensitive. In wild-type flies, behavioral impairment consistently occurred after 40 nmol/fly GHB whereas in *cheapdates*, even 10 nmol/fly was effective (Fig. 2). We measured whether the elimination of injected GHB differs between *cheapdate* and wild-type flies. Using our recently developed GC/MS methodology for GHB measurement, we found no difference in GHB elimination in these two fly strains (Fig. 3).

3.2. $GABA_B$ antagonism and $GABA_{B(1)}$ RNAi reduce the behavioral effects of GHB

Pretreating flies with 0.2 nmol/fly of the $GABA_B$ receptor antagonist CGP54626 inhibits the behavioral effects of 40 nmol/fly GHB (Table 2). On the other hand, pretreatment with the GHB receptor antagonist NCS-382 did not affect the behavioral consequences of GHB (Table 2). We also used our previously established RNAi method for knocking down $GABA_{B(1)}$ receptors in adult wild-type flies. GHB treatment was introduced 3 days after dsRNA injections. In control-dsRNA-injected flies, GHB produced the typical behavioral impairments that were absent in $GABA_{B(1)}$-dsRNA-injected flies (Fig. 4).

3.3. Repeated administration of GHB produces behavioral tolerance

We found that the previous exposure of flies to GHB reduces the behavior-impairing actions of a second GHB injection administered 4 h later (Fig. 5). In contrast, the previous exposure to GHB did not alter GHB elimination (Fig. 6).

3.4. Behavioral interactions between GHB and ethanol

Interestingly, the behavior-impairing effects of GHB were attenuated not only by previous exposure to GHB but also by previous exposure to ethanol (Fig. 5). When flies were first treated with ethanol and their response to subsequent ethanol administration was examined, we observed diminished behavior impairing activity following the second ethanol injection (i.e., a rapid ethanol tolerance). However, pretreatment with GHB did not alter the response of flies to subsequent ethanol injection (Fig. 7).

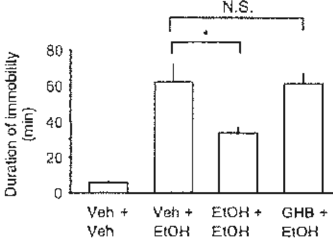

Fig. 7. Previous administration of ethanol but not GHB diminishes prolongation of immobility due to subsequent ethanol injection. Vehicle, ethanol (200 nmol/fly), and GHB (40 nmol/fly) were injected into wild-type flies 4 h prior to subsequent injection of vehicle or ethanol. The time to recovery (i.e., duration of immobility due to ethanol) was measured. Results are expressed as mean±S.E.M.; $*P<0.01$ ($n=16$).

4. Discussion

In this work, we demonstrated that pharmacological concentrations of GHB produce behavioral effects in adult *Drosophila* which require the presence and activation of $GABA_B$ receptors. The evidence for this was obtained by pharmacological antagonism of $GABA_B$ receptors and by RNAi-induced knockdown of $GABA_{B(1)}$ receptor subtypes. Both procedures were capable of inhibiting the behavioral effects of GHB. Our findings with in vivo experiments in *Drosophila* are consistent with observations from in vivo experiments with $GABA_{B(1)}$ receptor knockout mice (Kaupmann et al., 2003), and are somewhat at odds with the recent notion that GHB does not bind $GABA_B$ receptors in vitro in $GABA_B$ receptor-expressing HEK 293 cells (Wu et al., 2004). However, similar experiments in COS cells found that GHB is a weak agonist of recombinant $GABA_B$ receptors (Lingenhoehl et al., 1999). It is possible that the cell type-specific environment could contribute to recombinant metabotropic receptor functionality (Gabellini et al., 1994). Furthermore, the specific GHB binding in the brain of $GABA_{B(1)}$ receptor knockout mice was significantly lower than in wild-type mice (Wu et al., 2004), suggesting that a component of in vivo GHB binding, and possibly its pharmacological actions may involve $GABA_B$ receptors. On the other hand, the functional role of putative GHB receptors, particularly with respect to behavior, is unclear. We found that a GHB receptor antagonist, NCS-382, had no effect on the behavioral GHB actions in *Drosophila*. Others reported that NCS-382 did not block the behavioral actions of GHB in rats whereas these actions in rats were inhibited by the $GABA_B$ receptor antagonist CGP 35348 (Carter et al., 2003, 2004a,b).

Adult RNAi via dsRNA injection into adult insects is a useful tool for investigating the loss-of-function phenotypes that circumvent developmental alterations (Dzitoyeva et al., 2001; 2003; Blandin et al., 2002; Amdam et al., 2003; Goto et al., 2003; Farooqui et al., 2004). We found an inhibition of the behavioral effects of GHB with injectable $GABA_{B(1)}$ RNAi. These results, along with the data obtained with CGP 54626 inhibition of GHB effects, suggest that functional $GABA_B$ receptors are needed to produce behavioral GHB activity in *Drosophila*.

$GABA_B$ receptors, which mediate some of ethanol's behavioral effects in mice (Zaleski et al., 2001) and *Drosophila* (Dzitoyeva et al., 2003), are linked to complex transduction mechanisms and involve negative coupling to the cAMP pathway (Couve et al., 2004; Knight and Bowery, 1996). Regulation of the cAMP pathway is critical for

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modifying the sensitivity of *Drosophila* to ethanol. Thus, *cheapdate* mutants, which are characterized by defective cAMP signaling, are sensitive to ethanol (Moore et al., 1998). Using ethanol injections, we confirmed the increased sensitivity of *cheapdate* compared to wild-type flies in a dose range from 50–200 nmol ethanol/fly. The highest ethanol dose used (400 nmol/fly) was not toxic and prolonged immobility in a manner that was no longer sensitive to *cheapdate*-dependent mechanisms. Interestingly, we found that *cheapdate* mutants are also more sensitive to the behavior-impairing actions of GHB. The increased sensitivity to GHB does not appear to be caused by alterations in GHB metabolism. We found that both *cheapdate* and wild-type flies eliminate the injected GHB equally well. Although the exact mechanism for the increased ethanol sensitivity of *cheapdate* is not clear, Moore et al. (1998) found that this enhanced ethanol sensitivity can be reversed by treatment with agents that increase cAMP levels or protein kinase A (PKA) activity. Conversely, genetic or pharmacological reductions in PKA activity resulted in increased sensitivity to ethanol, providing functional evidence for the involvement of the cAMP signal transduction pathway in the behavioral response to impairing levels of ethanol. Our data extend this notion to the behavior-impairing effects of GHB. Considering the involvement of $GABA_B$ receptors in the behavioral effects of GHB, we investigated whether the content of $GABA_{B(1)}$ mRNA is altered in *cheapdate* and found no difference between these and wild-type flies (data not shown). It is possible that the cAMP-linked functioning of $GABA_B$ receptors is altered by the *cheapdate* mutation.

Rapid tolerance to repeated ethanol exposures was observed under various regimens of ethanol delivery to a number of different species including *Drosophila* (Scholz et al., 2000; Ghezzi et al., 2004). Repeated administration of GHB to rats leads to diminished GHB intoxication; i.e., tolerance (Bania et al., 2003). It has been proposed that the development of tolerance to ethanol (Zaleski et al., 2001; Dzitoyeva et al., 2003) and GHB (Eckermann et al., 2004) may involve $GABA_B$ receptors. Although our data in *Drosophila* indicate that $GABA_B$ receptors participate in the acute behavioral effects of GHB and ethanol, it does not appear that a single mechanism; e.g., a direct action of GHB on these receptors, is responsible for GHB-induced GHB tolerance and ethanol crosstolerance. Namely, although GHB pretreatment produced GHB tolerance it did not produce ethanol tolerance. On the other hand, ethanol pretreatment was able to produce both ethanol and GHB tolerance. Thus, it appears that in spite of many similarities between ethanol and GHB, the primary sites of their action may differ.

We can only speculate about the mechanisms regulated by $GABA_B$ receptors that modify the behavioral responses of *Drosophila* to ethanol (Dzitoyeva et al., 2003) and GHB (this study). The behavior-impairing effects of ethanol in flies can be modified by genetic manipulations that impair the function of insulin-producing cells or of the insulin-receptor signaling pathway (Corl et al., 2005). Interestingly, $GABA_B$ receptors contribute to the modulation of glucose-stimulated insulin secretion in rat pancreatic beta cells (Brice et al., 2002). It should be investigated whether an interaction of $GABA_B$ receptors with *Drosophila* insulin signaling plays any role in modifying the behavioral effects of ethanol and/or GHB.

In conclusion, our *Drosophila* model, similar to recent experiments with $GABA_{B(1)}$ receptor knockout mice (Kaupmann et al., 2003), indicates that $GABA_B$ receptors rather than NCS-382-sensitive GHB receptors mediate the acute locomotor-impairing effects of GHB. Although $GABA_B$ receptors also participate in the behavioral actions of ethanol, they do not appear to be involved in all of the behavioral interactions between GHB and ethanol, for example, in crosstolerance. A recently cloned GHB receptor (Andriamampandry et al., 2003) does not have a *Drosophila* orthologue. Whether *Drosophila* express a different GHB receptor should be explored.

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stimulatory or inhibitory peptides or, conceivably, activate precursor forms by limited cleavage. Alternatively, it could have a protective role by stopping inhibitory factors from gaining access to the luminal cells in the intact tissue. Interestingly, although oxytocin (which has powerful action on myoepithelial cells) can be hydrolysed by endopeptidase-24.11, it is a very poor substrate compared with peptides such as ANP and bradykinin, thus raising some doubts that this hormonal signal is terminated by the surface endopeptidase.

Several new antigens have lately been identified on the myoepithelial cell membrane.[27,28] Our hypothesis would predict that some of these antigens may well be other members of a battery of cell-surface enzymes that control the local milieu. Thus, the overexpression of the c-*erb*B-2 gene product on the lateral and basal membranes of breast carcinoma cells in a high proportion of intraduct carcinomas[29] would be consistent with this molecule's being a receptor for a paracrine growth factor (as yet unidentified) perhaps produced by the myoepithelial cells, or modified by them by means of their endopeptidase activity before its reaction with the tumour cells.

We therefore propose that cell-surface peptidases may have a key role in the control of growth and differentiation of many cellular systems by modulating the activity of peptide factors and regulating their access to adjacent cells. The hypothesis is open to direct experimental investigation since various well-characterised, non-toxic inhibitors,[5] acting specifically on several of these enzymes,[3] are available. These can be tested both in vitro and in vivo for their ability to alter growth and differentiation of different cell types in tissues with cell-surface peptidase activity.

Correspondence should be addressed to A. J. K., MRC Membrane Peptidase Research Group, Department of Biochemistry, University of Leeds, Leeds LS2 9JT.

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Clinical Pharmacology

GAMMA-HYDROXYBUTYRIC ACID FOR TREATMENT OF ALCOHOL WITHDRAWAL SYNDROME

L. GALLIMBERTI[1] G. CANTON[1]
N. GENTILE[1] M. FERRI[1]
M. CIBIN[1] S. D. FERRARA[2]
F. FADDA[3] G. L. GESSA[3]

Centro di Alcologia e Farmacodipendenza, General Hospital of Dolo, Venice;[1] Centre of Behavioural and Forensic Toxicology, University of Padova;[2] and Department of Neuroscience "Bernard B. Brodie", University of Cagliari,[3] Italy

Summary The effect of gamma-hydroxybutyric acid (GHB) on ethanol withdrawal syndrome in alcoholics was investigated in a randomised double-blind study. Patients with withdrawal symptoms were treated either with GHB (orally in a syrup preparation) (11 patients) or with the syrup alone (12). GHB treatment (50 mg/kg) led to a prompt reduction in withdrawal symptoms, such as tremors, sweating, nausea, depression, anxiety, and restlessness. The only side-effect was dizziness. GHB may be useful in the management of alcohol withdrawal syndrome in man.

INTRODUCTION

Gamma-hydroxybutyric acid (GHB), a constituent of the mammalian brain, is found in highest concentrations in the hypothalamus and basal ganglia.[1] Since there are central recognition sites with high affinity for GHB, this compound

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EFFECT OF GHB ON ALCOHOL WITHDRAWAL SYNDROME

Treatment group (no of patients)	Total score					
		After treatment (h)				
	30 min before treatment	1	2	3	5	7
GHB (11)	12·6 (6·1)	7·2 (3·9)*	4·2 (3·1)†	2·1 (1·6)†	1·5 (1·7)†	2·6 (1·3)†
Control (12)	11·8 (5·7)	11·8 (4·7)‡	11·3 (3·5)	12·6 (9·2)	13·6 (6·5)	14·7 (4·3)*

Values are means (SD).
*$p < 0·05$; †$p < 0·01$ (Pratt's test for comparison of scores before and after treatment).
‡$p < 0·05$ (Mann-Whitney test for comparison of control and GHB groups).

probably functions as a neurotransmitter or as a neuromodulator rather than as an incidental metabolite of gamma-aminobutyric acid (GABA).[2] GHB has been used as an intravenous hypnotic anaesthetic agent,[3] and in the treatment of sleep disturbances.[4] In narcolepsy GHB is given orally, at bedtime, to limit the number of rapid eye movement episodes during the night, and this reduces narcoleptic episodes during the day.[5]

In its lactone form, GHB inhibits voluntary ethanol consumption in rats that have a strong preference for ethanol.[6] GHB also suppresses ethanol withdrawal syndrome in rats that have been rendered physically dependent on ethanol by repeated ethanol administration.[7]

These considerations and the safety of GHB[4] led us to study the effect of this drug on alcohol withdrawal syndrome in alcoholics.

PATIENTS AND METHODS

Patients included in the study were alcoholics who met the DSM III-R criteria of alcohol withdrawal syndrome. Patients gave written consent. Patients were excluded if they had convulsions, delirium tremens, or concurrent severe illness, or if they abused other drugs, or were receiving antiepileptic treatment. On admission, patients were clinically examined and randomly allocated to one of two groups; one received 1 dose of oral GHB (50 mg/kg) dissolved in a black cherry syrup, and the other received a corresponding volume of syrup alone (control group). Both preparations were provided by CT, Sanremo, Italy. The patients did not know whether they were receiving GHB or vehicle. The GHB group consisted of 11 patients (8 men, 3 women) and their ages ranged from 31 to 63 years (mean 43·9). The control group consisted of 12 patients (8 men, 4 women) with a mean age of 43·5 years (range 28–59).

Clinical evaluations were done by the same investigator (G. L.) who was blinded to treatment group. On the morning after admission, each patient was examined 30 min before the dose of GHB was given, and 1, 2, 3, 5, and 7 h later. 6 main withdrawal symptoms were evaluated—ie, tremors, sweating, nausea, depression, anxiety, and restlessness. Each symptom was scored on a 4-point scale as follows: 0, not present; 1, mild; 2, moderate; and 3, severe. The sum of these points gave the total score of symptoms for each patient, the maximum being 18 points. Individual alcohol withdrawal symptoms were not compared because they varied greatly between patients. Instead the sum of the scores for each symptom were added together for each patient and the total score was used as an index of severity of withdrawal. Blood pressure and heart rate were also recorded every day. We used the word fluency test of Borkowski et al[8] to look for a possible sedative effect of GHB. Routine laboratory tests were carried out on admission and were repeated if there were any abnormalities. Standard routine therapy (diazepam, vitamins, and sodium valproate) was available for severe distress in both groups of patients, but this was not needed during the double-blind phase.

The Mann-Whitney U-test was used to test differences between the two treatment groups. A modified Wilcoxon test (Pratt's test) was applied for within-patient comparisons.

RESULTS

The mean scores of the two groups before treatment were similar—ie, 12·6 in the GHB group and 11·8 in the control group. In the GHB patients, there was a rapid decrease in mean score with a significant effect within 1 h. Nearly all withdrawal symptoms disappeared within 2 to 7 h of receiving the dose of GHB. By contrast, withdrawal scores of control patients did not decrease, and even significantly increased after 7 h (table). A small decrease in heart rate (10–13%), but no change in blood pressure, was observed after GHB treatment. There were no significant differences in the word fluency test between GHB patients and controls.

After completion of the double-blind phase of the study, the code was broken and control patients were assigned to a conventional treatment schedule, as indicated by their clinical state. Patients in the GHB group received further doses of the drug every 8 h up to the 3rd day. Subsequently, the total daily dose was reduced by 30% per day until the 7th day when GHB was discontinued. The mean withdrawal score of these patients, recorded in open study each morning before the first daily treatment, remained below 2.

7 of the 11 patients treated with GHB said that they had slight and transient dizziness about 30 min after the first drug administration; these symptoms disappeared spontaneously within 15 min. Dizziness with similar features recurred on the second day in 3 patients after the first morning dose of GHB. None of the control group reported dizziness. No other side-effects attributable to GHB were noted by the observer or the patients. None of the patients reported somnolence after GHB.

DISCUSSION

Despite the small number of patients, the results clearly indicated that GHB is effective for the suppression of withdrawal symptoms in alcoholics. GHB action has a rapid onset and seems to be without serious side-effects. Our findings agree with experimental data in rats: therefore, the mechanisms involved in ethanol dependence in rats may be similar to those in human beings. Thus, study of laboratory animals might help to clarify some of the neurochemical mechanisms of ethanol dependence in man.

The protective action of GHB against ethanol withdrawal in our patients was not due to sedative and hypnotic effects. Moreover, the GHB effect cannot be attributable to other central actions of the compound, such as inhibition of dopamine release[9,10] and increase in acetylcholine release,[11] because the mechanisms of these actions are not yet known. The protective effect of GHB may be due to its GABA-like action:[12,13] drugs which are effective clinically or in experimental ethanol withdrawal (eg, benzodiazepines, barbiturates, muscimol, amino-oxyacetic acid, progabide, and ethanol itself)[14-18] all have a direct or indirect GABA-like

action in the central nervous system, which eventually leads to an increase in the chloride transport across the chloride ion channels in the neuronal membrane (see refs 19, 20). Although the above drugs are known to potentiate transmission at the level of $GABA_A$ receptors, the nature of the GABAergic action of GHB is not clear.[21]

Finally, GHB may exert its protective effect by acting on its specific receptors in the brain. This hypothesis raises the important question of the possible role of such receptors in ethanol dependence.

Correspondence should be addressed to G. L. G., Department of Neuroscience, University of Cagliari, Via Porcell n.4, 09124 Cagliari, Italy.

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Oncology

AGE OF ONSET AND TYPE OF LEUKAEMIA

ANNA BUTTURINI[1] ROBERT PETER GALE[2]

Department of Pediatrics, University of Parma, Italy;[1] and Department of Medicine, Division of Hematology-Oncology, UCLA School of Medicine, Los Angeles, California, USA[2]

INTRODUCTION

LEUKAEMIA is a common cancer in people younger than 50 years old, especially children. Several types are described, including acute lymphoblastic leukaemia (ALL), and acute and chronic myelogenous leukaemia (AML and CML). ALL occurs predominantly in young children and adolescents, whereas CML is uncommon in young people (<20 years). AML occurs in infants, adolescents, and older people but not usually in young children.[1] Why do different leukaemias predominate at different ages? If specific leukaemogens cause certain types of leukaemia, and if the influence of these factors correlates with age, distinct leukaemias would be age-associated. An alternative hypothesis is that age at leukaemogenesis determines the type of leukaemia, irrespective of the specific leukaemogenic agent.[2] For example, exposure to the same leukaemogenic factor may cause ALL in a child but AML in an adult. These two hypotheses are not mutually exclusive and both might operate with different leukaemogenic factors.

Since the cause of most cases of leukaemia is unknown, it is difficult to decide between these alternatives. However, there are some instances in which either the cause of leukaemia or the host factors that predispose to leukaemia (other than age) are known. We now review the situations that might point to the pathogenesis of leukaemia.

EXOGENOUS AND HOST RISK FACTORS

Known exogenous causes of leukaemia in human beings are ionising radiation, mutagenic drugs and chemicals, and the HTLV-1 retrovirus.[1] There are several examples of radiation-induced leukaemogenesis, including the atomic bomb survivors, people exposed to diagnostic X-rays in utero, and people who have received radiation for malignant or non-malignant conditions.[3] Data about non-ionising radiation are controversial. The leukaemogenic effects of drugs and chemicals are most evident in people with cancer (usually Hodgkin's disease or ovarian cancer) who are receiving chemotherapy,[3] and in those exposed to benzene.[4] HTLV-1 is associated with the development of adult T-cell leukaemia (ATL) predominantly in Japan but also in other areas.[5] In addition, several host factors increase the likelihood that leukaemia will develop, including congenital disorders associated with chromosomal imbalances or instability such as Down syndrome and Fanconi's anaemia.[6,7]

To see whether age is an important determinant of the type of leukaemia that develops in human beings, we will consider the interaction of exogenous and host risk factors (other than age).

Radiation

Atomic bomb survivors[8] can be grouped into those who were exposed after birth and those who were exposed in utero. There is no evidence of an increased risk of leukaemia in the latter,[9] so we will focus on the former. The incidences of ALL, AML, and CML were all greatly increased in people exposed to radiations from the atomic bombs; the relative risks of getting leukaemia were 20 to 25-fold and were highest in those who were the youngest at the time of exposure. Also, young people had the shortest latent period before developing leukaemia. However, these data do not point to any correlation between age at exposure and type of

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Gamma-Hydroxybutyric Acid in the Treatment of Alcohol Dependence: A Double-Blind Study

Luigi Gallimberti, Mila Ferri, Santo Davide Ferrara, Fabio Fadda, and Gian Luigi Gessa

The effect of gamma-hydroxybutyric acid on alcohol consumption and alcohol craving in alcoholics was investigated in a randomized double-blind study versus placebo. Patients were treated as outpatients during a three month period either with gamma-hydroxybutyric acid (50 mg/kg/day, divided into three daily doses) or with placebo. Of the 82 alcoholics that entered the study, 71 completed it, 36 in the gamma-hydroxybutyric acid and 35 in the placebo group. Alcohol consumption was assessed by the subject's self report. At the 3rd month of treatment, 11 patients in the gamma-hydroxybutyric acid group referred to be abstinent and 15 referred controlled drinking; while in the placebo group only two and six patients referred abstinence and controlled drinking, respectively. Serum-gammaglutamyltransferase activity correlated with the admitted alcohol consumption. Gamma-hydroxybutyric acid treatment decreased alcohol craving during the 3 months of treatment. Transient side effects were noted by six patients on gamma-hydroxybutyric acid and two on placebo. The results suggest that gamma-hydroxybutyric acid may be useful in the treatment of alcohol dependence.

Key Words: GHB, Alcohol Dependence, Craving.

GAMMA-HYDROXYBUTYRIC ACID (GHB), a normal brain constituent originating from GABA metabolism, is considered to play a neurotransmitter and/or neuromodulatory role in the central nervous system (CNS).[1] Systemically administered, GHB exerts hypnotic and anesthetic effects in animals and in man, therefore it was introduced in the clinic as a general anesthetic and hypnotic agent.[2]

The mechanism by which GHB produces its central effect is not known. It has been suggested that GHB enhances GABAergic activity,[3] although evidence for a direct interference of GHB with GABA transmission is not available. Previous results from our laboratory have shown that GHB, in its lactone form, inhibits voluntary ethanol consumption in a rat line selectively bred for high preference for ethanol,[4] and that GHB suppresses ethanol withdrawal syndrome in rats rendered physically dependent on ethanol by forced ethanol administration.[5]

More recently in a double-blind study we found that GHB, given orally in nonhypnotic doses, is highly effective in suppressing the withdrawal symptomatology in alcoholics; the GHB effect has a rapid onset and the compound is devoid of adverse side effects.[6]

The above considerations and the relative safety of GHB led us to study the clinical efficacy of the compound in the treatment of alcohol dependence.

METHODS

Eighty-two alcoholic patients entered the study sequentially over a 1-year period after giving informed consent. Each patient was studied for 3 months in a double-blind versus placebo trial. Patients included in this study had a 5-year or more history of alcoholism defined according to the DMS III-R criteria. They had an average daily ethanol intake in excess of 150 g for the past 2 years or more. Exclusion criteria were a major psychiatric disorder other than alcohol dependence, cirrhosis of the liver, pregnancy, renal or heart failure, epilepsy. Within 8 hr of admission to the day-hospital, each subject underwent a full physical examination by a physician of the team and routine laboratory tests, including serum-gammaglutamyltransferase (S-GT), erythrocyte mean cell volume (E-MCV), and alcoholuria. Thereafter each subject was examined for depression, anxiety, and severity of alcoholism according to the rating scales reported in Table 1; each subject was randomly assigned to the drug or placebo group.

The active medication consisted of GHB dissolved in a black cherry syrup, in the concentration of 250 mg/ml. Placebo consisted of a cherry syrup with the same organoleptic characteristics as the active medication. GHB was administered orally at the dose of 50 mg/kg divided into three daily doses. The placebo group received the same volume of the syrup. Both the active medication and the placebo syrup were supplied by CT Laboratories, Sanremo, Italy.

Patients received the first medical interview and treatment in the day-hospital; thereafter they were followed up as outpatients and seen every day from 8 AM to 5 PM for the first 3 days and then at weekly intervals. Subjects were told by the physician that the goal of the treatment was to assess whether a drug that they were going to receive was able to decrease alcohol withdrawal symptomatology and alcohol craving, that they should try to abstain from alcohol ingestion, but that this was not mandatory for the study. The latter was aimed at assessing the real efficacy of the drug and any possible side effects.

At the weekly visit subjects provided a urine sample for measurement of alcohol concentration[7] and were interviewed by one of the physicians (L.G. or M.F.) about their alcohol intake and the intensity of alcohol craving. A self-reported alcohol intake was recorded as the mean number of standard drinks consumed per day and the percentage of days of abstinence.

Craving for alcohol was defined as the preoccupation with, thought about, and urge for alcohol. The intensity of alcohol craving was assessed with a questionnaire derived, with proper modifications, from Stunkard and Messick's questionnaire to measure dietary restraint, disinhibition, and hunger.[8] The questionnaire contained 11 items, each of which required a yes or no answer, corresponding to 1 or 0 points, respectively; therefore, the maximum craving score was 11 points.[9]

The self-reported alcohol consumption was correlated with the weekly measurements of alcoholuria and by any possible information obtained

From the Addiction Medicine Unit, USSL 21, Padova, Italy (L.G., M.F.); Centre of Behavioural and Forensic Toxicology, University of Padova, Italy (S.D.F.); "Bernard B. Brodie" Department of Neuroscience, University of Cagliari, Italy (G.L.G., F.F.).

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Reprint requests: Prof Gian Luigi Gessa, "Bernard B. Brodie" Department of Neuroscience, University of Cagliari, Via Porcell 4, 09124 CAGLIARI, Italy.

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from collaterals of the patients. Other laboratory tests were repeated at monthly intervals.

Physicians who performed treatments and medical interviews were unaware of the treatment administered.

RESULTS

Of the 82 subjects that entered the study, 71 completed the course. Of the 11 subjects who withdrew from the study, four did so for lack of compliance (in failing to report at one weekly visit: three in the placebo and one in the GHB group), three for dizziness and vertigo and one for headache (in the GHB group), one for gastric ulcer exacerbation, one for refusal to take the treatment, and one for nausea (in the placebo group). The relatively high percentage of subjects remaining in the study may be attributed to the intensive follow-ups. The placebo group consisted of 35 subjects, 24 men and 11 women; while the GHB group numbered 36 subjects, 23 men and 13 women. The two groups did not differ in age, initial S-GT, and E-MCV. In fact, the GHB group was aged 41 ± 15 years, S-GT 115 ± 108, E-MCV 97 ± 8 fl; while the placebo group was aged 40 ± 13 years, S-GT 118 ± 112, E-MCV 98 ± 6 fl (means ± SD).

As shown in Tables 1 and 2, subjects in the two groups did not differ for severity of alcoholism or alcohol intake, they were not depressed and their level of anxiety was relatively low.

Table 1. Characteristics of Subjects Undergoing Treatment with Placebo or Gamma-Hydroxybutyric Acid (GHB)

Variable (mean ± SD)	Subjects to receive		
	Placebo	GHB	*p*
Males	24	23	
Females	11	13	
Age	36.8 ± 15.6	38.1 ± 13.4	NS*
Years of alcoholism (DMS-III-R)	6.7 ± 4.9	7.1 ± 5.1	NS
VAST score†	26.4 ± 11.3	28.2 ± 14.7	NS
Anxiety score‡			
State	43.6 ± 11.4	48.4 ± 16.3	NS
Trait	45.3 ± 13.1	46.9 ± 10.2	NS
Depression score§	6.1 ± 5.8	5.3 ± 6.1	NS

p, Student's *t* test.

* NS, not significant.

† VAST, Veterans Alcoholism Screening Test.[16] The scores reported are referred to the past year.

‡ Spielberger's State and Trait Anxiety Scale scores range from 20 = no anxiety to 80 = extreme anxiety.[17]

§ Hamilton Depression Scale scores range from 0 = no depression to 60 = extreme depression.[18]

Table 2 shows the effect of GHB treatment on ethanol consumption, assessed as the mean number of drinks consumed per day and the percentage of days of abstinence. During the 3-month treatment period, in the placebo group there were no significant variations in both the number of daily drinks and in the abstinent days. On the other hand, the GHB-treated patients showed a decrease to about one half in the number of daily drinks and a 3-fold increase in the number of abstinent days.

As Table 3 shows, GHB significantly reduced alcohol craving. This effect was present within the 1st month of treatment and persisted throughout the treatment period. Placebo treatment produced a modest reduction in craving during the 1st month of treatment.

At the end of the 3rd month of treatment, on the basis of the self-reported ethanol consumption during the previous month, the subjects were assigned to one of three categories: abstinence, controlled drinking, and excessive drinking. Controlled drinking and excessive drinking were defined when the subject admitted ethanol consumption of less and more than 40 g/day, respectively.

As shown in Table 4, 11 and 15 out of the 36 subjects reported abstinence and controlled drinking in the GHB-treated group. On the other hand, in the placebo-treated group only two and six out of the 35 subjects showed abstinence and controlled drinking, respectively, while 27 subjects reported excessive drinking.

As Table 3 shows, S-GT values correlated with the admitted alcohol consumption. On the other hand no significant differences were observed in the E-MCV values before treatment and in the follow-up within groups as well as between the two groups. The alcoholuria correlated with the admitted alcohol consumption (data not shown) and the validity of the self report of alcohol intake was confirmed by information obtained from the patient's collaterals.

Adverse side effects were investigated using a standard questionnaire. Four of the patients on GHB and one on placebo complained of dizziness and vertigo after the first morning dose on the first 3 days of treatment, the symptomatology was transient, disappearing within 6 hr. Two patients on GHB and one on placebo complained of headache after the first morning dosage persisting for 3 to 4 hr. This symptomatology disappeared following the 3rd day of treatment.

No subject showed alterations in the renal, blood, and

Table 2. Effect of Gamma-Hydroxybutyric acid (GHB) on Ethanol Consumption in Alcoholics

Response	During the 3 months before treatment			During the 3 months of treatment period		
	Placebo	GHB	*p*	Placebo	GHB	*p*
Daily drinks (mean ± SEM)	11.4 ± 0.6	12.1 ± 0.5	NS	9.3 ± 0.7	4.7 ± 0.4	<0.01
% of abstinent days (mean ± SEM)	4.9 ± 0.4	5.6 ± 0.5	NS	8.4 ± 1.6	25.9 ± 3.1	<0.001

Each value is the mean ± SEM from 35 placebo and 36 GHB treated subjects. Values of alcohol intake prior to treatment were based on a single interview, while those during treatment were obtained by weekly interviews (means ± SEM). Therefore, the statistical significance of the results was calculated by comparing the values of GHB versus placebo, during the 3-month treatment period (Student's *t* test).

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Table 3. Effect of Gamma-Hydroxybutyric Acid on Ethanol Craving

Month of treatment	Craving score	
	Placebo	GHB
Prior to treatment	8.5 ± 0.3	8.9 ± 0.5
1st month	5.1 ± 0.6†	2.1 ± 0.1†
2nd month	7.5 ± 0.4	3.3 ± 0.4*,†
3rd month	7.6 ± 0.3	3.1 ± 0.6*,†

Data are the means ± SEM obtained by averaging the scores of the 4 weekly interviews during each month. Baseline scores were those of the first visit prior to treatment (maximum score 11). * $p < 0.001$ with respect to placebo value, † $p < 0.001$ with respect to basal value by Student's t test.

In consideration of the multiple comparisons, in order to protect against false-positive results, level of significance was fixed as follows $/n = 0.05/9 = 0.0055$; therefore, vaues of $p > 0.0054$ were considered statistically not significant.

Table 4. Correlation of Gamma-Hydroxybutyric Acid Effect on Alcohol Consumption, Serum Gamma-Glutamyltransferase (S-GT) and Erythrocyte Mean Cell Volume (E-MCV)

Condition of patients	Placebo			GHB		
	N	S-GT (I.U./L)	E-MCV (fl)	*N*	S-GT (I.U./L)	E-MCV (fl)
Before treatment						
Excessive drinking	35	118 ± 112	98 ± 6	36	115 ± 108	97 ± 8
Last month of treatment						
Abstinence	2	33, 48	97 ± 4	11	31 ± 38*	94 ± 4
Controlled drinking	6	53 ± 41*	98 ± 7	15	48 ± 61*	90 ± 8
Excessive drinking	27	118 ± 110	103 ± 6	10	113 ± 131	98 ± 7

Values are means ± SD.

Controlled and excessive drinking: admitted ethanol consumption during the preceding month period of less and more than 40 g/day, respectively. *N*, number of patients.

* $p < 0.01$ with respect to pretreatment value (Student's t test).

liver tests. Blood pressure and pulse rate did not change significantly after either placebo or GHB treatment. Scores for depression and anxiety did not change significantly either in the placebo or GHB-treated patients during the 3-month treatment period (results not shown).

COMMENT

The present study shows that GHB is effective in reducing ethanol consumption and ethanol craving in alcoholics. Ethanol consumption was assessed as the patient's self report and the reduction was measured as a reduction both in the number of drinks per day and in the percentage of days of abstinence during the 3-month treatment period.

Moreover, the combination of the number of patients reporting abstinence and those reporting controlled drinking was considered to be an indicator of the treatment's success. According to this parameter, the success score with GHB was higher than 70% of the subjects at the end of the 3rd month of treatment, while it was about 20% with placebo. The validity of self-reported data was supported by laboratory tests (S-GT) and weekly urine analyses for alcohol.

It is likely that GHB-induced reduction in ethanol consumption is the consequence of its reducing effect on alcohol craving. The latter effect is consistent with previous observations showing that the compound is effective in suppressing the ethanol withdrawal syndrome in alcoholics[6]; craving being considered a symptom of protracted abstinence[10] and the major stimulus for relapses into ethanol abuse. The finding that GHB inhibits ethanol craving suggests a possible association of the drug with disulfiram, which is known to prevent ethanol consumption by a negative reaction but fails to reduce craving.

As mentioned above, we found that GHB suppresses voluntary ethanol consumption in rats selected for high ethanol preference[4] and reduces ethanol withdrawal syndrome in rats physically dependent on ethanol.[5] Therefore, our clinical results are not only of practical, but also of general theoretical interest, since they stress the predictive relevance of the experimental model for clinical research.

Experimental studies suggest that GHB administration interferes with the activity of dopamine,[11] serotonin,[12] acetylcholine,[13] opioids,[14] and GABA.[15] At present it is unknown which of these interactions bears some relevance for the suppressant effect on ethanol consumption and craving.

Moreover, since GHB is a normal brain constituent and has many of the characteristics of neurotransmitter and/or neuromodulator,[1] the possible relevance of changes in the content and activity of endogenous GHB in the pathogenesis of alcoholism might be considered.

Finally, the possibility exists that GHB might act by mimicking the central effects of ethanol. Indeed, ethanol moiety is present in the structure of GHB and the latter shares with ethanol different pharmacological and neurochemical characteristics. Moreover tolerance to ethanol is extended to GHB.[5] Should the latter hypothesis be validated, the rationale for using GHB in the treatment of alcoholism would be the same as that of using methadone in heroin addiction.

Whatever the exact mechanism of action of GHB, our results indicate that GHB deserves more extensive investigation as a clinically useful drug in the treatment of alcoholism.

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ANNOUNCEMENT

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Gamma-Hydroxybutyric Acid for Treatment of Opiate Withdrawal Syndrome

Luigi Gallimberti, M.D., Mauro Cibin, M.D., Piero Pagnin, M.D., Roberta Sabbion, M.D., Pier Paolo Pani, M.D., Roberto Pirastu, M.D., Santo Davide Ferrara, M.D., and Gian Luigi Gessa, M.D.

In a double-blind placebo-controlled trial, gamma-hydroxybutyric acid (GHB) (25 mg/kg orally) suppressed most of the withdrawal symptomatology in 14 heroin addicts and 13 methadone-maintained subjects. The GHB effect was prompt (within 15 minutes) and persisted for between 2 and 3 hours. Subsequently, the same patients received GHB in an open study every 2 to 4 hours for the first 2 days and 4 to 6 hours for the following 6 days: most abstinence signs and symptoms remained suppressed and patients reported felling well. Urine analysis failed to detect any presence of opiate metabolites. No withdrawal symptomatology recurred after 8 days of treatment when GHB was suspended, and patients were challenged with an intravenous injection of 0.4 mg naloxone. The results indicate that GHB may be useful in the management of opiate withdrawal. [Neuropsychopharmacology 9:77–81, 1993]

KEY WORDS: *Gamma-hydroxybutyric acid; Heroin; Methadone; Opiates; Withdrawal syndrome; Opiate dependence*

Gamma-hydroxybutyric acid (GHB), an endogenous constituent of the mammalian brain, is found in the highest concentrations in the hypothalamus and basal ganglia (Snead and Morely 1981). Because there are specific high-affinity binding sites for GHB in the central nervous system (CNS), and the compound is located principally in synaptosomes—from which it is released in a Ca^{2+}-dependent process—GHB is considered to function either as a neurotransmitter or neuromodulator rather than as an incidental metabolite of gamma-aminobutyric acid (GABA) (Mandel et al. 1987).

Gamma-hydroxybutyric acid has been used as an intravenous hypnotic and anesthetic agent (Mamelak et al. 1977) and in the treatment of narcolepsy. In the latter condition, GHB is given orally at bedtime to improve nocturnal sleep quality, thus reducing cataplexy episodes during the day (Mamelak et al. 1986). Previous work in animals has shown that GHB both inhibits voluntary ethanol consumption in rats having a strong preference for ethanol and suppresses the ethanol withdrawal syndrome in rats physically dependent on ethanol (Fadda et al. 1989a, 1989b).

More recently, in a double-blind study we found that GHB, given orally in nonhypnotic doses, is highly effective in suppressing the withdrawal symptomatology in alcoholics; the GHB effect being rapid and devoid of negative side effects (Gallimberti et al. 1989). By using GHB in the management of the withdrawal syndrome in a number of alcoholics who concomitantly abused heroin, we observed that GHB not only suppressed the alcohol withdrawal symptomatology

From the Addiction Treatment Service (LG, RS) (SERT, USL 21), Padova; Neurotoxicology Unit, General Hospital of Dolo (MC), Venice; Third Medicine Division, General Hospital of Padova (PP), Padova; Centre of Behavioural and Forensic Toxicology, IML, University of Padova (SDF), Padova; and Bernard B. Brodie Department of Neuroscience, University of Cagliari and Addiction Treatment Service (PPP, RP, GLG) (SERT, USL 21), Cagliari, Italy.

Address correspondence to Prof. Gian Luigi Gessa, Bernard B. Brodie Department of Neuroscience, University of Cagliari, Via Porcell 4, I-09124 Cagliari, Italy.

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but, even more effectively, that of heroin. The present study was undertaken to clarify, in a double-blind condition, whether GHB was effective in suppressing the withdrawal syndrome in heroin- and methadone-dependent subjects.

SUBJECTS AND METHODS

The subjects participating the study were 22 male heroin users, with a mean age of 27.3 years (range 22 to 33 years), with a clear history of daily use of heroin for over 3 years (3 to 6 years) claiming a high degree of opiate dependence; and 19 male subjects, with an age ranging from 24 to 31 years (mean 28 years) undergoing a methadone maintenance treatment program at the Addiction Treatment Service (SERT, USL 21), Cagliari.

Methadone-maintained subjects were receiving a stabilized dose of 30 to 60 mg/day of methadone for at least 6 months prior to hospitalization. All subjects were interested in discontinuing opiate consumption and gave informed consent to the study. The present study required hospitalization for a period of 8 days. Subjects were hospitalized on the morning of the day following their last consumption of opiates. On admission each subject underwent a medical and psychiatric examination, routine laboratory tests, urine screening analysis for opiate metabolites, amphetamine, cocaine, benzodiazepines, barbiturates, cannabinoids, and alcohol.

The first test revealed that all heroin-dependent subjects had urinary opiate metabolites. All subjects under methadone maintenance presented methadone metabolites, three of whom revealed also other opiate metabolites. Small concentrations of alcohol and benzodiazepine metabolites were found in the urine of both groups of patients. After admission, methadone-maintained subjects were left with no opiate administration for 24 hours and the rating of withdrawal symptomatology was started at 8 AM on the second day of hospitalization. Fifteen of the 22 heroin-dependent subjects showed overt signs of abstinence on the morning of the day of admission; therefore, the rating of withdrawal symptomatology for these subjects was made on the morning of the same day of admission. The other seven heroin-dependent subjects, who showed objective signs of abstinence after 6 PM on the day of admission, received 1 or 2 intramuscular doses of 15 mg of morphine to ease their discomfort (no morphine was given after midnight) and their withdrawal symptomatology was scored on the second day of hospitalization starting at 8 AM as for methadone-maintained subjects.

Withdrawal symptomatology was evaluated by one of the investigators (either RP or PPP) blind to the treatment conditions. Twenty-one items associated with the withdrawal syndrome were rated as present (1) or absent (0), according to Gold et al. (1978); the maximum score attainable was 21. Symptoms considered included: craving, nausea, anorexia, anxiety and restlessness, aching bones and muscles, insomnia or yen sleep, and hot and cold flashes. Signs considered comprised: tremors, yawning, vomiting, diarrhea, perspiration, lacrimination, rhinorrhea, increased respiration rate and depth, goose flesh, mydriasis, spontaneous orgasm, increased temperature, tachycardia, and increased blood pressure.

Withdrawal scoring was made every 30 minutes for 3 hours prior to treatment (baseline period) and at various times afterwards, as indicated in the Results Section. The experiment was randomized and double blind; subjects, nurse, and physician observer were unaware of the substance administered. Active medication, consisting of GHB 17% solution in a black cherry syrup, and an identical placebo were provided by CT (San Remo, Italy). Subjects took GHB orally at the dose of 25 mg/kg or the placebo syrup. Treatments were given by a nurse who was not otherwise involved in the study. The Mann-Whitney U-test was used to test differences between placebo and GHB treatment. A modified Wilcoxon test (Pratt's test) was applied for within-patient comparison.

RESULTS

All methadone- and heroin-dependent subjects showed signs and symptoms of abstinence that increased during the 3 hours preceding treatment. Table 1 shows the reported withdrawal scores obtained during the 30 minutes preceding treatment and at various times after treatment. The administration of GHB (25 mg/kg) reduced most of the withdrawal signs and symptoms both in heroin- and methadone-dependent subjects. The GHB effect had a rapid onset but short duration. Thus, the global withdrawal score was significantly reduced within 15 minutes and remained maximally suppressed between 30 minutes and 2 hours after treatment. At the third hour, withdrawal scores tended to increase once again. Table 2 shows the effect of GHB on specific items of the opiate withdrawal scale. It appears that GHB was effective in reducing all the signs and symptoms of opiate withdrawal, except for diarrhea and insomnia, which were resistant to the GHB effect. All patients on GHB referred to relief of subjective distress. Placebo had no significant effect on any of the withdrawal items; therefore, at the end of the double-blind trial, these patients were assigned to a more conventional detoxification treatment with clonidine or methadone.

On the other hand, patients on GHB continued

Table 1. Effect GHB on Opiate Withdrawal Syndrome

Drug Withdrawn (No. Subjects)	Treatment	Before Treatment	Total Withdrawal Score (Minutes After Treatment)						8th Day of Treatment[d]	
			15	30	60	90	120	180	Prior to Naloxone	After Naloxone
Heroin (14)	GHB	15.1 ± 1.5	4.8 ± 0.9[a]	2.5 ± 1.9[a]	2.1 ± 0.8[b]	1.6 ± 1.2	1.3 ± 0.1[b]	5.1 ± 1.9[b]	1.2 ± 0.3	2.3 ± 0.2
Heroin (8)	Placebo	16.7 ± 1.0	15.3 ± 1.8[c]	13.7 ± 2.1[c]	18.1 ± 2.1[c]	16.1 ± 2.4[c]	20.0 ± 3.1	19.3 ± 2.1[c]		
Methadone (13)	GHB	13.4 ± 2.0	5.1 ± 2.3[a]	2.8 ± 2.4[a]	1.4 ± 1.6[b]	2.1 ± 2.1	1.9 ± 1.2[b]	3.3 ± 1.3[b]	2.1 ± 0.2	2.2 ± 0.1
Methadone (6)	Placebo	12.3 ± 1.7	12.7 ± 1.4[c]	13.4 ± 1.2[c]	12.7 ± 2.5[c]	15.2 ± 1.1[c]	16.1 ± 1.8[c]	15.9 ± 1.7[c]		

[a] $p < .01$; [b] $p < .001$ (Pratt's test for comparison of scores before and after treatment).
[c] $p < .01$ (Mann-Whitney test for comparison of placebo and GHB group).
[d] Tested 30 minutes prior to and 10 minutes after naloxone, 0.4 mg (IV).
Total withdrawal scores are mean ± SEM.

this treatment receiving the drug in an open study every 2 to 4 hours on the first 2 days and every 4 to 6 hours the following 6 days, as indicated by the pharmacokinetics of GHB (Lettieri and Fung 1979, Ferrara et al. 1992). Every day, withdrawal signs and symptoms were recorded before each dose. The withdrawal score remained reduced throughout the trial. On the eighth day, GHB administration was suspended, subjects were observed during a period of 5 to 6 hours, and then received an intravenous injection of 0.4 mg of naloxone. No withdrawal signs and symptoms occurred before and after naloxone treatment (see Table 1). Urine analysis performed every other day during the trial failed to detect the presence of opiate metabolites. Three methadone-dependent and two heroin-dependent subjects reported transient dizziness or vertigo on the second and/or third day following the first morning dose of GHB; these symptoms were usually well tolerated. No other side effects attributable to this compound were noted either by the observer or the subjects themselves. None of the subjects reported somnolence after GHB.

DISCUSSION

The standard method used in the management of heroin withdrawal is the substitution of methadone for heroin and gradual dose reduction until complete abstinence is obtained. More recently the alpha-2 agonist, clonidine, has been introduced to facilitate withdrawal from opioids. Although clonidine is effective in reducing many of the autonomic components of the withdrawal syndrome, it fails to suppress its subjective components, such as craving, lethargy, insomnia, and restlessness. Moreover, it is not uncommon for clonidine to produce major side effects, such as sedation and severe hypotension (see Jaffe 1987). Consequently, finding a nonopioid drug capable of suppressing withdrawal symptomatology without causing negative side effects is of prime importance. Our results indicate that GHB is effective in suppressing the opiate withdrawal syndrome in humans. The GHB effect has a rapid onset but short duration. These features are in accordance with the pharmacokinetics of GHB, indicating that the compound is readily absorbed after oral administration and is rapidly eliminated (Lettieri and Fung 1979; Ferrara et al. 1992). Due to the short duration of its effect, GHB should be administered at frequent intervals. This is a limitation of an otherwise effective and well-tolerated compound.

The mechanism by which GHB suppresses opiate withdrawal syndrome is not known. A number of mechanisms may be invoked but none can be convincingly accepted. It is possible to discount the sedative effect of the compound because no sedation was pro-

Table 2. Effect of GHB on Specific Signs and Symptoms on Gold's Withdrawal Scale

Sign/Symptom	Percent of Subjects Presenting a Given Sign/Symptom at Various Times After GHB		
	Before Treatment	1 Hour	3 Hours
Craving	100	26	37
Nausea	89	15	23
Anorexia	59	33	21
Anxiety/restlessness	92	46	58
Aching bones and muscles	96	38	31
Insomnia or yen sleep	96	70	83
Hot and cold flashes	74	18	22
Tremors	71	15	7
Yawning	81	0	0
Vomiting	18	0	0
Diarrhea	51	43	53
Perspiration	78	0	0
Lacrimation	52	0	0
Rhinorrhea	39	0	0
Increased respiration rate and depth	63	0	0
Goose flesh	71	15	28
Mydriasis	50	0	0
Spontaneous orgasm	0	0	0
Increased temperature	41	8	13
Tachycardia	85	15	21
Increased blood pressure	96	15	30

Data for heroin- (n = 14) and methadone- (n = 13) dependent subjects were pooled. The 21 items of the scale were rated as present (1) or absent (0) according to Gold et al. (1978).

duced by those doses of GHB suppressing opiate withdrawal. Experimental evidence indicates that GHB interferes with the activity of serotonin (Spano and Przegalinski 1973), acetylcholine (Sethy et al. 1976), GABA (Snead and Nichols 1987), and dopamine (DA) (Gessa et al. 1968) in the CNS. The interference of GHB with DAergic transmission might be more relevant for its suppressant effect on the withdrawal syndrome. Indeed, recent studies have shown that both ethanol and morphine withdrawal syndromes are associated with profound inhibition of DA output in the nucleus accumbens and in the ventral caudate nucleus, as measured by brain microdialysis (Rossetti et al. 1990, 1991; Acquas et al. 1991).

Contrary to what was observed following anesthetic doses of GHB (Walters et al. 1973), we have recently found that nonanesthetic doses of the compound activate the firing rate of DAergic neurons (Diana et al. 1991), and Cheramy et al. (1977) have reported that GHB increases DA release from the caudate nucleus of cats. Because the increase in DA output is considered to play an important role in the rewarding effects of morphine and alcohol (Di Chiara and Imperato 1988), it is reasonable to hypothesize that the fall in DA output might be involved in the negative symptoms of withdrawal and, vice versa, a stimulation of DA output might be implicated in the suppressant effect of GHB on withdrawal symptomatology.

Alternatively, it should also be considered that GHB is a normal brain constituent, which seems to function as a neurotransmitter or neuromodulator (Mandel et al. 1987). Therefore possible changes in the endogenous content and activity of this compound in the pathogenesis of withdrawal from opiates and alcohol are worth specific investigation. Whatever the mechanism of action of GHB, its efficacy in suppressing both opiate and alcohol withdrawal syndrome is of practical importance, because a combination of opiate and alcohol abuse is not uncommon. Results in preparation from our group have shown that GHB prevents the emergence of opiate withdrawal signs and symptoms evoked by naloxone in opiate-dependent patients.

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ORIGINAL PAPER

Luigi Gallimberti · Fabrizio Schifano · Giovanni Forza
Lorella Miconi · S. Davide Ferrara

Clinical efficacy of gamma-hydroxybutyric acid in treatment of opiate withdrawal

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Abstract This paper describes the role of gamma-hydroxybutyric acid (GHB) in the treatment of opiate withdrawal syndrome. In the two patients described, after having abruptly withdrawn from long-term methadone treatment, GHB was orally administered (each dose given every 4–6 h) for 8–9 days. The GHB showed both a high efficacy (some mild and transient symptoms attributable to opiate withdrawal were observed, but only in the first days of therapy) and a good tolerability (no clinical phenomena interpreted as GHB side effects were found). These results could be of interest in improving the pharmacological treatment of drug addiction.

Key words Gamma-hydroxybutyrate · Opiate dependence · Opiate withdrawal · Drug addiction

Introduction

In opiate addiction pharmacological treatment of withdrawal symptoms is often the first approach. The more effective this initial treatment is and the fewer its side effects, the more the patient will be induced to continue treatment. Gamma-hydroxybutyric acid (GHB) is a normal component of the nervous system in mammals. It was first marketed in Italy (under the name of Alcover, by Laboratory C.T. of San Remo) in 1992 as a sodium salt. Preclinical pharmacological studies had shown that in its lactone form GHB inhibits voluntary ethanol consumption in a rat line selectively bred for high preference for ethanol, and that GHB suppresses ethanol withdrawal syndrome in rats made physically dependent on ethanol by forced ethanol administration [1–3].

In humans these results were supported by our group. In a randomized double-blind study [4] a sample of patients showing alcohol-withdrawal syndrome were treated either with GHB (150 mg/kg/day in a syrup preparation; 11 patients) or with a syrup placebo (12 patients). The GHB treatment led to a prompt reduction in withdrawal symptoms such as tremor, sweating, nausea, depression, anxiety and restlessness. The only side effect was dizziness.

More recently the effect of GHB on alcohol consumption and craving in alcoholics was investigated in a randomized double-blind study [5]. A total of 71 outpatients completed the 3-month trial, either with GHB (50 mg/kg/day) or placebo. During the 3-month treatment period, in the placebo group there were no significant variations in either the number of daily drinks or in the abstinent days. The GHB-treated patients, on the other hand, showed a decrease by one-half in the number of daily drinks, and a 3-fold increase in the number of abstinent days. The GHB treatment significantly decreased alcohol craving during the 3 months of treatment. Transient side effects (dizziness and headache in the first days of treatment) were noted by a few patients on GHB.

Some of our previous (unpublished) observations on patients with both alcohol and multiple-drug abuse were so encouraging that we wished to try out GHB treatment on opiate-dependent subjects. This paper reports two clinical cases in which GHB treatment was effective in controlling opiate-withdrawal syndrome. In Italy GHB may only be given to alcohol addicts, therefore a special authorization was obtained from the Italian Ministry of Health for the prescription of GHB to opiate addicts. Both patients were informed about this experimental-drug trial and gave their informed written consent.

Patients and methods

Case 1

The first patient, a 30-year-old unmarried female, began injecting heroin at the age of 15 years. On many occasions she had unsuc-

L. Gallimberti
Addiction Treatment Unit, Via Giustiniani, 2, I-35122 Padua, Italy
F. Schifano (✉)* · L. Miconi
Addiction Treatment Unit, I-35143 Padua, Italy
* SerT, Via dei Colli 4, I-35143 Padua, Italy
G. Forza
Addiction Treatment Unit, Viale Spellanzon, 55, I-31015 Conegliano (TV), Italy
S. D. Ferrara
Centre of Behavioural and Forensic Toxicology, University of Padua, Via Giustiniani, 2, I-35122 Padua, Italy

cessfully tried to reduce or suspend the use of opiates and had marked tolerance to them. Moreover, in early adolescence she had often been truant from school, told lies and stole. On examination she was found to be impulsive, could not hold a job, was irritable and aggressive and had frequently come into conflict with the law. According to the DSM-III-R she fit the diagnostic criteria for opiate addiction and antisocial-personality disorder. Since January 1991 she had been on methadone (mean dose 60 mg/day) with only partially successful results: Urine tests showed the frequent presence of morphine. She was then hospitalized for "washing out" preparatory to entering a residential therapeutic community.

Approximately 25 h after the first dose of methadone (60 mg) withdrawal symptoms of moderate severity appeared (gooseflesh, sweating, psychomotor agitation, alternate hot flushes and cold sweats, muscle contraction and mydriasis), scoring 30 on Wang's scale [6]. She was given an initial GHB dose of 50 mg/kg, and 15 min later all symptoms had completely disappeared (score: 0 on Wang's scale; overall feelings of subjective well-being). This dose was repeated every 4 h for 7 days, and then every 6 h for 2 days. The GHB treatment was suspended on the 9th day, and a naloxone test (0.4 mg i.v.) was given: No symptoms were observed. The patient began naltrexone therapy (50 mg/day orally) on that day and was discharged in good physical condition, suitable for entry into the therapeutic community. During her hospital stay she never reported particularly important withdrawal symptoms or fits of opiate craving, as measured daily by a 10-cm visual analogue scale (where 0 was absence of craving and 10 its maximum), except on one occasion (day 3 of GHB therapy, 2 h after a dose), when indomethacin had to be injected i.m. for extensive muscle pain. Urine tests during hospitalization never revealed any intake of psychoactive substances extraneous to the treatment procedure.

Case 2

The second patient, a 24-year-old male, began using heroin continuously at the age of 19 years. Since July 1989 he had spent most of his time trying to obtain the drug, had greatly reduced his social and work activities, and had marked tolerance. According to the DSM-III-R he fit the diagnostic criteria for opiate addiction, but did not meet any diagnostic criteria on axis 2. Since January 1992 he has been on methadone (fixed dose of 50 mg/day), and urine tests have occasionally been positive for morphine, but not for other psychotropic substances. He was hospitalized for a brief "washing out" preparatory to entering a residential therapeutic community.

Approximately 30 h after the last dose of methadone, when withdrawal symptoms appeared (sweating, psychomotor agitation, gooseflesh, extensive muscle pain, diarrhea and abdominal cramps of moderate severity; score of 25 on Wang's scale), an initial GHB dose of 30 mg/kg was given. Approximately 20 min later all withdrawal symptoms had completely disappeared (score: 0 on Wang's scale; overall sensation of subjective well-being and no craving for opiates). This dose was repeated every 4 h. Two days after the beginning of GHB treatment three evacuations (semiliquid faeces) were observed, but no antidiarrhea treatment had to be given. During his hospital stay no fits of opiate craving (measured as in case 1) or other disturbances linked to opiate withdrawal symptoms were observed. The GHB treatment was suspended on the 8th day of therapy and a challenge test with naloxone (0.4 mg i.v.) was given: No symptoms were observed. During the entire hospital stay urine tests did not reveal any opiates or other substances of abuse. The patient was then discharged and entered the therapeutic community in good physical condition.

Discussion

The previously mentioned clinical cases indicate that GHB can satisfactorily control withdrawal symptoms and opiate craving, both frequent causes of early drop-out during hospitalization. Treatment with GHB, with careful consideration of its pharmacokinetic properties [7], thus deserves attention, because its control over withdrawal symptoms and craving is not associated with the side effects often produced by other drugs more frequently used on a clinical basis (e.g. clonidine causes hypotension; benzodiazepines and neuroleptics have marked sedative effects; nonsteroid analgesics cause gastric pain). With regard to the dosage for the two patients described (300 mg/kg/day, subsequently reduced to 200 mg/kg/day in case 1, and 180 mg/kg/day in case 2) it seems that under our controlled clinical use the same dose of GHB caused different responses in different patients and different responses in the same person at different times. Again, it must be emphasized that the dosage that can satisfactorily control opiate withdrawal symptoms seems, from these preliminary observations, higher than the dose (150 mg/kg/day) needed to control alcohol-withdrawal symptoms [4].

There could be doubts as to the possible abuse liability of GHB. In this respect, the United States Food and Drug Administration (FDA) issued an advisory warning that GHB use outside of FDA-approved physician-supervised protocols was unsafe and illicit [8], given that some acute (but not lethal) poisonings attributed to GHB had been reported to the FDA, and that GHB has been marketed illicitly to bodybuilders for weight control, as a sleeping aid and as a food supplement instead of L-tryptophan. However, our long-term clinical experience with this drug in alcoholics confirms findings in these two patients: abrupt cessation of GHB administration even after long-term use does not involve the occurrence of symptoms attributable to withdrawal phenomena [5]. We have also never recorded any patient behaviour attributable to GHB abuse. Further preclinical and clinical trials may clarify the pharmacodynamics of GHB so as to shed some light on the biology of drug addiction [9].

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Flumazenil effects on growth hormone response to gamma-hydroxybutyric acid

G. Gerra[1], R. Caccavari[1], B. Fontanesi[1], A. Marcato[1], G. Fertonani Affini[1], D. Maestri[2], P. Avanzini[2], R. Lecchini[2], R. Delsignore[3] and A. Mutti[4]

[1]*Servizio Tossicodipendenze, USL 4, Via Guasti S. Cecilia 3, 43100 Parma,* [2]*Laboratorio di Analisi Chimico-Cliniche, Ospedale di Parma,* [3]*Clinica Medica Generale and* [4]*Cattedra di Medicina del Lavoro, Università degli Studi di Parma, Italy*

Correspondence to: G. Gerra at above address

Gamma-hydroxybutyric acid (GHBA) has been recently introduced for alcohol detoxication but few data are available concerning the central mechanism of action of this gamma-aminobutyric acid (GABA) catabolite. GHBA ability to stimulate growth hormone (GH) and prolactin (PRL) secretion has been reported: the involvement of GABA, dopamine or serotonin systems acting on pituitary hormones has been hypothesized. In the present study we investigated GH and PRL responses to GHBA with or without flumazenil (a benzodiazepine receptor antagonist) i.v. pretreatment. Our study included nine male healthy volunteers (aged 23.2 ± 2.5 years) who were submitted to three tests in random order: (1) oral GHB administration; (2) oral GHBA and i.v. flumazenil administration; (3) oral placebo and i.v. saline administration. Blood samples for GH and PRL assays were collected during the three tests at −15, 0, 15, 30, 45, 60 and 90 min. GHBA induced a significant increase in GH plasma levels; flumazenil pretreatment antagonized GHBA action on GH secretion. No changes were obtained with placebo and saline administration. A subpopulation of GABA receptors or GHBA-specific receptors seems to be involved in GHBA action. The benzodiazepine receptor antagonist flumazenil was able to influence the sensitivity and the neuroendocrine consequences of GHBA binding site stimulation.

Keywords: Dopamine – Flumazenil – Gamma-aminobutyric acid – Gamma-hydroxybutyric acid – Growth hormone – Prolactin

INTRODUCTION

Gamma-hydroxybutyric acid (GHBA), a physiologic metabolite of gamma-aminobutyric acid (GABA; Gold and Roth, 1977; Maitre *et al.*, 1983), has been used as an i.v. hypnotic anaesthetic agent (Laborit *et al.*, 1960) and in the treatment of sleep disturbances (Mamelak *et al.*, 1986).

GHBA inhibits voluntary ethanol consumption in rats that show a preference for ethanol (Fadda *et al.*, 1983), and suppresses ethanol withdrawal in rats physically dependent on ethanol (Fadda *et al.*, 1989). GHBA effectiveness in the treatment of ethanol withdrawal in alcoholic patients has recently been demonstrated (Gallimberti *et al.*, 1989). The action of GHBA on ethanol withdrawal may be due to its GABA-like activity (Anden and Stock, 1973; Snead and Nicholas, 1987) but the nature of the GABAergic action of GHBA remains unclear.

Contrasting findings have been reported on the ability of GHBA to influence monoaminergic pathways (Fuxe *et al.*, 1975). Some authors suggest that GHBA may decrease dopamine release (Roth and Suhr, 1970), but other studies have reported a rise in dopamine concentration in the brain (Hutchins *et al.*, 1972) and in the dopaminergic nerve terminals following GHBA administration (Stock *et al.*, 1973).

GABA and GABA metabolites are involved in the control of pituitary hormone secretion (Racagni *et al.*, 1982). Growth hormone (GH) and prolactin (PRL) plasma level increases have been observed after GHBA injection in a small sample of healthy volunteers (Takahara *et al.*, 1977). We investigated pituitary hormone response to GHBA oral administration in humans with the aim of indirectly evaluating the effects of GHBA on GABAergic and dopaminergic systems. Since the benzodiazepine receptor antagonist flumazenil (Amrein *et al.*, 1987) has been demonstrated to influence $GABA_A$ receptor sensitivity (Polc and Ducic, 1991), our experiment also included GH and PRL responses to GHBA in healthy subjects pretreated with flumazenil.

METHODS

Nine healthy male subjects aged 23.2 years (S.D. 2.5 years) were included in the study. All the subjects gave

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their informed consent: the experimental nature of the study was clearly explained to them. All subjects were within 10% of their ideal body weight; physical training was not allowed in the 48 h prior to the test.

None of the subjects was affected by endocrine, metabolic, heart, liver, renal or other disease. Psychiatric evaluation for depressive or other major affective disorders with the Minnesota Multiphasic Personality Inventory (MMPI, of Hathaway and McKinley) gave negative results. Subjects were not tobacco smokers and they had limited alcohol intake during meals (15-20 g alcohol daily). They had taken no drugs during the previous month before the tests.

All subjects were given three tests in random order; 4 days separated the tests.

Test A

After an overnight fast and bed rest the subject was seated on a chair and an indwelling cannula was inserted into a forearm vein at 08.30 h. Two basal blood samples were taken at 08.45 and 09.00 h. GHBA (Alcover CT Sanremo, Italy) 1.5 g was then administered orally; physiological saline solution (250 ml) was slowly injected i.v. during the test, and blood samples were taken at 15, 30, 45, 60 and 90 min after the administration of the drug.

Test B

Blood samples were collected using the same procedure and at the same time as in Test A. GHBA 1.5 g was administered orally at 09.00 h and flumazenil (Anexate Roche) 1 mg dissolved in physiological saline solution (250 ml) was injected i.v. during the test (10 μg/min). Flumazenil injection began 15 min before GHBA administration.

Test C

Blood samples were collected using the same procedure and at the same time as for Tests A and B. Placebo was administered orally at 09.00 h and physiological saline solution (250 ml) was slowly injected i.v. Before GHBA or placebo administration and every 15 min following administration blood pressure, heart rate and clinical adverse effects were evaluated by the same observer. Blood samples were then centrifuged and the plasma kept frozen until analysis. GH and PRL plasma levels were measured by specific radioimmunoassay using commercial kits (Sorin, Italy). The sensitivities of the methods were 0.2 and 0.5 ng/ml, respectively.

Statistical analysis

Statistical analysis was based on the paired Student's *t*-test and analysis of variance (ANOVA).

RESULTS

The mean ± S.E.M. baseline GH in the three tests was 5.3 ± 0.8, 4.1 ± 0.3 and 4.4 ± 0.3 ng/ml (in the GHBA, saline and GHBA-flumazenil tests, respectively): there

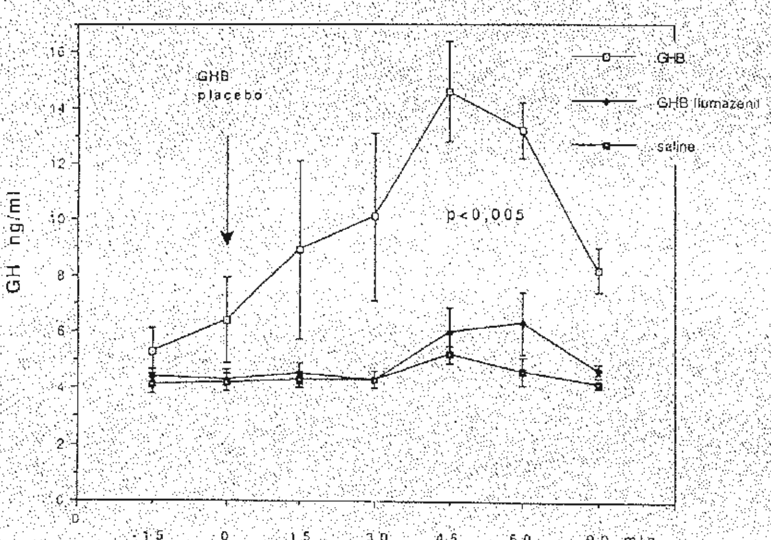

FIG. 1. Growth hormone plasma levels (mean ± SE) after GHBA (1.5 mg, orally) —□—, after GHBA (1.5 mg, orally) and flumazenil infusion (1 mg in saline solution i.v., 10 μg/min) —●— and after saline only —■— in healthy volunteers.

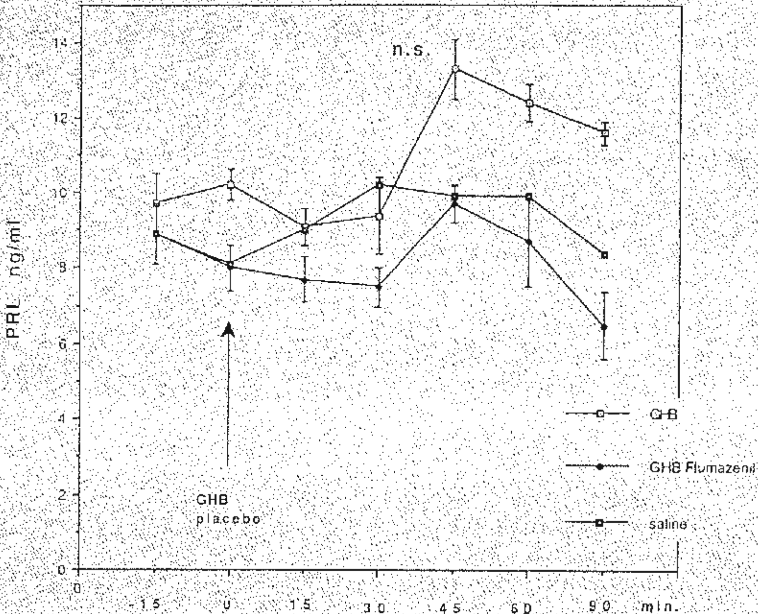

FIG. 2. Prolactin plasma levels (mean ± SE) after GHBA (1.5 mg, orally) —□—, after GHBA (1.5 mg, orally) and flumazenil infusion (1 mg in saline solution i.v.: 10 μg/min) —●— and after saline only —■— in healthy volunteers.

were no significant differences in basal GH levels among the three tests. The corresponding values for baseline PRL in the three tests were 9.7 ± 0.9, 8.9 ± 0.8 and 8.9 ± 0.8 ng/ml (in the GHBA, saline and GHBA-flumazenil tests, respectively), with no significant differences among the three tests.

GH plasma levels increased significantly [df 8, F(3.52), $p < 0.005$] after GHBA administration (Δ_{max} 9.3 ± 1.0 ng/ml at 45 min; Test A), whereas they showed a slight and statistically non-significant [df 8, F(0.65), $p > 0.05$] increase after GHBA during flumazenil infusion (Δ_{max} 1.9 ± 0.8 ng/ml at 60 min; Test B).

GH responses to GHBA only and GHBA-flumazenil were significantly different ($p < 0.05$), whereas saline and placebo administration induced no significant change in GH plasma levels (Test C; Fig. 1).

PRL plasma levels showed a slight and non-significant [df 8, F(1.39), $p > 0.05$] increase after GHBA administration (Δ_{max} 3.6 ± 0.6 ng/ml at 45 min; Test A). GHBA and flumazenil together (Test B) and saline and placebo (Test C) induced no significant change in PRL plasma levels. Likewise, PRL responses to GHBA in Test A and to GHBA and flumazenil in Test B were not significantly different (Fig. 2).

Blood pressure and heart rate were not influenced by GHBA or flumazenil administration.

All subjects tolerated the oral administration of GHBA and flumazenil injection without major side effects; no mood changes, anxiety, sedation or behavioural symptoms became evident during the tests.

DISCUSSION

Our experiment showed that an increase in GH plasma levels is induced by GHBA administration, thus confirming previous studies on its effects on pituitary hormones (Racagni *et al.*, 1982). The mechanism of action on GH-secreting cells is difficult to interpret. According to initial studies, GHBA may influence pituitary cells, interfering with the serotoninergic system (Takahara *et al.*, 1977; Spano and Przegalinsky, 1973). The involvement of the GABA complex has been hypothesized in view of the ability of bicuculline (a GABAergic antagonist) to antagonize GHBA effects on GH secretion (Vijayan and McCann, 1978). The stimulating role of GABAergic drugs on GH secretion in humans has been widely demonstrated (Cavagnini *et al.*, 1980). GABA-induced GH release could also be mediated through the dopaminergic system, for example pimozide (a dopamine antagonist) is able to inhibit GABA effects on GH secretion (Cocchi *et al.*, 1980).

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A variety of studies have reported that GHBA reduces dopamine release, thus increasing brain dopamine concentration (Gessa *et al.*, 1966; Roth *et al.*, 1973). Since dopamine has been demonstrated to stimulate GH secretion (Muller, 1979), such a reduction in dopamine release cannot be responsible for GHBA-induced changes in GH. Furthermore, our data showing the absence of effect of GHBA on PRL plasma levels indicate that these oral doses of GHBA do not influence the dopaminergic system. However, at higher doses (2.5 g i.v.) GHBA may increase PRL levels (Takahara *et al.*, 1977).

More recently an increasing body of evidence has been reported which does not support the hypothesis of GHBA GABAergic action: GHBA fails to alter the function of the $GABA_A$ receptor complex (Serra *et al.*, 1991), and *in vivo* administration of GHBA did not modify the chloride channel function coupled with the $GABA_A$ receptor (Serra *et al.*, 1989). Different subunits (alpha, beta, gamma) may be responsible for the heterogeneous nature of GABA and the benzodiazepine receptor complex (Levitan *et al.*, 1988). The effects of GHBA may be due to the involvement of a subpopulation of these receptors (Serra *et al.*, 1991); a possible binding of GHBA on specific receptors has also been suggested (Gallimberti *et al.*, 1989). In any case, studies with picrotoxin, a drug modulating the function of the GABA receptor complex, showed that GHBA and GABA receptors may share certain moieties (Snead *et al.*, 1992).

In our experiment pretreatment with the benzodiazepine antagonist flumazenil significantly decreased GH response to GHBA oral administration. This finding is consistent with the theory postulating an interference of GHBA on the GABA receptor complex, or on a receptor subpopulation in close relation with benzodiazepine receptors. Flumazenil has indeed been shown to antagonize the effects of bicuculline (a GABA antagonist) on the GABA receptor complex (Polc and Ducic, 1991). The activity of benzodiazepine receptor antagonists in counteracting GHBA-induced GH secretion must be interpreted with caution. However it is unlikely that flumazenil itself causes a decrease in GH levels since we have found no changes in plasma GH following flumazenil administration (unpublished data).

A possible partial agonist activity of GHBA on benzodiazepine receptors cannot be excluded. An alternative explanation is that GHBA is releasing an endogenous ligand of the benzodiazepine receptor that is causing GH secretion. However, to our knowledge no data are available to support these mechanisms. Other authors suggest that GHBA may act through the endogenous opioid system, since in the brain GHBA raises dynorphin levels (Mamelak, 1989) and modulates the activity of opioid interneurons (Hechler *et al.*, 1991). The activation of the opioid system could be responsible for GH secretion increase (Locatelli *et al.*, 1978), but this mechanism of action does not explain the ability of flumazenil to inhibit the effect of GHBA on pituitary function, and further studies need to be performed.

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GAMMA-HYDROXYBUTYRIC ACID IN THE TREATMENT OF ALCOHOL DEPENDENCE

G.L. GESSA* and L. GALLIMBERTI**
*"Bernard B. Brodie" Department of Neuroscience, University of Cagliari, Cagliari, Italy; ** Drug Abuse Unit, USSL 21, Padova, Italy.

Gamma-hydroxybutyric acid (GHB), a normal brain constituent originating from GABA metabolism, is considered to play a neurotransmitter and/or neuromodulatory role in the CNS.[1] Systemically administered, GHB exerts hypnotic and anesthetic effects in animals and in man, therefore it was introduced in the clinic as a general anesthetic and hypnotic agent.[2]

Previous results from our laboratory have shown that GHB, in its lactone form, inhibits voluntary ethanol consumption in a rat line selectively bred for high preference for ethanol,[3] and that GHB suppresses ethanol withdrawal syndrome in rats rendered physically dependent on ethanol by forced ethanol administration.[4]

More recently in a double blind study we found that GHB, given orally in non hypnotic doses, is highly effective in suppressing the withdrawal symptomatology in alcoholics; the GHB effect has a rapid onset and the compound is devoid of adverse side effects.[5]

The above considerations and the relative safety of GHB led us to study the clinical efficacy of the compound in the treatment of alcohol dependence.

We investigated the effect of gamma-hydroxybutyric acid on alcohol consumption and alcohol craving in alcoholics in a randomized double-blind study versus placebo. Alcohol consumption was assessed by the subject's self report. Patients were treated as outpatients during a three month period either with gamma-hydroxybutyric acid (50 mg/kg/day, divided into three daily doses) or with placebo. Of the 82 alcoholics that entered the study 71 completed it; 36 in the gamma-hydroxybutyric acid and 35 in the placebo group. At the 3rd month of treatment 11 patients in the gamma-hydroxybutyric acid group referred to be abstinent and 15 referred controlled drinking; while in the placebo group only 2 and 6 patients referred abstinence and controlled drinking, respectively. Serum-gammaglutamyltransferase activity correlated with the admitted alcohol consumption. Gamma-hydroxybutyric acid treatment decreased alcohol craving during the 3 months of treatment. Transient side effects were noted by 6 patients on gamma-hydroxybutyric acid and 2 on placebo.

Experimental studies suggest that GHB administration interferes with the activity of dopamine,[6] serotonin,[7] acetylcholine,[8] opioids[9] and GABA.[10] At present it is unknown which of these interactions bears some relevance for the suppressant effect on ethanol consumption and craving.

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Moreover, since GHB is a normal brain constituent and has many of the characteristics of neurotransmitter and/or neuromodulator,[1] the possible relevance of changes in the content and activity of endogenous GHB in the pathogenesis of alcoholism might be considered.

Finally, the possibility exists that GHB might act by mimicking the central effects of ethanol. Indeed, ethanol moiety is present in the structure of GHB and the latter shares with ethanol different pharmacological and neurochemical characteristics. Moreover tolerance to ethanol is extended to GHB.[4] Should the latter hypothesis be validated, the rationale for using GHB in the treatment of alcoholism would be the same as that of using methadone in heroin addiction.

Whatever the exact mechanism of action of GHB, our results indicate that GHB deserves more extensive investigation as a clinically useful drug in the treatment of alcoholism.

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CHAPTER 3

DRUG METABOLISM

Frank J. Gonzalez and Robert H. Tukey

How Humans Cope with Exposure to Xenobiotics. The ability of humans to metabolize and clear drugs is a natural process that involves the same enzymatic pathways and transport systems that are utilized for normal metabolism of dietary constituents. Humans come into contact with scores of foreign chemicals or xenobiotics (substances foreign to the body) through exposure to environmental contaminants as well as in our diets. Fortunately, humans have developed a means to rapidly eliminate xenobiotics so they do not cause harm. In fact, one of the most common sources of xenobiotics in the diet is from plants that have many structurally diverse chemicals, some of which are associated with pigment production and others that are actually toxins (called phytoallexins) that protect plants against predators. A common example is poisonous mushrooms that have many toxins that are lethal to mammals, including amanitin, gyromitrin, orellanine, muscarine, ibotenic acid, muscimol, psilocybin, and coprine. Animals must be able to metabolize and eliminate such chemicals in order to consume vegetation. While humans can now choose their dietary source, a typical animal does not have this luxury and as a result is subject to its environment and the vegetation that exists in that environment. Thus, the ability to metabolize unusual chemicals in plants and other food sources is critical for survival.

Drugs are considered xenobiotics and most are extensively metabolized in humans. It is worth noting that many drugs are derived from chemicals found in plants, some of which had been used in Chinese herbal medicines for thousands of years. Of the prescription drugs in use today for cancer treatment, many derive from plant species (*see* Chapter 51); investigating folklore claims led to the discovery of most of these drugs. It is therefore not surprising that animals utilize a means for disposing of human-made drugs that mimics the disposition of chemicals found in the diet. This capacity to metabolize xenobiotics, while mostly beneficial, has made development of drugs very time consuming and costly due in large part to (1) interindividual variations in the capacity of humans to metabolize drugs, (2) drug-drug interactions, and (3) species differences in expression of enzymes that metabolize drugs. The latter limits the use of animal models in drug development.

A large number of diverse enzymes have evolved in animals that apparently only function to metabolize foreign chemicals. As will be discussed below, there are such large differences among species in the ability to metabolize xenobiotics that animal models cannot be relied npon to predict how humans will metabolize a drug. Enzymes that metabolize xenobiotics have historically been called drug-metabolizing enzymes, although they are involved in the metabolism of many foreign chemicals to which humans are exposed. Dietary differences among species during the course of evolution could account for the marked species variation in the complexity of the drug-metabolizing enzymes.

Today, most xenobiotics to which humans are exposed come from sources that include environmental pollution, food additives, cosmetic products, agrochemicals, processed foods, and drugs. In general, these are lipophilic chemicals, that in the absence of metabolism would not be efficiently eliminated, and thus would accumulate in the body, resulting in toxicity. With very few exceptions, all xenobiotics are subjected to one or multiple pathways that constitute the phase 1 and phase 2 enzymatic systems. As a general paradigm, metabolism serves to convert these hydrophobic chemicals into derivatives that can easily be eliminated through the urine or the bile.

In order to be accessible to cells and reach their sites of action, drugs generally must possess physical properties that allow them to move down a concentration gradient

into the cell. Thus, most drugs are hydrophobic, a property that allows entry through the lipid bilayers into cells where drugs interact with their target receptors or proteins. Entry into cells is facilitated by a large number of transporters on the plasma membrane (*see* Chapter 2). This property of hydrophobicity would render drugs difficult to eliminate, since in the absence of metabolism, they would accumulate in fat and cellular phospholipid bilayers in cells. The xenobiotic-metabolizing enzymes convert drugs and xenobiotics into compounds that are hydrophilic derivatives that are more easily eliminated through excretion into the aqueous compartments of the tissues. Thus, the process of drug metabolism that leads to elimination plays a major role in diminishing the biological activity of a drug. For example, *(S)-phenytoin*, an anticonvulsant used in the treatment of epilepsy, is virtually insoluble in water. Metabolism by the phase 1 cytochrome P450 isoenzymes (CYPs) followed by phase 2 uridine diphosphate-glucuronosyltransferase (UGT) enzymes produces a metabolite that is highly water soluble and readily eliminated from the body (Figure 3–1). Metabolism also terminates the biological activity of the drug. In the case of phenytoin, metabolism also increases the molecular weight of the compound, which allows it to be eliminated more efficiently in the bile.

While xenobiotic-metabolizing enzymes are responsible for facilitating the elimination of chemicals from the body, paradoxically these same enzymes can also convert certain chemicals to highly reactive toxic and carcinogenic metabolites. This occurs when an unstable intermediate is formed that has reactivity toward other compounds fonnd in the cell. Chemicals that can be converted by xenobiotic metabolism to cancer-causing derivatives are called carcinogens. Depending on the structure of the chemical substrate, xenobiotic-metabolizing enzymes produce electrophilic metabolites that can react with nucleophilic cellular macromolecules such as DNA, RNA, and protein. This can cause cell death and organ toxicity. Reaction of these electrophiles with DNA can sometimes result in cancer through the mutation of genes such as oncogenes or tumor suppressor genes. It is generally believed that most human cancers are due to exposure to chemical carcinogens. This potential for carcinogenic activity makes testing the safety of drug candidates of vital importance. Testing for potential cancer-causing activity is particularly critical for drugs that will be used for the treatment of chronic diseases. Since each species has evolved a unique combination of xenobiotic-metabolizing enzymes, nonprimate rodent models cannot be solely used during drug development for testing the safety of new drug candidates targeted for human diseases. Nevertheless, testing in rodent models such as mice and rats can usually identify potential carcinogens.

Phenytoin — Highly lipophilic

↓ CYP

4-Hydroxyphenytoin — Slightly soluble in water

↓ UGT + UDP-GA

4-Hydroxyphenytoin-β-D-glucuronide — Very soluble in water

Figure 3–1. ***Metabolism of phenytoin by phase 1 cytochrome P450 (CYP) and phase 2 uridine diphosphate-glucuronosyltransferase (UGT).*** CYP facilitates 4-hydroxylation of phenytoin to yield 5-(-4-hydroxyphenyl)-5-phenylhydantoin (HPPH). The hydroxy group serves as a substrate for UGT that conjugates a molecule of glucuronic acid using UDP-glucuronic acid (UDP-GA) as a cofactor. This converts a very hydrophobic molecule to a larger hydrophilic derivative that is eliminated *via* the bile.

The Phases of Drug Metabolism. Xenobiotic metabolizing enzymes have historically been grouped into the phase 1 reactions, in which enzymes carry out oxidation, reduction, or hydrolytic reactions, and the phase 2 reactions, in which enzymes form a conjugate of the substrate (the phase 1 product) (Table 3–1). The phase 1 enzymes lead to the introduction of what are called functional groups, resulting in a modification of the drug, such that it now carries an –OH, -COOH, -SH, -O- or NH_2 group. The addition of functional groups does little to increase the water solubility of the drug, but can dra-

Table 3–1
Xenobiotic Metabolizing Enzymes

ENZYMES	REACTIONS
Phase 1 "oxygenases"	
Cytochrome P450s (P450 or CYP)	C and O oxidation, dealkylation, others
Flavin-containing monooxygenases (FMO)	N, S, and P oxidation
Epoxide hydrolases (mEH, sEH)	Hydrolysis of epoxides
Phase 2 "transferases"	
Sulfotransferases (SULT)	Addition of sulfate
UDP-glucuronosyltransferases (UGT)	Addition of glucuronic acid
Glutathione-S-transferases (GST)	Addition of glutathione
N-acetyltransferases (NAT)	Addition of acetyl group
Methyltransferases (MT)	Addition of methyl group
Other enzymes	
Alcohol dehydrogenases	Reduction of alcohols
Aldehyde dehydrogenases	Reduction of aldehydes
NADPH-quinone oxidoreductase (NQO)	Reduction of quinones

mEH and sEH are microsomal and soluble epoxide hydrolase. UDP, uridine diphosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

matically alter the biological properties of the drug. Phase 1 metabolism is classified as the functionalization phase of drug metabolism; reactions carried out by phase 1 enzymes usually lead to the inactivation of an active drug. In certain instances, metabolism, usually the hydrolysis of an ester or amide linkage, results in bioactivation of a drug. Inactive drugs that undergo metabolism to an active drug are called prodrugs. An example is the antitumor drug *cyclophosphamide*, which is bioactivated to a cell-killing electrophilic derivative (*see* Chapter 51). Phase 2 enzymes facilitate the elimination of drugs and the inactivation of electrophilic and potentially toxic metabolites produced by oxidation. While many phase 1 reactions result in the biological inactivation of the drug, phase 2 reactions produce a metabolite with improved water solubility and increased molecular weight, which serves to facilitate the elimination of the drug from the tissue.

Superfamilies of evolutionarily related enzymes and receptors are common in the mammalian genome; the enzyme systems responsible for drug metabolism are good examples. The phase 1 oxidation reactions are carried out by CYPs, flavin-containing monooxygenases (FMO), and epoxide hydrolases (EH). The CYPs and FMOs are composed of superfamilies of enzymes. Each superfamily contains multiple genes. The phase 2 enzymes include several superfamilies of conjugating enzymes. Among the more important are the glutathione-S-transferases (GST), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), *N*-acetyltransferases (NAT), and methyltransferases (MT). These conjugation reactions usually require the substrate to have oxygen (hydroxyl or epoxide groups), nitrogen, and sulfur atoms that serve as acceptor sites for a hydrophilic moiety, such as glutathione, glucuronic acid, sulfate, or an acetyl group, that is covalently conjugated to an acceptor site on the molecule. The example of phase 1 and phase 2 metabolism of phenytoin is shown in Figure 3–1. The oxidation by phase 1 enzymes either adds or exposes a functional group, permitting the products of phase 1 metabolism to serve as substrates for the phase 2 conjugating or synthetic enzymes. In the case of the UGTs, glucuronic acid is delivered to the functional group, forming a glucuronide metabolite that is now more water soluble with a higher molecular weight that is targeted for excretion either in the urine or bile. When the substrate is a drug, these reactions usually convert the original drug to a form that is not able to bind to its target receptor, thus attenuating the biological response to the drug.

Sites of Drug Metabolism. Xenobiotic metabolizing enzymes are found in most tissues in the body with the highest levels located in the tissues of the gastrointestinal tract (liver, small and large intestines). Drugs that are orally administered, absorbed by the gut, and taken to the

liver, can be extensively metabolized. The liver is considered the major "metabolic clearing house" for both endogenous chemicals (*e.g.*, cholesterol, steroid hormones, fatty acids, and proteins), and xenobiotics. The small intestine plays a crucial role in drug metabolism since most drugs that are orally administered are absorbed by the gut and taken to the liver through the portal vein. The high concentration of xenobiotic-metabolizing enzymes located in the epithelial cells of the GI tract is responsible for the initial metabolic processing of most oral medications. This should be considered the initial site for first-pass metabolism of drugs. The absorbed drug then enters the portal circulation for its first pass through the liver, where metabolism may be prominent, as it is for β adrenergic receptor antagonists, for example. While a portion of active drug escapes this first-pass metabolism in the GI tract and liver, subsequent passes through the liver result in more metabolism of the parent drug until the agent is eliminated. Thus, drugs that are poorly metabolized remain in the body for longer periods of time and their pharmacokinetic profiles show much longer elimination half-lives than drugs that are rapidly metabolized. Other organs that contain significant xenobiotic-metabolizing enzymes include the tissues of the nasal mucosa and lung, which play important roles in the first-pass metabolism of drugs that are administered through aerosol sprays. These tissues are also the first line of contact with hazardous chemicals that are airborne.

Within the cell, xenobiotic-metabolizing enzymes are found in the intracellular membranes and in the cytosol. The phase 1 CYPs, FMOs, and EHs, and some phase 2 conjugating enzymes, notably the UGTs, are all located in the endoplasmic reticulum of the cell (Figure 3–2). The endoplasmic reticulum consists of phospholipid bilayers organized as tubes and sheets throughout the cytoplasm of the cell. This network has an inner lumen that is physically distinct from the rest of the cytosolic components of the cell and has connections to the plasma membrane and nuclear envelope. This membrane localization is ideally suited for the metabolic function of these enzymes: hydrophobic molecules enter the cell and become embedded in the lipid bilayer where they come into direct contact with

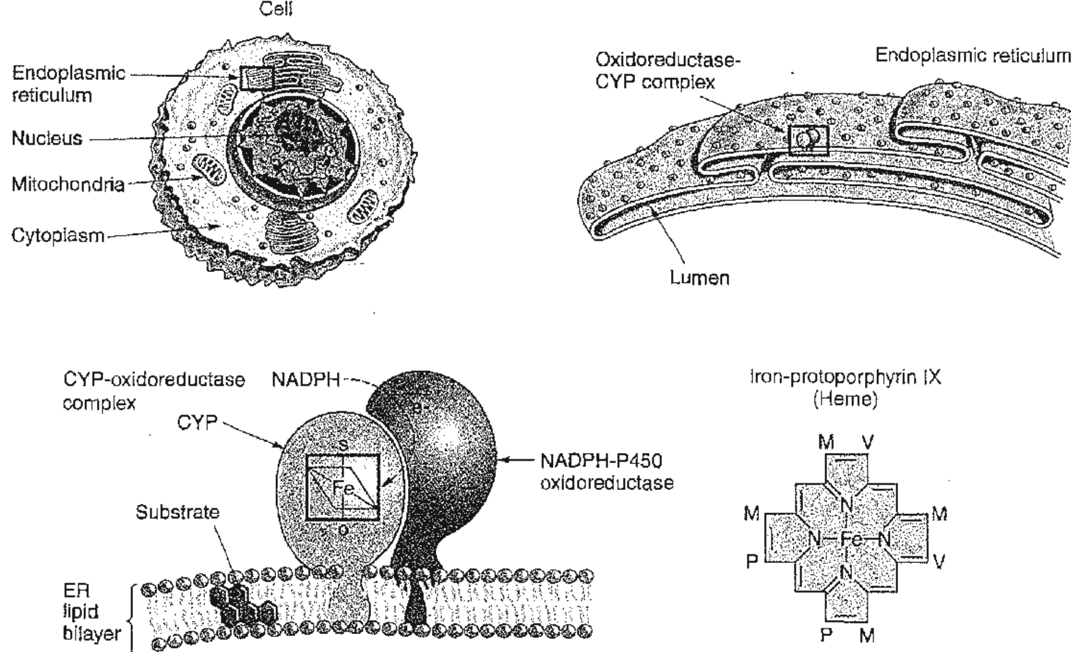

Figure 3–2. *Location of CYPs in the cell.* The figure shows increasingly microscopic levels of detail, sequentially expanding the areas within the black boxes. CYPs are embedded in the phospholipid bilayer of the endoplasmic reticulum (ER). Most of the enzyme is located on the cytosolic surface of the ER. A second enzyme, NADPH-cytochrome P450 oxidoreductase, transfers electrons to the CYP where it can, in the presence of O_2, oxidize xenobiotic substrates, many of which are hydrophobic and dissolved in the ER. A single NADPH-CYP oxidoreductase species transfers electrons to all CYP isoforms in the ER. Each CYP contains a molecule of iron-protoporphyrin IX that functions to bind and activate O_2. Substituents on the porphyrin ring are methyl (M), propionyl (P), and vinyl (V) groups.

the phase 1 enzymes. Once subjected to oxidation, drugs can be conjugated in the membrane by the UGTs or by the cytosolic transferases such as GST and SULT. The metabolites can then be transported out of the cell through the plasma membrane where they are deposited into the bloodstream. Hepatocytes, which constitute more than 90% of the cells in the liver, carry out most drug metabolism and can produce conjugated substrates that can also be transported though the bile canalicular membrane into the bile from which they are eliminated into the gut (*see* Chapter 2).

The CYPs. The CYPs are a superfamily of enzymes, all of which contain a molecule of heme that is noncovalently bound to the polypeptide chain (Figure 3–2). Many other enzymes that use O_2 as a substrate for their reactions contain heme. Heme is the oxygen-binding moiety, also found in hemoglobin, where it functions in the binding and transport of molecular oxygen from the lung to other tissues. Heme contains one atom of iron in a hydrocarbon cage that functions to bind oxygen in the CYP active site as part of the catalytic cycle of these enzymes. CYPs use O_2, plus H^+ derived from the cofactor-reduced nicotinamide adenine dinucleotide phosphate (NADPH), to carry out the oxidation of substrates. The H^+ is supplied through the enzyme NADPH-cytochrome P450 oxidoreductase. Metabolism of a substrate by a CYP consumes one molecule of molecular oxygen and produces an oxidized substrate and a molecule of water as a by-product. However, for most CYPs, depending on the nature of the substrate, the reaction is "uncoupled," consuming more O_2 than substrate metabolized and producing what is called activated oxygen or O_2^-. The O_2^- is usually couverted to water by the enzyme superoxide dismutase.

Among the diverse reactions carried out by mammalian CYPs are *N*-dealkylation, *O*-dealkylation, aromatic hydroxylation, *N*-oxidation, *S*-oxidation, deamination, and dehalogenation (Table 3–2). More than 50 individual CYPs have been identified in humans. As a family of enzymes, CYPs are involved in the metabolism of dietary and xenobiotic agents, as well as the synthesis of endogenous compounds such as steroids and the metabolism of bile acids, which are degradation by-products of cholesterol. In contrast to the drug-metabolizing CYPs, the CYPs that catalyze steroid and bile acid synthesis have very specific substrate preferences. For example, the CYP that produces estrogen from testosterone, CYP19 or aromatase, cau metabolize only testosterone and does not metabolize xenobiotics. Specific inhibitors for aromatase, such as *anastrozole,* have been developed for use in the treatment of estrogen-dependent tumors (*see* Chapter 51). The synthesis of bile acids from cholesterol occurs in the liver, where, subsequent to CYP-catalyzed oxidation, the bile acids are conjugated and transported through the bile duct and gallbladder into the small intestine. CYPs involved in bile acid production have strict substrate requirements and do not participate in xenobiotic or drug metabolism.

The CYPs that carry out xenobiotic metabolism have a tremendous capacity to metabolize a large number of structurally diverse chemicals. This is due both to multiple forms of CYPs and to the capacity of a single CYP to metabolize many structurally distinct chemicals. A single compound can also be metabolized, albeit at different rates, by different CYPs. In addition, CYPs can metabolize a single compound at different positions on the molecule. In contrast to enzymes in the body that carry out highly specific reactions involved in the biosynthesis and degradation of important cellular constituents in which there is a single substrate and one or more products, or two simultaneous substrates, the CYPs are considered promiscuous in their capacity to bind and metabolize multiple substrates (Table 3–2). This property, which is due to large and fluid substrate binding sites in the CYP, sacrifices metabolic turnover rates; CYPs metabolize substrates at a fraction of the rate of more typical enzymes involved in intermediary metabolism and mitochondrial electron transfer. As a result, drugs have, in general, half-lives of the order of 3 to 30 hours, while endogenous compounds have half-lives of the order of seconds or minutes (*e.g.*, dopamine and insulin). Even though CYPs have slow catalytic rates, their activities are sufficient to metabolize drugs that are administered at high concentrations in the body. This unusual feature of extensive overlapping substrate specificities by the CYPs is one of the underlying reasons for the predominance of drug-drug interactions. When two coadministered drugs are both metabolized by a single CYP, they compete for binding to the enzyme's active site. This can result in the inhibition of metabolism of one or both of the drugs, leading to elevated plasma levels. If there is a narrow therapeutic index for the drugs, the elevated serum levels may elicit unwanted toxicities. Drug-drug interactions are among the leading causes of adverse drug reactions.

The CYPs are the most actively studied of the xenobiotic metabolizing enzymes since they are responsible for metabolizing the vast majority of therapeutic drugs. CYPs are complex and diverse in their regulation and catalytic activities. Cloning and sequencing of CYP complementary DNAs, and more recently total genome sequencing, have revealed the existence of 102 putatively functional genes and 88 pseudogenes in the mouse, and 57 putatively functional

Table 3–2
Major Reactions Involved in Drug Metabolism

REACTION		EXAMPLES
I. *Oxidative reactions*		
N-Dealkylation	$RNHCH_3 \rightarrow RNH_2 + CH_2O$	Imipramine, diazepam, codeine, erythromycin, morphine, tamoxifen, theophylline, caffeine
O-Dealkylation	$ROCH_3 \rightarrow ROH + CH_2O$	Codeine, indomethacin, dextromethorphan
Aliphatic hydroxylation	$RCH_2CH_3 \rightarrow RCHOHCH_3$	Tolbutamide, ibuprofen, phenobarbital, meprobamate, cyclosporine, midazolam
Aromatic hydroxylation	R-benzene → R-benzene epoxide (O) → R-phenol (OH)	Phenytoin, phenobarbital, propanolol, ethinyl estradiol, amphetamine, warfarin
N-Oxidation	$RNH_2 \rightarrow RNHOH$; $R_1R_2NH \rightarrow R_1R_2N{-}OH$	Chlorpheniramine, dapsone, meperidine
S-Oxidation	$R_1R_2SH_2 \rightarrow R_1R_2S{=}O$	Cimetidine, chlorpromazine, thioridazine, omeprazole
Deamination	$RCH(NH_2)CH_3 \rightarrow R{-}C(OH)(NH_2){-}CH_3 \rightarrow R{-}C(=O){-}CH_3 + NH_3$	Diazepam, amphetamine
II. *Hydrolysis reactions*		
	R,R-benzene epoxide (O) → R,R-benzene diol (OH, OH)	Carbamazepine
	R_1COR_2 (C=O) $\rightarrow R_1COOH \rightarrow R_2OH_2$	Procaine, aspirin, clofibrate, meperidine, enalapril, cocaine
	R_1CNR_2 (C=O) $\rightarrow R_1COOH \rightarrow R_2NH_2$	Lidocaine, procainamide, indomethacin
III. *Conjugation reactions*		
Glucuronidation	R + UDP-glucuronic acid (COOH, OH, OH, OH, O–UDP) → glucuronide (COOH, O—R, OH, OH, OH) + UD	Acetaminophen, morphine, oxazepam, lorazepam

(Continued)

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Table 3–2
Major Reactions Involved in Drug Metabolism (Continued)

	REACTION	EXAMPLES
Sulfation	PAPS + ROH → $R-O-SO_2-OH$ + PAP (PAPS: 3'-phosphoadenosine-5' phosphosulfate; PAP: 3'-phosphoadenosine-5'-phosphate)	Acetaminophen, steroids, methyldopa
Acetylation	$CoAS-CO-CH_3 + RNH_2 \rightarrow RNH-CO-CH_3 + CoA\text{-}SH$	Sulfonamides, isoniazid, dapsone, clonazepam (*see* Table 3–3)
Methylation	RO-, RS-, RN- + AdoMet → RO-CH_3 + AdoHomCys	L-Dopa, methyldopa, mercaptopurine, captopril
Glutathione conjugation	GSH + R → R-GSH	Adriamycin, fosfomycin, busulfan

genes and 58 pseudogenes in humans. These genes are grouped, based on amino acid sequence similarity, into a large number of families and subfamilies. CYPs are named with the root CYP followed by a number designating the family, a letter denoting the subfamily, and another number designating the CYP form. Thus, CYP3A4 is family 3, subfamily A, and gene number 4. While several CYP families are involved in the synthesis of steroid hormones and bile acids, and the metabolism of retinoic acid and fatty acids, including prostaglandins and eicosanoids, a limited number of CYPs (15 in humans) that fall into families 1 to 3 are primarily involved in xenobiotic metabolism (Table 3–1). Since a single CYP can metabolize a large number of structurally diverse compounds, these enzymes can collectively metabolize scores of chemicals found in the diet, environment, and administered as drugs. In humans, 12 CYPs (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) are known to be important for metabolism of xenobiotics. The liver contains the greatest abundance of xenobiotic-metabolizing CYPs, thus ensuring efficient first-pass metabolism of drugs. CYPs are also expressed throughout the GI tract, and in lower amounts in lung, kidney, and even in the CNS. The expression of the different CYPs can differ markedly as a result of dietary and environmental exposure to inducers, or through interindividual changes resulting from heritable polymorphic differences in gene structure, and tissue-specific expression patterns can impact on overall drug metabolism and clearance. The most active CYPs for drug metabolism are those in the CYP2C, CYP2D, and CYP3A subfamilies. CYP3A4 is the most abundantly expressed and involved in the metabolism of about 50% of clinically used drugs (Figure 3–3A). The CYP1A, CYP1B, CYP2A, CYP2B, and CYP2E subfamilies are not significantly involved in the metabolism of therapeutic drugs, but they do catalyze the metabolic activation of many protoxins and procarcinogens to their ultimate reactive metabolites.

There are large differences in levels of expression of each CYP between individuals as assessed both by clinical pharmacologic studies and by analysis of expression in human liver samples. This large interindividual variability in CYP expression is due to the presence of genetic polymorphisms and differences in gene regulation (*see* below). Several human CYP genes exhibit polymorphisms, including *CYP2A6*, *CYP2C9*, *CYP2C19*, and *CYP2D6*. Allelic variants have been found in the *CYP1B1* and *CYP3A4* genes, but they are present at low frequencies in humans and appear not to have a major role in interindividual levels of expression of these enzymes. However, homozygous mutations in the *CYP1B1* gene are associated with primary congenital glaucoma.

Drug-Drug Interactions. Differences in the rate of metabolism of a drug can be due to drug interactions. Most commonly, this occurs when two drugs (*e.g.*, a statin and a macrolide antibiotic or antifungal) are coadministered and are metabolized by the same enzyme. Since most of these drug-drug interactions are due to CYPs, it becomes important to determine the identity of the CYP that metabolizes a particular drug and to avoid coadministering drugs that are metabolized by the same enzyme. Some drugs can also inhibit CYPs independently of being substrates for a CYP. For example, the common antifungal agent, *ketoconazole* (NIZORAL), is a potent inhibitor of CYP3A4 and other CYPs, and coadministration of ketoconazole with the anti-HIV viral protease inhibitors reduces the clearance of the protease inhibitor and increases its plasma concentration and the risk of toxicity. For most drugs, descriptive information found on the package insert lists the CYP that carries out its metabolism and the potential for drug interactions. Some drugs are CYP inducers that can induce not only their own metabolism, but also induce metabolism of other coadministered drugs (*see* below and Figure 3–13). Steroid hormones and herbal products such as St. John's wort can increase hepatic levels of CYP3A4, thereby increasing the metabolism of

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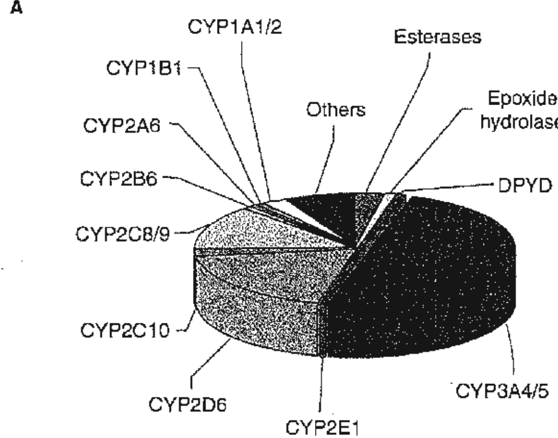
A



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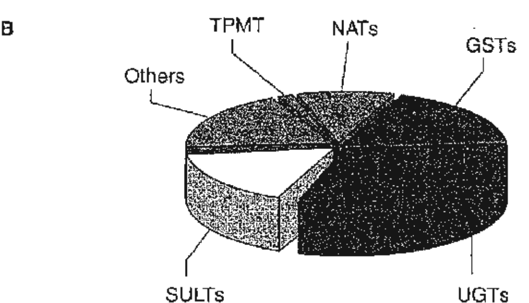

Figure 3–3. ***The fraction of clinically used drugs metabolized by the major phase 1 and phase 2 enzymes.*** The relative size of each pie section represents the estimated percentage of drugs metabolized by the major phase 1 (panel *A*) and phase 2 (panel *B*) enzymes, based on studies in the literature. In some cases, more than a single enzyme is responsible for metabolism of a single drug. CYP, cytochrome P450; DPYD, dihydropyrimidine dehydrogenase; GST, glutathione-S-transferase; NAT, N-acetyltransferase; SULT, sulfotransferase, TPMT, thiopurine methyltransferase; UGT, UDP-glucuronosyltransferase.

many orally administered drugs. Drug metabolism can also be influenced by diet. CYP inhibitors and inducers are commonly found in foods and in some cases these can influence the toxicity and efficacy of a drug. Components found in grapefruit juice (*e.g.*, naringin, furanocoumarins) are potent inhibitors of CYP3A4, and thus some drug inserts recommend not taking medication with grapefruit juice because it could increase the bioavailability of a drug.

Terfenadine, a once popular antihistamine, was removed from the market because its metabolism was blocked by CYP3A4 substrates such as *erythromycin* and grapefruit juice. Terfenadine is actually a prodrug that requires oxidation by CYP3A4 to its active metabolite, and at high doses the parent compound caused arrhythmias. Thus, elevated levels of parent drug in the plasma as a result of CYP3A4 inhibition caused ventricular tachycardia in some individuals, which ultimately led to its withdrawal from the market. In addition, interindividual differences in drug metabolism are significantly influenced by polymorphisms in CYPs. The CYP2D6 polymorphism has led to the withdrawal of several clinically used drugs (*e.g.*, *debrisoquine* and *perhexiline*) and the cautious use of others that are known CYP2D6 substrates (*e.g.*, *encainide* and *flecainide* [antiarrhythmics], *desipramine* and *nortriptyline* [antidepressants], and *codeine*).

Flavin-Containing Monooxygenases (FMOs). The FMOs are another superfamily of phase 1 enzymes involved in drug metabolism. Similar to CYPs, the FMOs are expressed at high levels in the liver and are bound to the endoplasmic reticulum, a site that favors interaction with and metabolism of hydrophobic drug substrates. There are six families of FMOs, with FMO3 being the most abundant in liver. FMO3 is able to metabolize nicotine as well as H_2-receptor antagonists (*cimetidine* and *ranitidine*), antipsychotics (*clozapine*), and antiemetics (*itopride*). A genetic deficiency in this enzyme causes the fish-odor syndrome due to a lack of metabolism of trimethylamine *N*-oxide (TMAO) to trimethylamine (TMA); in the absence of this enzyme, TMAO accumulates in the body and causes a socially offensive fish odor. TMAO is found at high concentrations, up to 15% by weight, in marine animals where it acts as an osmotic regulator. FMOs are considered minor contributors to drug metabolism and they almost always produce benign metabolites. In addition, FMOs are not induced by any of the xenobiotic receptors (*see* below) or easily inhibited; thus, in distinction to CYPs, FMOs would not be expected to be involved in drug-drug interactions. In fact, this has been demonstrated by comparing the pathways of metabolism of two drugs used in the control of gastric motility, itopride and *cisapride*. Itopride is metabolized by FMO3 while cisapride is metabolized by CYP3A4; thus, itopride is less likely to be involved in drug-drug interactions than is cisapride. CYP3A4 participates in drug-drug interactions through induction and inhibition of metabolism, whereas FMO3 is not induced or inhibited by any clinically used drugs. It remains a possibility that FMOs may be of importance in the development of new drugs. A candidate drug could be designed by introducing a site for FMO oxidation with the knowledge that selected metabolism and pharmacokinetic properties could be accurately calculated for efficient drug-based biological efficacy.

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Hydrolytic Enzymes. Two forms of *epoxide hydrolase* carry out hydrolysis of epoxides produced by CYPs. The soluble epoxide hydrolase (sEH) is expressed in the cytosol while the microsomal epoxide hydrolase (mEH) is localized to the membrane of the endoplasmic reticulum. Epoxides are highly reactive electrophiles that can bind to cellular nucleophiles found in protein, RNA, and DNA, resulting in cell toxicity and transformation. Thus, epoxide hydrolases participate in the deactivation of potentially toxic derivatives generated by CYPs. There are a few examples of the influence of mEH on drug metabolism. The antiepileptic drug *carbamazepine* is a prodrug that is converted to its pharmacologically active derivative, carbamazepine-10,11-epoxide by CYP. This metabolite is efficiently hydrolyzed to a dihydrodiol by mEH, resulting in inactivation of the drug (Figure 3–4). Inhibition of mEH can cause an elevation in plasma concentrations of the active metabolite, causing side effects. The tranquilizer *valnoctamide* and anticonvulsant *valproic acid* inhibit mEH, resulting in clinically significant drug interactions with carbamazepine. This has led to efforts to develop new antiepileptic drugs such as *gabapentin* and *levetiracetam* that are metabolized by CYPs and not by EHs.

The *carboxylesterases* comprise a superfamily of enzymes that catalyze the hydrolysis of ester- and amide-containing chemicals. These enzymes are found in both the endoplasmic reticulum and the cytosol of many cell types and are involved in detoxification or metabolic activation of various drugs, environmental toxicants, and carcinogens. Carboxylesterases also catalyze the activation of prodrugs to their respective free acids. For example, the prodrug and cancer chemotherapeutic agent *irinotecan* is a *camptothecin* analog that is bioactivated by plasma and intracellular carboxylesterases to the potent topoisomerase inhibitor SN-38 (Figure 3–5).

$CONH_2$

CYP

$CONH_2$

mEH

OH

OH

$CONH_2$

Figure 3–4. ***Metabolism of carbamazepine by CYP and microsomal epoxide hydrolase (mEH).*** Carbamazepine is oxidized to the pharmacologically-active metabolite carbamazepine-10,11-epoxide by CYP. The epoxide is converted to a trans-dihydrodiol by mEH. This metabolite is biologically inactive and can be conjugated by phase 2 enzymes.

CH_2CH_3

Irinotecan

H_3C OH O

H_2O CES2

CH_2CH_3

N—C=O + HO

SN-38

H_3C OH O

UDP-glucuronic acid UGT1A1/7 UDP

COOH

CH_2CH_3

SN-38 glucuronide

H_3C OH O

Figure 3–5. ***Metabolism of irinotecan (CPT-11).*** The prodrug CPT-11 is initially metabolized by a serum esterase (CES2) to the topoisomerase inhibitor SN-38, which is the active camptothecin analog that slows tumor growth. SN-38 is then subject to glucuronidation, which results in loss of biological activity and facilitates elimination of the SN-38 in the bile.

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Conjugating Enzymes. There are a large number of phase 2 conjugating enzymes, all of which are considered to be synthetic in nature since they result in the formation of a metabolite with an increased molecular mass. Phase 2 reactions also terminate the biological activity of the drug. The contributions of different phase 2 reactions to drug metabolism are shown in Figure 3–3B. Two of the phase 2 reactions, glucuronidation and sulfation, result in the formation of metabolites with a significantly increased water-to-lipid partition coefficient, resulting in hydrophilicity and facilitating their transport into the aqueous compartments of the cell and the body. Glucuronidation also markedly increases the molecular weight of the compound, a modification that favors biliary excretion. While sulfation and acetylation terminate the biological activity of drugs, the solubility properties of these metabolites are altered through minor changes in the overall charge of the molecule. Characteristic of the phase 2 reactions is the dependency on the catalytic reactions for cofactors such as UDP-glucuronic acid (UDP-GA) and 3′-phosphoadenosine-5′-phosphosulfate (PAPS), for UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT), respectively, which react with available functional groups on the substrates. The reactive functional groups are often generated by the phase 1 CYPs. All of the phase 2 reactions are carried out in the cytosol of the cell, with the exception of glucuronidation, which is localized to the luminal side of the endoplasmic reticulum. The catalytic rates of phase 2 reactions are significantly faster than the rates of the CYPs. Thus, if a drug is targeted for phase 1 oxidation through the CYPs, followed by a phase 2 conjugation reaction, usually the rate of elimination will depend upon the initial (phase 1) oxidation reaction. Since the rate of conjugation is faster and the process leads to an increase in hydrophilicity of the drug, phase 2 reactions are generally considered to assure the efficient elimination and detoxification of most drugs.

Glucuronidation. Among the more important of the phase 2 reactions in the metabolism of drugs is that catalyzed by UDP-glucuronosyltransferases (UGTs) (Figure 3–3B). These enzymes catalyze the transfer of glucuronic acid from the cofactor UDP-glucuronic acid to a substrate to form β-D-glucopyranosiduronic acids (glucuronides), metabolites that are sensitive to cleavage by β-glucuronidase. The generation of glucuronides can be formed through alcoholic and phenolic hydroxyl groups, carboxyl, sulfuryl, and carbonyl moieties, as well as through primary, secondary, and tertiary amine linkages. Examples of glucuronidation reactions are shown in Table 3–2 and Figure 3–5. The structural diversity in the many different types of drugs and xenobiotics that are processed through glucuronidation assures that most clinically efficacious therapeutic agents will be excreted as glucuronides.

There are 19 human genes that encode the UGT proteins. Nine are encoded by the *UGT1* locus and 10 are encoded by the *UGT2* family of genes. Both families of proteins are involved in the metabolism of drugs and xenobiotics, while the UGT2 family of proteins appears to have greater specificity for the glucuronidation of endogenous substances such as steroids. The UGT2 proteins are encoded by unique genes on chromosome 4 and the structure of each gene includes six exons. The clustering of the UGT2 genes on the same chromosome with a comparable organization of the regions encoding the open reading frames is evidence that gene duplication has occurred, a process of natural selection that has resulted in the multiplication and eventual diversification of the potential to detoxify the plethora of compounds that are targeted for glucuronidation.

The nine functional UGT1 proteins are all encoded by the *UGT1* locus (Figure 3–6), which is located on chromosome 2. The *UGT1* locus spans nearly 200 kb, with over 150 kb encoding a tandem array of cassette exonic regions that encode approximately 280 amino acids of the amino terminal portion of the UGT1A proteins. Four exons are located at the 3′ end of the locus that encode the carboxyl 245 amino acids that combine with one of the consecutively numbered array of first exons to form the individual *UGT1* gene products. Since exons 2 to 5 encode the same sequence for each UGT1A protein, the variability in substrate specificity for each of the UGT1A proteins results from the significant divergence in sequence encoded by the exon 1 regions. The 5′ flanking region of each first-exon cassette contains a fully functional promoter capable of initiating transcription in an inducible and tissue-specific manner.

From a clinical perspective, the expression of UGT1A1 assumes an important role in drug metabolism since the glucuronidation of bilirubin by UGT1A1 is the rate-limiting step in assuring efficient bilirubin clearance, and this rate can be affected by both genetic variation and competing substrates (drugs). Bilirubin is the breakdown product of heme, 80% of which originates from circulating hemoglobin and 20% from other heme-containing proteins such as the CYPs. Bilirubin is

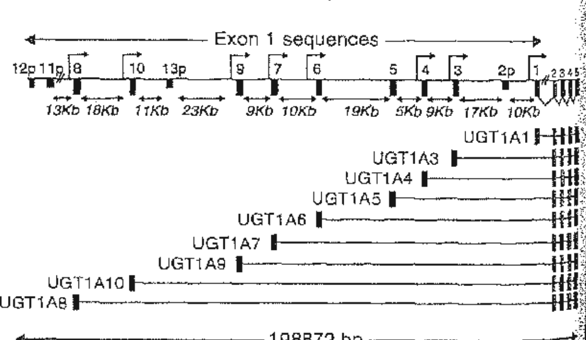

Figure 3–6. ***Organization of the UGT1A Locus.*** Transcription of the *UGT1A* genes commences with the activation of PolII, which is controlled through tissue-specific events. Conserved exons 2 to 5 are spliced to each respective exon 1 sequence resulting in the production of unique *UGT1A* sequences. The *UGT1A* locus encodes nine functional proteins.

hydrophobic, associates with serum albumin, and must be metabolized further by glucuronidation to assure its elimination. The failure to efficiently metabolize bilirubin by glucuronidation leads to elevated serum levels and a clinical symptom called hyperbilirubinemia or jaundice. There are more than 50 genetic lesions in the *UGT1A1* gene that can lead to inheritable unconjugated hyperbilirubinemia. Crigler-Najjar syndrome type I is diagnosed as a complete lack of bilirubin glucuronidation, while Crigler-Najjar syndrome type II is differentiated by the detection of low amounts of bilirubin glucuronides in duodenal secretions. Types I and II Crigler-Najjar syndrome are rare, and result from genetic polymorphisms in the open reading frames of the *UGT1A1* gene, resulting in abolished or highly diminished levels of functional protein.

Gilbert's syndrome is a generally benign condition that is present in up to 10% of the population; it is diagnosed clinically because circulating bilirubin levels are 60% to 70% higher than those seen in normal subjects. The most common genetic polymorphism associated with Gilbert's syndrome is a mutation in the *UGT1A1* gene promoter, which leads to reduced expression levels of UGT1A1. Subjects diagnosed with Gilbert's syndrome may be predisposed to adverse drug reactions resulting from a reduced capacity to metabolize drugs by UGT1A1. If a drug undergoes selective metabolism by UGT1A1, competition for drug metabolism with bilirubin glucuronidation will exist, resulting in pronounced hyperbilirubinemia as well as reduced clearance of metabolized drug. *Tranilast* [N-(3′4′-demethoxycinnamoyl)-anthranilic acid] is an investigational drug used for the prevention of restenosis in patients that have undergone transluminal coronary revascularization (intracoronary stents). Tranilast therapy in patients with Gilbert's syndrome has been shown to lead to hyperbilirubinemia as well as potential hepatic complications resulting from elevated levels of tranilast.

Gilbert's syndrome also alters patient responses to irinotecan. Irinotecan, a prodrug used in chemotherapy of solid tumors (*see* Chapter 51), is metabolized to its active form, SN-38, by serum carboxylesterases (Figure 3–5). SN-38, a potent topoisomerase inhibitor, is inactivated by UGT1A1 and excreted in the bile (Figures 3–7 and 3–8). Once in the lumen of the intestine, the SN-38 glucuronide undergoes cleavage by bacterial *β*-glucuronidase and re-enters the circulation through intestinal absorption. Elevated levels of SN-38 in the blood lead to hematological toxicities characterized by leukopenia and neutropenia, as well as damage to the intestinal epithelial cells (Figure 3–8), resulting in acute and life-threatening diarrhea. Patients with Gilbert's syndrome who are receiving irinotecan therapy are predisposed to the hematological and gastrointestinal toxicities resulting from elevated serum levels of SN-38, the net result of insufficient UGT1A activity and the consequent accumulation of a toxic drug in the GI epithelium.

The UGTs are expressed in a tissue-specific and often inducible fashion in most human tissues, with the highest concentration of enzymes found in the GI tract and liver. Based upon their physicochemical properties, glucuronides are excreted by the kidneys into the urine or through active transport processes through the apical surface of the liver hepatocytes into the bile ducts where they are transported to the duodenum for excretion with components of the bile. Most of the bile acids that are glucuronidated are reabsorbed back to the liver for reutilization by "enterohepatic

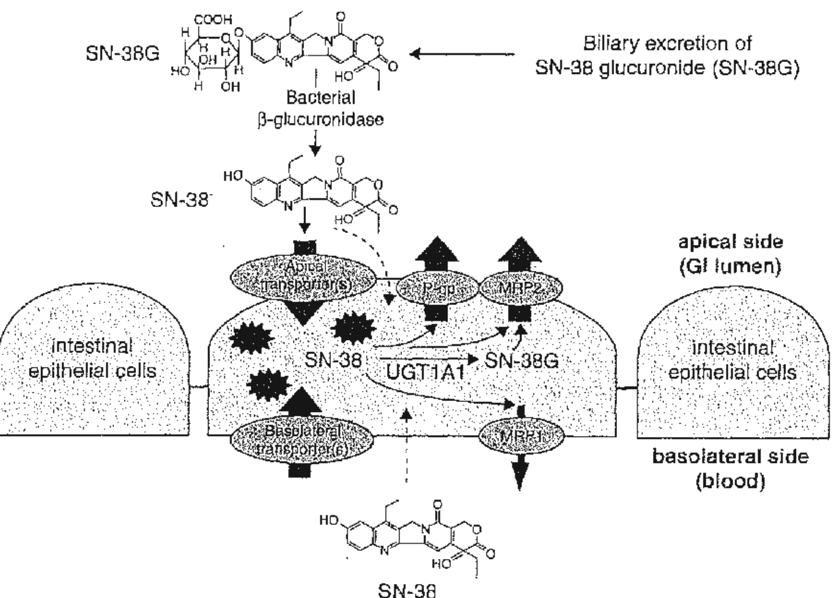

Figure 3–7. ***Routes of SN-38 transport and exposure to intestinal epithelial cells.*** SN-38 is transported into the bile following glucuronidation by liver UGT1A1 and extrahepatic UGT1A7. Following cleavage of luminal SN-38 glucuronide (SN-38G) by bacterial *β*-glucuronidase, reabsorption into epithelial cells can occur by passive diffusion (indicated by the dashed arrows entering the cell) as well as by apical transporters. Movement into epithelial cells may also occur from the blood by basolateral transporters. Intestinal SN-38 can efflux into the lumen through P-glycoprotein (P-gp) and multidrug resistance protein 2 (MRP2) and into the blood *via* MRP1. Excessive accumulation of the SN-38 in intestinal epithelial cells, resulting from reduced glucuronidation, can lead to cellular damage and toxicity (Tukey *et al.*, 2002).

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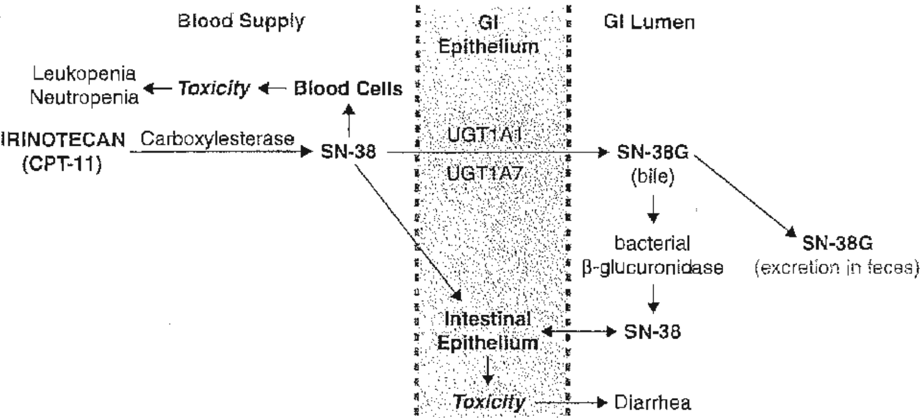

Figure 3–8. ***Cellular targets of SN-38 in the blood and intestinal tissues.*** Excessive accumulation of SN-38 can lead to blood toxicities such as leukopenia and neutropenia, as well as damage to the intestinal epithelium. These toxicities are pronounced in individuals that have reduced capacity to form the SN-38 glucuronide, such as patients with Gilbert's syndrome. Note the different body compartments and cell types involved (Tukey *et al.*, 2002).

recirculation"; many drugs that are glucuronidated and excreted in the bile can re-enter the circulation by this same process. The β-D-glucopyranosiduronic acids are targets for β-glucuronidase activity found in resident strains of bacteria that are common in the lower GI tract, liberating the free drug into the intestinal lumen. As water is reabsorbed into the large intestine, free drug can then be transported by passive diffusion or through apical transporters back into the intestinal epithelial cells, from which the drug can then re-enter the circulation. Through portal venous return from the large intestine to the liver, the reabsorption process allows for the re-entry of drug into the systemic circulation (Figures 3–7 and 3–8).

Sulfation. The sulfotransferases (SULTs) are located in the cytosol and conjugate sulfate derived from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the hydroxyl groups of aromatic and aliphatic compounds. Like all of the xenobiotic metabolizing enzymes, the SULTs metabolize a wide variety of endogenous and exogenous substrates. In humans, 11 SULT isoforms have been identified, and, based on evolutionary projections, have been classified into the SULT1 (SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SYLT1C4, SULT1E1), SULT2 (SULT2A1, SULT2B1-v1, SULT2B1-v2), and SULT4 (SULT4A1) families. SULTs play an important role in normal human homeostasis. For example, SULT1B1 is the predominant form expressed in skin and brain, carrying out the catalysis of cholesterol and thyroid hormones. Cholesterol sulfate is an essential metabolite in regulating keratinocyte differentiation and skin development. SULT1A3 is highly selective for catecholamines, while estrogens are sulfated by SULT1E1 and dehydroepiandrosterone (DHEA) is selectively sulfated by SULT2A1. In humans, significant fractions of circulating catecholamines, estrogens, iodothyronines, and DHEA exist in the sulfated form.

The different human SULTs display a variety of unique substrate specificities. The SULT1 family isoforms are considered to be the major forms involved in drug metabolism, with SULT1A1 being the most important and displaying extensive diversity in its capacity to catalyze the sulfation of a wide variety of structurally heterogeneous xenobiotics. The isoforms in the SULT1 family have been recognized as phenol SULTs, since they have been characterized to catalyze the sulfation of phenolic molecules such as *acetaminophen*, *minoxidil*, and *17α-ethinyl estradiol*. While two SULT1C isoforms exist, little is known about their substrate specificity toward drugs, although SULT1C4 is capable of sulfating the hepatic carcinogen N-OH-2-acetylaminofluorene. Both SULT1C2 and SULT1C4 are expressed abundantly in fetal tissues and decline in abundance in adults, yet little is known about their substrate specificities. SULT1E catalyzes the sulfation of endogenous and exogenous steroids, and has been found localized in liver as well as in hormone-responsive tissues such as the testis, breast, adrenal gland, and placenta.

The conjugation of drugs and xenobiotics is considered primarily a detoxification step, assuring that these agents are compartmented into the water compartments of the body for targeted elimination. However, drug metabolism through sulfation often leads to the generation of chemically reactive metabolites, where the sulfate is electron withdrawing and may be heterolytically cleaved, leading to the formation of an electrophilic cation. Most examples of the generation by sulfation of a carcinogenic or toxic response in animal or test mutagenicity assays have been documented with chemicals derived from the environment or from food mutagens generated from well-cooked meat. Thus, it is important to understand whether genetic linkages can be made by

associating known human SULT polymorphisms to cancer episodes that are felt to originate from environmental sources. Since SULT1A1 is the most abundant in human tissues and displays broad substrate specificity, the polymorphic profiles associated with this gene and the onset of various human cancers is of considerable interest. An appreciation of the structure of the proteins of the SULT family will aid in drug design and advance an understanding of the linkages relating sulfation to cancer susceptibility, reproduction, and development. The SULTs from the SULT1 and SULT2 families were among the first xenobiotic-metabolizing enzymes to be crystallized and the data indicate a highly conserved catalytic core (Figure 3–9A). The structures reveal the role of the co-substrate PAPS in catalysis, identifying the conserved amino acids that facilitate the 3′ phosphate's role in sulfuryl transfer to the protein and in turn to the substrate (Figure 3–9B). Crystal structures of the different SULTs indicate that while conservation in the PAPS binding region is maintained, the organization of the substrate binding region differs, helping to explain the observed differences in catalytic potential displayed with the different SULTs.

Glutathione Conjugation. The glutathione-S-transferases (GSTs) catalyze the transfer of glutathione to reactive electrophiles, a function that serves to protect cellular macromolecules from interacting with electrophiles that contain electrophilic heteroatoms (-O, -N, and -S) and in turn protects the cellular environment from damage. The co-substrate in the reaction is the tripeptide glutathione, which is synthesized from γ-glutamic acid, cysteine, and glycine (Figure 3–10). Glutathione exists in the cell as oxidized (GSSG) or reduced (GSH), and the ratio of GSH:GSSG is critical in maintaining a cellular environment in the reduced state. In addition to affecting xenobiotic conjugation with GSH, a severe reduction in GSH content can predispose cells to oxidative damage, a state that has been linked to a number of human health issues.

In the formation of glutathione conjugates, the reaction generates a thioether linkage with drug or xenobiotic to the cysteine moiety of the tripeptide. Characteristically, all GST substrates contain an electrophilic atom and are hydrophobic, and by nature will associate with cellular proteins. Since the concentration of glutathione in cells is usually very high, typically ~7 μmol/g of liver, or in the 10 mM range, many drugs and xenobiotics can react nonenzymatically with glutathione. However, the GSTs have been found to occupy up to 10% of the total cellular protein concentration, a property that assures efficient conjugation of glutathione to reactive electrophiles. The high concentration of GSTs also provides the cells with a sink of cytosolic protein, a property that facilitates noncovalent and sometimes covalent interactions with compounds that are not substrates for glutathione conjugation. The cytosolic pool of GSTs, once identified as *ligandin*, has been shown to bind steroids, bile acids, bilirubin, cellular hormones, and environmental toxicants, in addition to complexing with other cellular proteins.

Over 20 human GSTs have been identified and divided into two subfamilies: the cytosolic and the microsomal forms. The major differences in function between the microsomal and cytosolic GSTs reside in the selection of substrates for conjugation; the cytosolic forms have more importance in the metabolism of drugs and xenobiotics, whereas the microsomal GSTs are important in the endogenous metabolism of leukotrienes and prostaglandins. The cytosolic GSTs are divided into seven classes termed alpha (GSTA1 and 2), mu (GSTM1 through 5), omega (GSTO1), pi (GSTP1), sigma (GSTS1), theta (GSTT1 and 2), and zeta (GSTZ1). Those in the alpha and mu classes can form heterodimers, allowing for a large number of active transferases to form. The cytosolic forms of GST catalyze conjugation, reduction, and isomerization reactions.

The high concentrations of GSH in the cell, as well as the overabundance of GSTs, means that few reactive molecules escape detoxification. However, while there appears to be an overcapacity of enzyme and reducing equivalents, there is always concern that some reactive intermediates will escape detoxification, and by nature of their electrophilicity, will bind to cellular components and cause toxicity. The potential for such an occurrence is heightened if GSH is depleted or if a specific form of GST is polymorphic. While it is difficult to deplete cellular GSH levels, therapeutic agents that require large doses to be clinically efficacious have the greatest potential to lower cellular GSH levels. Acetaminophen, which is normally metabolized by glucuronidation and sulfation, is also a substrate for oxidative metabolism by CYP2E1, which generates the toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). An overdose of acetaminophen can lead to depletion of cellular GSH levels, thereby increasing the potential for NAPQI to interact with other cellular components. Acetaminophen toxicity is associated with increased levels of NAPQI and tissue necrosis.

Like many of the enzymes involved in drug and xenobiotic metabolism, all of the GSTs have been shown to be polymorphic. The mu (GSTM1*0) and theta (GSTT1*0) genotypes express a null phenotype; thus, individuals that are polymorphic at these loci are predisposed to toxicities by agents that are selective substrates for these GSTs. For example, the GSTM1*0 allele is observed in 50% of the Caucasian population and has been linked genetically to human malignancies of the lung, colon, and bladder. Null activity in the GSTT1 gene has been associated with adverse side effects and toxicity in cancer chemotherapy with cytostatic drugs; the toxicities result from insufficient clearance of the drugs *via* GSH conjugation. Expression of the null genotype can be as high as 60% in Chinese and Korean populations. Therapies may alter efficacies, with an increase in severity of adverse side effects.

While the GSTs play an important role in cellular detoxification, their activities in cancerous tissues have been linked to the development of drug resistance toward chemotherapeutic agents that are both substrates and nonsubstrates for the GSTs. Many anticancer drugs are effective because they initiate cell death or apoptosis, which is linked to the activation of mitogen-activated protein (MAP) kinases such as JNK and p38. Investigational studies have demonstrated that overexpression of GSTs is associated with resistance to apoptosis and the inhibition of MAP kinase activity. In a variety of tumors, the levels of GSTs are overexpressed, which

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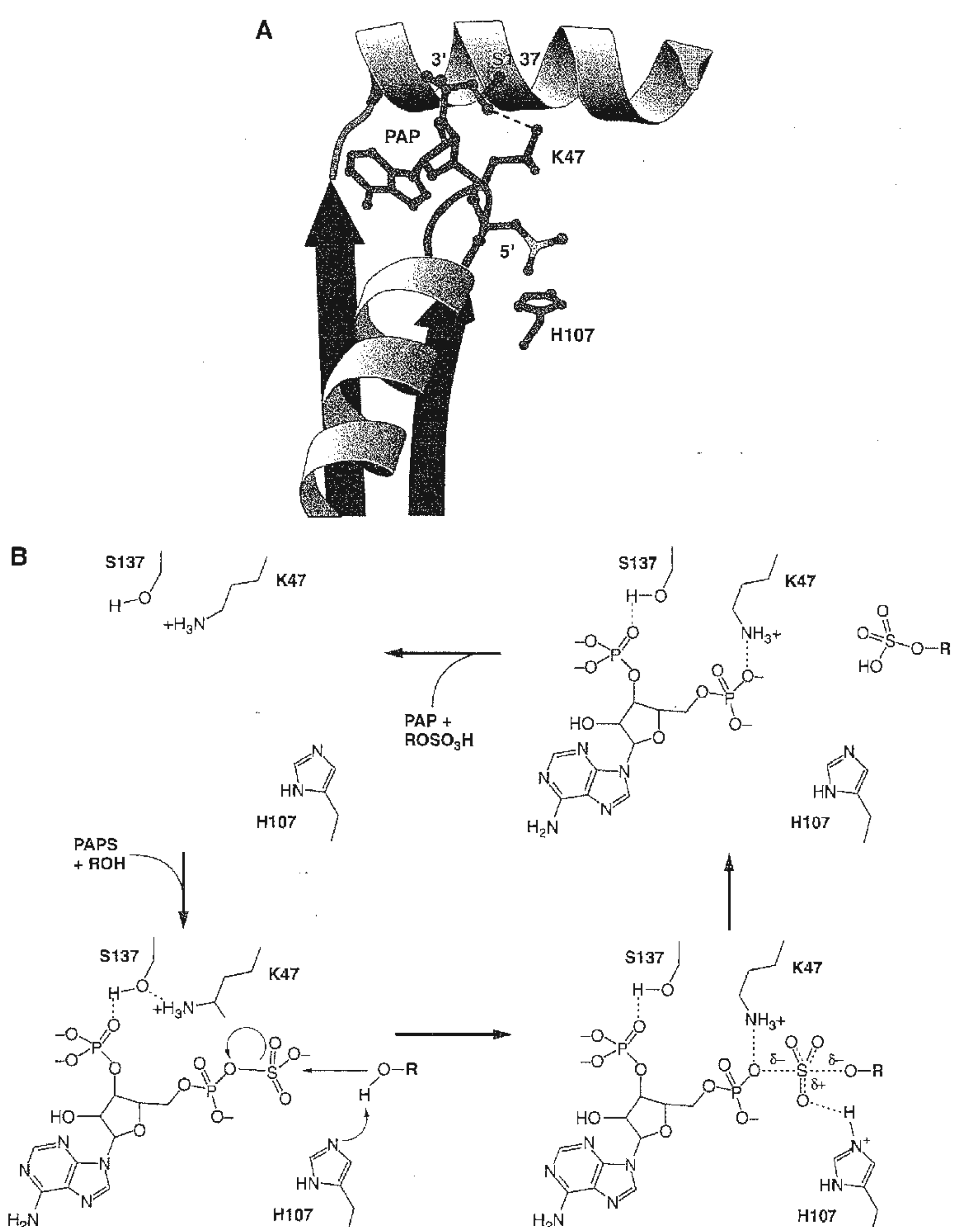

Figure 3–9. ***The proposed reaction mechanism of sulfuryl transfer catalyzed by the sulfotransferases (SULTs).*** *A.* Shown in this figure are the conserved strand-loop-helix and strand-turn-helix structure of the catalytic core of all SULTs where PAPS and xenobiotics bind. Shown is the hydrogen bonding interaction of PAPS with Lys[47] and Ser[137] with His[107] which complexes with substrate (xenobiotic). ***B.*** The proposed reaction mechanism shows the transfer of the sulfuryl group from PAPS to the OH-group on the substrate and the interactions of the conserved SULT residues in this reaction. (For additional information *see* Negishi *et al.*, 2001.)

X (substrate) → GST → GSH → X-GSH

Figure 3–10. Glutathione as a co-substrate in the conjugation of a drug or xenobiotic (X) by glutathione-S-transferase (GST).

leads to a reduction in MAP kinase activity and reduced efficacy of chemotherapy. Taking advantage of the relatively high levels of GST in tumor cells, inhibition of GST activity has been exploited as a therapeutic strategy to modulate drug resistance by sensitizing tumors to anticancer drugs. TLK199, a glutathione analog, serves as a prodrug that undergoes activation by plasma esterases to a GST inhibitor, TLK117, which potentiates the toxicity of different anticancer agents (Figure 3–11). Alternatively, the elevated level of GST activity in cancerous cells has been utilized to develop prodrugs that can be activated by the GSTs to form electrophilic intermediates. TLK286 is a substrate for GST that undergoes a β-elimination reaction, forming a glutathione conjugate and a nitrogen mustard (Figure 3–12) that is capable of alkylating cellular nucleophiles, resulting in antitumor activity.

N-Acetylation. The cytosolic N-acetyltransferases (NATs) are responsible for the metabolism of drugs and environmental agents that contain an aromatic amine or hydrazine group. The addition of the acetyl group from the cofactor acetyl-coenzyme A often leads to a metabolite that is less water soluble because the potential ionizable amine is neutralized by the covalent addition of the acetyl group. NATs are among the most polymorphic of all the human xenobiotic drug-metabolizing enzymes.

The characterization of an acetylator phenotype in humans was one of the first hereditary traits identified, and was responsible for the development of the field of pharmacogenetics (*see* Chapter 4). Following the discovery that isonicotinic acid hydrazide (*isoniazid*) could be used in the cure of tuberculosis, a significant proportion of the patients (5% to 15%) experienced toxicities that ranged from numbness and tingling in their fingers to CNS damage. After finding that isoniazid was metabolized by acetylation and excreted in the urine, researchers noted that individuals suffering from the toxic effects of the drug excreted the largest amount of unchanged drug and the least amount of acetylated isoniazid. Pharmacogenetic studies led to the classification of "rapid" and "slow" acetylators, with the "slow" phenotype being predisposed to toxicity. Purification and characterization of N-acetyltransferase and the eventual cloning of its RNA provided sequence characterization of the gene for slow and fast acetylators, revealing polymorphisms that correspond to the "slow" acetylator phenotype. There are two functional NAT genes in humans, *NAT1* and *NAT2*. Over 25 allelic variants of *NAT1* and *NAT2* have been characterized, and in individuals in whom acetylation of drugs is compromised, homozygous genotypes for at least two variant alleles are required to predispose a patient to lowered drug metabolism. Polymorphism in the *NAT2* gene and its association with the slow acetylation of isoniazid was one of the first completely characterized genotypes shown to impact drug metabolism, thereby linking pharmacogenetic phenotype to a genetic polymorphism. Although nearly as many mutations have been identified in the *NAT1* gene as the *NAT2* gene, the frequency of the *slow* acetylation patterns are attributed mostly to polymorphism in the *NAT2* gene.

A list of drugs that are subject to acetylation and their known toxicities are listed in Table 3–3. The therapeutic relevance of NAT polymorphisms is in avoiding drug-induced toxicities. The adverse drug response in a slow acetylator resembles a drug overdose; thus, reducing the dose or increasing the dosing interval is recommended. Drugs containing an aromatic amine or a hydrazine group

TLK199 (prodrug)

H_2O → cellular esterases → $HOCH_2CH_3$

TLK177 (active GST inhibitor)

Figure 3–11. Activation of TLK199 by cellular esterases to the glutathione-S-transferase (GST) inhibitor TLK117. (For additional information, *see* Townsend and Tew, 2003.)

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Figure 3–12. ***Generation of the reactive alkylating agent following the conjugation of glutathione to TLK286.*** GST interacts with the prodrug and GSH analog, TLK286, *via* a tyrosine in the active site of GST. GSH portion is shown in blue. The interaction promotes β-elimination and cleavage of the prodrug to a vinyl sulfone and an active alkylating fragment. (*See* Townsend and Tew, 2003.)

exist in many classes of clinically used drugs, and if a drug is known to be subject to drug metabolism through acetylation, confirming an individual's phenotype can be important. For example, *hydralazine*, a once popular orally active antihypertensive (vasodilator) drug, is metabolized by NAT2. The administration of therapeutic doses of hydralazine to a slow acetylator can result in extreme hypotension and tachycardia. Several drugs, such as the sulfonamides, that are known targets for acetylation have been implicated in idiosyncratic hypersensitivity reactions; in such instances, an appreciation of a patient's acetylating phenotype is particularly important. Sulfonamides are transformed into hydroxylamines that interact with cellular proteins, generating haptens that can elicit autoimmune responses. Individuals who are slow acetylators are predisposed to drug-induced autoimmune disorders.

Tissue-specific expression patterns of NAT1 and NAT2 have a significant impact on the fate of drug metabolism and the potential for eliciting a toxic episode. NAT1 seems to be ubiquitously expressed among most human tissues, whereas NAT2 is found in liver and the GI tract. Characteristic of both NAT1 and NAT2 is the ability to form N-hydroxy–acetylated metabolites from bicyclic aromatic hydrocarbons, a reaction that leads to the nonenzymatic release of the acetyl group and the generation of highly reactive nitrenium ions. Thus, N-hydroxy acetylation is thought to activate certain environmental toxicants. In contrast, direct N-acetylation of the environmentally generated bicyclic aromatic amines is stable and leads to detoxification. Individuals who are NAT2 fast acetylators are able to efficiently metabolize and detoxify bicyclic aromatic amine through liver-dependent acetylation. Slow acetylators (NAT2 deficient), however, accumulate bicyclic aromatic amines, which then become substrates for CYP-dependent N-oxidation. These N-OH metabolites are eliminated in the urine. In tissues such as bladder epithelium, NAT1 is highly expressed and can efficiently catalyze the N-hydroxy acetylation of bicyclic aromatic amines, a process that leads to de-acetylation and the formation of the mutagenic nitrenium ion, especially in NAT2-deficient subjects. Epidemiological studies have shown that slow acetylators are predisposed to bladder cancer if exposed environmentally to bicyclic aromatic amines.

Methylation. In humans, drugs and xenobiotics can undergo O-, N-, and S-methylation. The identification of the individual methyltransferase (MT) is based on the substrate and methyl conjugate. Humans express three N-methyltransferases, one catechol-O-methyltransferase (COMT) a phenol-O-methyltransferase (POMT), a thiopurine S-methyltransferase (TPMT), and a thiol methyl-

Table 3–3
Indications and Unwanted Side Effects of Drugs Metabolized by N-Acetyltransferases

DRUG	INDICATION	MAJOR SIDE EFFECTS
Acebutolol	Arrhythmias, hypertension	Drowsiness, weakness, insomnia
Amantadine	Influenza A, parkinsonism	Appetite loss, dizziness, headache, nightmares
Aminobenzoic acid	Skin disorders, sunscreens	Stomach upset, contact sensitization
Aminoglutethimide	Adrenal cortex carcinoma, breast cancer	Clumsiness, nausea, dizziness, agranulocytosis
Aminosalicylic acid	Ulcerative colitis	Allergic fever, itching, leukopenia
Amonafide	Prostate cancer	Myelosuppression
Amrinone	Advanced heart failure	Thrombocytopenia, arrhythmias
Benzocaine	Local anesthesia	Dermatitis, itching, rash, methemoglobinemia
Caffeine	Neonatal respiratory distress syndrome	Dizziness, insomnia, tachycardia
Clonazepam	Epilepsy	Ataxia, dizziness, slurred speech
Dapsone	Dermatitis, leprosy, AIDS-related complex	Nausea, vomiting, hyperexcitability, methemoglobinemia, dermatitis
Dipyrone, metamizole	Analgesic	Agranulocytosis
Hydralazine	Hypertension	Hypotension, tachycardia, flush, headache
Isoniazid	Tuberculosis	Peripheral neuritis, hepatotoxicity
Nitrazepam	Insomnia	Dizziness, somnolence
Phenelzine	Depression	CNS excitation, insomnia, orthostatic hypotension, hepatotoxicity
Procainamide	Ventricular tachyarrhythmia	Hypotension, systemic lupus erythematosus
Sulfonamides	Antibacterial agents	Hypersensitivity, hemolytic anemia, fever, lupuslike syndromes

For details, *see* Meisel, 2002.

transferase (TMT). All of the MTs exist as monomers and use S-adenosyl-methionine (SAM; AdoMet) as the methyl donor. With the exception of a signature sequence that is conserved among the MTs, there is limited conservation in sequence, indicating that each MT has evolved to display a unique catalytic function. Although the common theme among the MTs is the generation of a methylated product, substrate specificity is high and distinguishes the individual enzymes.

Nicotinamide N-methyltransferase (NNMT) methylates serotonin and tryptophan, and pyridine-containing compounds such as nicotinamide and nicotine. Phenylethanolamine N-methyltransferase (PNMT) is responsible for the methylation of the neurotransmitter norepinephrine, forming epinephrine; the histamine N-methyltransferase (HNMT) metabolizes drugs containing an imidazole ring such as that found in histamine. COMT methylates neurotransmitters containing a catechol moiety such as dopamine and norepinephrine, drugs such as *methyldopa*, and drugs of abuse such as *ecstasy* (MDMA; 3,4-methylenedioxymethamphetamine).

From a clinical perspective, the most important MT may be TPMT, which catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds, including the thiopurine drugs *azathioprine* (AZA), *6-mercaptopurine* (6-MP), and *thioguanine*. AZA and 6-MP are used for the management of inflammatory bowel disease (*see* Chapter 38) as well as autoimmune disorders such as systemic lupus erythematosus and rheumatoid arthritis. Thioguanine is used in acute myeloid leukemia, and 6-MP is used worldwide for the treatment of childhood acute lymphoblastic leukemia (*see* Chapter 51). Because TPMT is responsible for the detoxification of 6-MP, a genetic deficiency in TPMT can result in severe toxicities in patients taking these drugs. When given orally at clinically established doses, 6-MP serves as a prodrug that is metabolized by hypoxanthine guanine phosphoribosyl transferase (HGPRT) to 6-thioguanine nucleotides (6-TGNs), which become incorporated into DNA and RNA, resulting in arrest of DNA replication and cytotoxicity. The toxic side effects arise when a lack of 6-MP methylation by TPMT causes a build-up of 6-MP, resulting in the generation of toxic levels of 6-TGNs. The identification of the inactive TPMT alleles and the development of a genotyping test to identify homozygous carriers of the defective allele have now made it possible to identify individuals who may be predisposed to the toxic side effects of 6-MP therapy. Simple adjustments in the patient's dosage regimen have been shown to be a life-saving intervention for those with TPMT deficiencies.

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The Role of Xenobiotic Metabolism in the Safe and Effective Use of Drugs. Any compound entering the body must be eliminated through metabolism and excretion *via* the urine or bile/feces. This mechanism keeps foreign compounds from accumulating in the body and possibly causing toxicity. In the case of drugs, metabolism results in the inactivation of their therapeutic effectiveness and facilitates their elimination. The extent of metabolism can determine the efficacy and toxicity of a drug by controlling its biological half-life. Among the most serious considerations in the clinical use of drugs are adverse drug reactions. If a drug is metabolized too quickly, it rapidly loses its therapeutic efficacy. This can occur if specific enzymes involved in metabolism are overly active or are induced by dietary or environmental factors. If a drug is metabolized too slowly, the drug can accumulate in the bloodstream; as a consequence, the pharmacokinetic parameter AUC (area under the plasma concentration–time curve) is elevated and the plasma clearance of the drug is decreased. This increase in AUC can lead to overstimulation of some target receptors or undesired binding to other receptors or cellular macromolecules. An increase in AUC often results when specific xenobiotic-metabolizing enzymes are inhibited, which can occur when an individual is taking a combination of different therapeutic agents and one of those drugs targets the enzyme involved in drug metabolism. For example, the consumption of grapefruit juice with drugs taken orally can inhibit intestinal CYP3A4, blocking the metabolism of many of these drugs. The inhibition of specific CYPs in the gut by dietary consumption of grapefruit juice alters the oral bioavailability of many classes of drugs, such as certain antihypertensives, immunosuppressants, antidepressants, antihistamines, and the statins, to name a few. Among the components of grapefruit juice that inhibit CYP3A4 are naringin and furanocoumarins.

While environmental factors can alter the steady-state levels of specific enzymes or inhibit their catalytic potential, these phenotypic changes in drug metabolism are also observed clinically in groups of individuals that are genetically predisposed to adverse drug reactions because of pharmacogenetic differences in the expression of xenobiotic-metabolizing enzymes (*see* Chapter 4). Most of the xenobiotic-metabolizing enzymes display polymorphic differences in their expression, resulting from heritable changes in the structure of the genes. For example, as discussed above, a significant population was found to be hyperbilirubinemic, resulting from a reduction in the ability to glucuronidate circulating bilirubin due to a lowered expression of the *UGT1A1* gene (Gilbert's syndrome). Drugs that are subject to glucuronidation by UGT1A1, such as the topoisomerase inhibitor SN-38 (Figures 3–5, 3–7, and 3–8), will display an increased AUC because individuals with Gilbert's syndrome are unable to detoxify these drugs. Since most cancer chemotherapeutic agents have a very narrow therapeutic index, increases in the circulating levels of the active form can result in significant toxicities. There are a number of genetic differences in CYPs that can have a major impact on drug therapy.

Nearly every class of therapeutic agent has been reported to initiate an adverse drug response (ADR). In the United States, the cost of such response has been estimated at $100 billion and to be the cause of over 100,000 deaths annually. It has been estimated that 56% of drugs that are associated with adverse responses are subjected to metabolism by the xenobiotic-metabolizing enzymes, notably the CYPs, which metabolize 86% of these compounds. Since many of the CYPs are subject to induction as well as inhibition by drugs, dietary factors, and other environmental agents, these enzymes play an important role in most ADRs. Thus, it has become mandatory that before a new drug application (NDA) is filed with the Food and Drug Administration, the route of metabolism and the enzymes involved in the metabolism must be known. As a result, it has become routine practice in the pharmaceutical industry to establish which enzymes are involved in metabolism of a drug candidate and to identify the metabolites and determine their potential toxicity.

Induction of Drug Metabolism. Xenobiotics can influence the extent of drug metabolism by activating transcription and inducing the expression of genes encoding drug-metabolizing enzymes. Thus, a foreign compound may induce its own metabolism, as may certain drugs. One potential consequence of this is a decrease in plasma drug concentration over the course of treatment, resulting in loss of efficacy, as the auto-induced metabolism of the drug exceeds the rate at which new drug enters the body. A list of ligands and the receptors through which they induce drug metabolism is shown in Table 3–4. A particular receptor, when activated by a ligand, can induce the transcription of a battery of target genes. Among these target genes are certain CYPs and drug transporters. Thus, any drug that is a ligand for a receptor that induces CYPs and transporters could lead to drug interactions. Figure 3–13 shows the scheme by which a drug may interact with nuclear receptors to induce its own metabolism.

The aryl hydrocarbon receptor (AHR) is a member of a superfamily of transcription factors with diverse roles in mammals, such as a regulatory role in the development of the mammalian CNS and modulating the response to chemical and oxidative stress. This

Table 3–4
Nuclear Receptors That Induce Drug Metabolism

RECEPTOR	LIGANDS
Aryl hydrocarbon receptor (AHR)	Omeprazole
Constitutive androstane receptor (CAR)	Phenobarbital
Pregnane X receptor (PXR)	Rifampin
Farnesoid X receptor (FXR)	Bile acids
Vitamin D receptor	Vitamin D
Peroxisome proliferator-activated receptor (PPAR)	Fibrates
Retinoic acid receptor (RAR)	*all-trans*-Retinoic acid
Retinoid X receptor (RXR)	9-*cis*-Retinoic acid

superfamily of transcription factors includes Per and Sim, two transcription factors involved in development of the CNS, and the hypoxia-inducible factor 1α (HIF1α) that activates genes in response to low cellular O_2 levels. The AHR induces expression of genes encoding CYP1A1 and CYP1A2, two CYPs that are able to metabolically activate chemical carcinogens, including environmental contaminants and carcinogens derived from food. Many of these substances are inert unless metabolized by CYPs. Thus, induction of these CYPs by a drug could potentially result in an increase in the toxicity and carcinogenicity of procarcinogens. For example, *omeprazole*, a proton pump inhibitor used to treat gastric and duodenal ulcers (*see* Chapter 36), is a ligand for the AHR and can induce CYP1A1 and CYP1A2, with the possible consequences of toxin/carcinogen activation as well as drug-drug interactions in patients receiving agents that are substrates for either of these CYPs.

Another important induction mechanism is due to type 2 nuclear receptors that are in the same superfamily as the steroid hormone receptors. Many of these receptors, identified on the basis of their structural similarity to steroid hormone receptors, were originally termed "orphan receptors," because no endogenous ligands were known to interact with them. Subsequent studies revealed that some of these receptors are activated by xenobiotics, including drugs. The type 2 nuclear receptors of most importance to

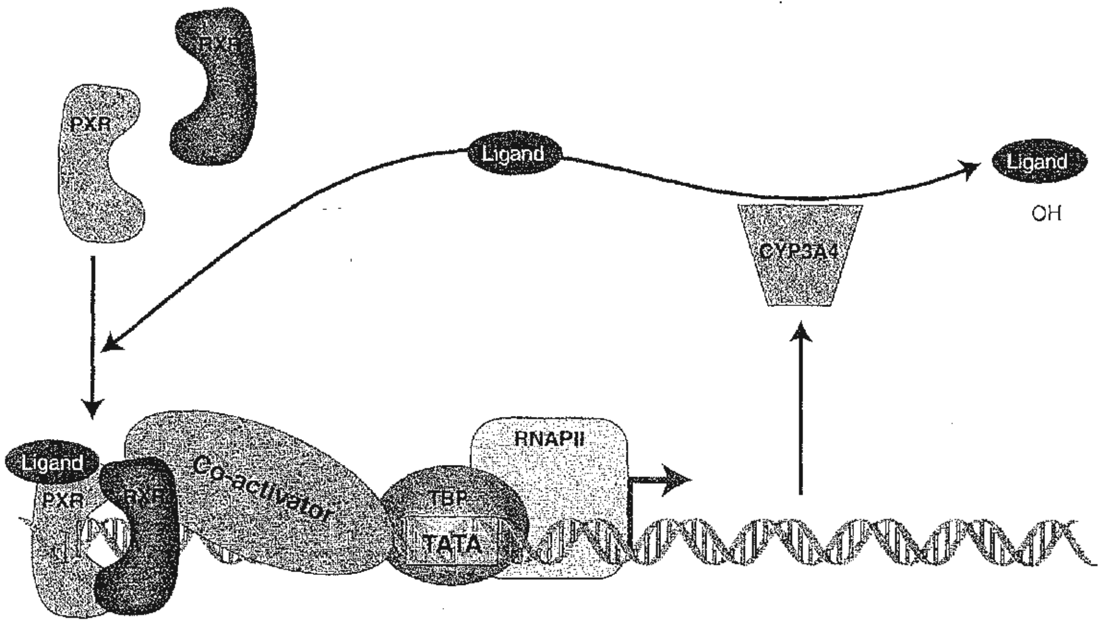

Figure 3–13. ***Induction of drug metabolism by nuclear receptor–mediated signal transduction.*** When a drug such as atorvastatin (Ligand) enters the cell, it can bind to a nuclear receptor such as the pregnane X receptor (PXR). PXR then forms a complex with the retinoid X receptor (RXR), binds to DNA upstream of target genes, recruits coactivator (which binds to the TATA box binding protein, TBP), and activates transcription. Among PXR target genes are CYP3A4, which can metabolize the atorvastatin and decrease its cellular concentration. Thus, atorvastatin induces its own metabolism. Atorvastatin undergoes both ortho and para hydroxylation. (*See* Handschin and Meyer, 2003.)

drug metabolism and drug therapy include the pregnane X receptor (PXR), constitutive androstane receptor (CAR), and the peroxisome proliferator activated receptor (PPAR). PXR, discovered based on its ability to be activated by the synthetic steroid pregnane 16α-carbonitrile, is activated by a number of drugs including, antibiotics (*rifampicin* and *troleandomycin*), Ca^{2+} channel blockers (*nifedipine*), statins (*mevastatin*), antidiabetic drugs (*troglitazone*), HIV protease inhibitors (*ritonavir*), and anticancer drugs (*paclitaxel*). Hyperforin, a component of St. John's wort, an over-the-counter herbal remedy used for depression, also activates PXR. This activation is thought to be the basis for the increase in failure of oral contraceptives in individuals taking St. John's wort: activated PXR is an inducer of CYP3A4, which can metabolize steroids found in oral contraceptives. PXR also induces the expression of genes encoding certain drug transporters and phase 2 enzymes including SULTs and UGTs. Thus, PXR facilitates the metabolism and elimination of xenobiotics, including drugs, with notable consequences (Figure 3–13).

The nuclear receptor CAR was discovered based on its ability to activate genes in the absence of ligand. Steroids such as *androstanol*, the antifungal agent *clotrimazole*, and the antiemetic *meclizine* are inverse agonists that inhibit gene activation by CAR, while the pesticide 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, the steroid 5β-pregnane-3,20-dione, and probably other endogenous compounds, are agonists that activate gene expression when bound to CAR. Genes induced by CAR include those encoding several CYPs (CYP2B6, CYP2C9, and CYP3A4), various phase 2 enzymes (including GSTs, UGTs, and SULTs), and drug and endobiotic transporters. CYP3A4 is induced by both PXR and CAR and thus its level is highly influenced by a number of drugs and other xenobiotics. In addition to a potential role in inducing the degradation of drugs including the over-the-counter analgesic acetaminophen, this receptor may function in the control of bilirubin degradation, the process by which the liver decomposes heme.

Clearly, PXR and CAR have a capacity for binding a great variety of ligands. As with the xenobiotic-metabolizing enzymes, species differences also exist in the ligand specificities of these receptors. For example, rifampicin activates human PXR but not mouse or rat PXR. Meclizine preferentially activates mouse CAR but inhibits gene induction by human CAR. These findings further establish that rodent model systems do not reflect the response of humans to drugs.

The peroxisome proliferator activated receptor (PPAR) family is composed of three members, α, β, and γ. PPARα is the target for the fibrate class of hyperlipidemic drugs, including the widely prescribed *gemfibrozil* and *fenofibrate*. While activation of PPARα results in induction of target genes encoding fatty acid metabolizing enzymes that result in lowering of serum triglycerides, it also induces CYP4 enzymes that carry out the oxidation of fatty acids and drugs with fatty acid–containing side chains, such as *leukotriene* and *arachidonic acid* analogs.

Role of Drug Metabolism in the Drug Development Process. There are two key elements associated with successful drug development: efficacy and safety. Both depend on drug metabolism. It is necessary to determine how and by which enzymes a potential new drug is metabolized. This knowledge allows prediction of whether the compound may cause drug-drug interactions or be susceptible to marked interindividual variation in metabolism due to genetic polymorphisms.

Historically, drug candidates have been administered to rodents at doses well above the human target dose in order to predict acute toxicity. For drug candidates to be used chronically in humans, such as for lowering serum triglycerides and cholesterol or for treatment of type 2 diabetes, long-term carcinogenicity studies are carried out in rodent models. For determination of metabolism, the compound is subjected to analysis by human liver cells or extracts from these cells that contain the drug-metabolizing enzymes. Such studies determine how humans will metabolize a particular drug, and to a limited extent, predict the rate of metabolism. If a CYP is involved, a panel of recombinant CYPs can be used to determine which CYP predominates in the metabolism of the drug. If a single CYP, such as CYP3A4, is found to be the sole CYP that metabolizes a drug candidate, then a decision can be made about the likelihood of drug interactions. Interactions become a problem when multiple drugs are simultaneously administered, for example in elderly patients, who on a daily basis may take prescribed antiinflammatory drugs, cholesterol-lowering drugs, blood pressure medications, a gastric acid suppressant, an anticoagulant, and a number of over-the-counter medications. Ideally, the best drug candidate would be metabolized by several CYPs so that variability in expression levels of one CYP or drug-drug interactions would not significantly impact its metabolism and pharmacokinetics.

Similar studies can be carried out with phase 2 enzymes and drug transporters in order to predict the metabolic fate of a drug. In addition to the use of recombinant human xenobiotic-metabolizing enzymes in predicting drug metabolism, human receptor–based (PXR and CAR) systems should also be used to determine whether a particular drug candidate could be a ligand for PXR, CAR, or PPARα.

Computer-based computational (*in silico*) prediction of drug metabolism may also be a prospect for the future, since the structures of several CYPs have been determined, including those of CYPs 2A6, 2C9, and 3A4. These structures may be used to predict metabolism of a drug candidate by fitting the compound to the enzyme's active site and determining oxidation potentials of sites on the molecule. However, the structures, determined by x-ray analysis

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of crystals of enzyme-substrate complexes, are static, whereas enzymes are flexible, and this vital distinction may be limiting. The large size of the CYP active sites, which permits them to metabolize many different compounds, also renders them difficult to model. The potential for modeling ligand or activator for the nuclear receptors also exists with limitations similar to those discussed for the CYPs. With refinement of structures and more powerful modeling software, *in silico* drug metabolism may be a reality in the future.

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The ABC of Solute Carriers

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Andrew P. Halestrap · David Meredith

The *SLC16* gene family—from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond

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Abstract The monocarboxylate cotransporter (MCT) family now comprises 14 members, of which only the first four (MCT1–MCT4) have been demonstrated experimentally to catalyse the proton-linked transport of metabolically important monocarboxylates such as lactate, pyruvate and ketone bodies. *SLC16A10* (T-type amino-acid transporter-1, TAT1) is an aromatic amino acid transporter whilst the other members await characterization. MCTs have 12 transmembrane domains (TMDs) with intracellular N- and C-termini and a large intracellular loop between TMDs 6 and 7. MCT1 and MCT4 require a monotopic ancillary protein, CD147, for expression of functional protein at the plasma membrane. Lactic acid transport across the plasma membrane is fundamental for the metabolism of and pH regulation of all cells, removing lactic acid produced by glycolysis and allowing uptake by those cells utilizing it for gluconeogenesis (liver and kidney) or as a respiratory fuel (heart and red muscle). The properties of the different MCT isoforms and their tissue distribution and regulation reflect these roles.

Keywords Lactate · Intracellular pH · Glycolysis · monocarboxylate transporter (MCT)

Historical perspective

Originally thought to be via non-ionic diffusion of the free acid, it was only after the demonstration that lactate and pyruvate transport into human erythrocytes is strongly inhibited by α-cyano-4-hydroxycinnamate (CHC) and organomercurials that a specific monocarboxylate transport mechanism was recognised (see [13, 55]). The different characteristics of monocarboxylate transporter observed after extensive characterisation in erythrocytes, cardiac myocytes and hepatocytes led to the proposal that a family of MCTs might exist [55]. The molecular identity of the first member of this family, MCT1, was established by parallel studies in two laboratories. In this laboratory, specific labelling studies in rat and rabbit erythrocytes [54] followed by purification and N-terminal sequencing [56] identified a 40- to 50-kDa protein. The N-terminus of this protein is identical to a putative 12-TMD transporter (MEV) of unknown function previously cloned by the group of Goldstein and Brown from a mutated Chinese hamster ovary cell line that exhibited enhanced mevalonate uptake [30]. Subsequently they demonstrated that the wild-type protein catalysed proton-linked pyruvate and lactate transport activity and named it monocarboxylate transporter-1 (MCT1) [33]. They proceeded to clone human MCT1 [18] and then, by screening a rat liver cDNA library, a related protein, MCT2, that is strongly expressed in the liver [19]. During investigations on X-chromosome inactivation, gene sequencing revealed another MCT family member, originally called XPCT [for X-linked, proline, glutamic acid, serine, threonine (PEST)-containing transporter] and now renamed MCT8 [35]. MCT3 was identified in the chicken retinal pigment epithelium [22, 70] whilst, in this laboratory, four more members of the MCT family, now named MCT4, MCT5, MCT6 and MCT7, were identified [58]. Very recently, a sodium- and proton-independent aromatic amino acid transporter, TAT1, was identified as a member of the MCT superfamily [31, 32]. MCT9 was identified purely from analysis of the human genomic expressed sequence tag (EST) databases [24] and here we report the existence of four new family members, identified in a similar manner, which we will refer to as MCT11, MCT12, MCT13 and MCT14. It should be noted that because of early confusions in the nomenclature, the MCT and *SLC16A* numbering of the family do not coincide but are correctly annotated in Table 1. The topology of the MCT

A. P. Halestrap (✉)
Department of Biochemistry,
University of Bristol, Bristol, BS8 1TD, UK
e-mail: A.Halestrap@Bristol.ac.uk
Tel.: +44-117-9288582, Fax: +44-117-9288724

D. Meredith
Department of Human Anatomy and Genetics,
University of Oxford, South Parks Road, Oxford, OX1 3QX, UK

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Table 1 *SLC16*—the monocarboxylate transporter family (*C* cotransporter, *E* exchanger, *F* facilitated transporter, *O* orphan transporter *G* genetic defect)

Human gene name	Protein name	Aliases	Predominant substrates	Transport type/coupling ions	Tissue distribution and cellular/subcellular expression	Link to disease[#]	Human gene locus	Sequence accession ID	Splice variants and their specific features
SLC16A1	MCT1		Lactate, pyruvate, ketone bodies	C/H^+ or E/monocarboxylate	Ubiquitous	Muscle weakness (exercise intolerance) G	1p13.2	NM_003051	
SLC16A2	MCT8	XPCT (*was MCT7)	T3, T4 (unpublished)	F	Liver, heart, brain, thymus, intestine, ovary, prostate, pancreas, placenta		Xq13.2	NM_006517	
SLC16A3	MCT4	(*was MCT3)	Lactate, pyruvate, ketone bodies	C/H^+	Skeletal muscle, chondrocytes, leukocytes, testis, lung, placenta, heart		17q25.3	NM_004207	
SLC16A4	MCT5	(*was MCT4)	O		Brain, muscle, liver, kidney, lung, ovary, placenta, heart		1p13.3	NM_004696	
SLC16A5	MCT6	(*was MCT5)	O		Kidney, muscle, brain, heart, pancreas, prostate, lung, placenta		17q25.1	NM_004695	
SLC16A6	MCT7	(*was MCT6)	O		Brain, pancreas, muscle		17q24.2	NM_004694	
SLC16A7	MCT2		Pyruvate, lactate, ketone bodies	C/H^+	Kidney, brain		12q14.1	NM_004731	
SLC16A8	MCT3	REMP	Lactate	C/H^+ (pH dependent but cotransport not confirmed experimentally)	Retinal pigment epithelium, choroid plexus		22q13.1	NM_013356	
SLC16A9	MCT9			O	Endometrium, testis, ovary, breast, brain, kidney, adrenal, retina		10q21.2	BN000144	
SLC16A10	TAT1		Aromatic amino acids (W, Y, F, L-DOPA)	F	Kidney, intestine, muscle, placenta, heart		6q21-q22	NM_018593	
SLC16A11	MCT11			O	Skin, lung, ovary, breast, lung, pancreas, retinal pigment epithelium, choroid plexus		17p13.2	NM_153357	
SLC16A12	MCT12			O	Kidney		10q23.3	ENSG00000152779	
SLC16A13	MCT13			O	Breast, bone marrow stem cells		17p13.1	BN000145	
SLC16A14	MCT14			O	Brain, heart, ovary, breast, lung, pancreas retinal pigment epithelium, choroid plexus		2q36.3	BN000146	

* Prior to publication of [66]

[#] Acquired defect

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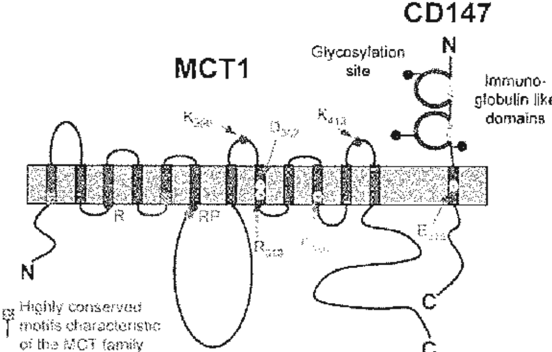


Fig. 1 The proposed topology of the monocarboxylate transporter (*MCT*) family members. CD147, the ancillary protein that associates with MCT1 and MCT4, is also shown. The N- and C-termini and the large loop between TMDs 6 and 7 show the greatest variation between family members, whilst the TMDs themselves are highly conserved. Critical residues identified in MCT1 and two highly conserved motifs characteristic of the MCT family are included, and further discussed in the section "Biochemical and structural aspects"

family is shown in Fig. 1 and discussed in detail below (section "Biochemical and structural aspects").

Functional characteristics of the family

MCT1 catalyses either net transport of one monocarboxylate with one proton or the exchange of one carboxylate for another [13]. Detailed kinetic analysis (see [55]) suggests that an ordered binding to the carrier of H^+ (K_m 0.2 μM, equivalent to a pK of 6.7 for the accepting group) followed by L-lactate (K_m 4–7 mM) precedes a conformational change that translocates the substrate across the membrane. This is followed by release and the return of the empty substrate binding site to the external surface. This last step is rate-limiting for net lactic acid transport across the membrane, as illustrated by trans-stimulation experiments [13]. The translocation cycle is freely reversible, with the kinetic parameters for lactic acid influx and efflux thermodynamically constrained according to the Haldane equation $(V_{max}/K_m)_{influx}=(V_{max}/K_m)_{efflux}$. Transport can be stimulated by decreasing the pH from 8 to 6 on the *cis*-side (primarily through a decrease in the K_m for lactate) or by raising the pH on the opposite side of the membrane (via an increase in the V_{max} of transport that stimulates the rate at which the unloaded carrier re-orientates in the membrane) [55].

MCT2, MCT3 and MCT4 also transport monocarboxylates with a proton [7], but the detailed kinetic mechanisms of these isoforms have not been studied. TAT1 has been shown to transport aromatic amino acids in a sodium-independent manner that also appears to be independent of protons [31]. To date, there are no published data on other MCT isoforms.

Distinctive feature of members of the MCT family

MCT1 (*SLC16A1*)

MCT1 is found in the great majority of tissues of all species studied, with no evidence for splice variants. Characterisation of endogenous MCT1 in erythrocytes [13], and MCT1 expressed in *Xenopus* oocytes [9, 40], has revealed that it transports short chain (C-2 to C-5) unbranched aliphatic monocarboxylates such as acetate (K_m 3.5 mM) and propionate (K_m 1.5 mM). Substitutions on C2 and C3 (excluding amino- and amido-) are tolerated or even preferred (e.g. pyruvate (K_m 0.7 mM), L-lactate (K_m 3–5 mM), acetoacetate (K_m 4–6 mM) and D-β-hydroxybutyrate (K_m 10–12 mM). Formate is a very poor substrate whilst bicarbonate, dicarboxylates, tricarboxylates and sulphonates are not transported. The carrier is stereoselective for 2-hydroxy-substituted monocarboxylates (e.g. L- over D-lactate >10-fold) but not for 2-chloropropionate and β-hydroxybutyrate.

Inhibitors of MCT1 can be divided into three categories:

(i) Bulky or aromatic monocarboxylates are competitive inhibitors, including 2-oxo-4-methylpentanoate, phenyl-pyruvate and derivatives of α-cyanocinnamate such as α-cyano-4-hydroxycinnamate (CHC). These are the most potent inhibitors of this class with K_i values of 50–500 μM. It is important to note that although CHC is often thought of as a specific inhibitor of MCT1, it also inhibits the mitochondrial pyruvate transporter with a K_i of <5 μM, as well as the anion exchanger AE1.

(ii) A range of amphiphilic compounds of widely divergent structure act as potent inhibitors ($K_{0.5}$ 1–10 μM) although they also inhibit AE1 and other membrane transport processes. These include bioflavanoids (e.g. quercetin and phloretin) and anion transport inhibitors such as 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and niflumic acid.

(iii) Some 4,4′-substituted stilbene-2,2′-disulphonates [such as 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS) and 4,4′-dibenzamidostilbene-2,2′-disulphonate (DBDS)] act as reversible inhibitors of MCT1 in erythrocytes, although with much lower affinity than for AE1. Inhibition by DIDS eventually becomes irreversible on prolonged incubation, reflecting covalent modification of the transporter [53]. MCT1 is inhibited irreversibly by a range of thiol and amino reagents and is especially sensitive to the organomercurial thiol reagent *p*-chloromercuribenzene sulphonate (pCMBS). More extensive data on the substrate and inhibitor specificity may be found elsewhere [13, 55].

MCT2 (*SLC16A7*)

MCT2 appears to demonstrate substantial species differences in both its amino acid sequence and tissue distribution [27], although some of the reported differences may reflect lack of specificity exhibited by commercial antibodies [3]. Northern blot analysis and inspection of the human EST database suggests that MCT2 is expressed poorly, if at all, in major human tissues [58], whilst in mouse, rat and hamster Northern and Western blot analysis and immunofluorescence microscopy have demonstrated MCT2 expression in liver, kidney, brain and sperm tails both and in hamster also in skeletal muscle and heart [19, 27]. Where MCT2 is expressed together with MCT1 its exact location within the tissue is different [3, 19, 20, 51, 52], suggesting a unique functional role as discussed below (see Physiological implications). There is no evidence for splice variants of the protein although alternative splicing in the 5′- and 3′-untranslated region (UTR) has been shown [27, 39]. Like MCT1, MCT2 (expressed in *Xenopus* oocytes) catalyses the proton-linked transport of a range of monocarboxylates, but with a considerably higher affinity than MCT1. K_m values for pyruvate and L-lactate are about 0.1 and 0.7 mM respectively [7, 39]. MCT2 is also more sensitive than MCT1 to inhibition by a range of inhibitors including CHC, DBDS and DIDS, but is insensitive to pCMBS [7, 19].

MCT3 (*SLC16A8*)

MCT3 has a unique distribution, being confined to the basal membrane of the retinal pigment epithelium and choroid plexus epithelia [50, 70], in contrast to the apically located MCT1 [2]. No splice variants of MCT3 are known, but in the case of chicken MCT3 there are two different mRNAs, resulting from different promoter usage. These are involved in temporal rather than spatial regulation [69]. When expressed in yeast, MCT3 transports L-lactate with a K_m of about 6 mM and is insensitive to CHC, phloretin and pCMBS, but detailed information on the substrate and inhibitor specificity is lacking [22]. It should be noted that MCT4 (below) was originally called MCT3 [58] on the basis of its sequence homology rather than function or localisation [50, 66].

MCT4 (*SLC16A3*)

MCT4 is expressed widely both at the mRNA and protein levels and particularly strongly in glycolytic tissues such as white skeletal muscle fibres, astrocytes, white blood cells, chondrocytes and some mammalian cell lines [4, 14, 43, 58, 66]. This has led us to propose that it may be of particular importance in tissues that rely on high levels of glycolysis to meet their energy needs [24, 66]. Indeed, in the rat MCT4 is expressed in the neonatal heart, which is more glycolytic in its energy metabolism than the adult heart where MCT4 is absent [25, 66]. MCT4 is also expressed strongly in placenta, which exports lactic acid rapidly from the foetal to the maternal circulation [58]. MCT4 expressed in *Xenopus* oocytes exhibits a much lower affinity for most substrates and inhibitors than MCT1, with K_m and K_i values some 5–10 times higher [14, 40]. Thus K_m values for L-lactate and pyruvate are 28 and 150 mM respectively and little inhibition by DIDS or CHC is observed at concentrations giving >50% inhibition of MCT1.

TAT1 (*SLC16A10*)

TAT1 is expressed strongly in intestine (basolateral membrane of the epithelial cells), placenta and liver in rat, whereas in humans skeletal muscle and kidney are the major sites of expression with lower amounts in heart, placenta and intestine [31, 32]. When expressed in *Xenopus* oocytes, both human and rat TAT1 transport aromatic amino acids in a sodium- and proton-independent manner with K_m values (millimolar, with rat values in parentheses) for L-phenylalanine, D-phenylalanine, L-tyrosine, L-tryptophan and L-DOPA of 0.75 (7.0), 2.3, 0.64 (2.6), 0.45 (3.7) and 1.21 (6.4) respectively. Monocarboxylates such as lactate and pyruvate are not substrates. No data are available on the sensitivity of TAT1 to inhibitors.

Other MCT family members

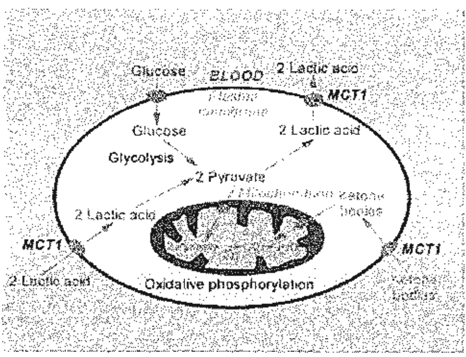
No data are available on the properties of MCT5, MCT6 and MCT7 (*SLC16A4–SLC16A6*) although their distribution in human tissues has been subject to Northern blot analysis [58]. Recently, in collaboration with Theo Visser's laboratory, we have demonstrated that, when expressed in *Xenopus* oocytes, MCT8, the MCT isoform most closely related to TAT1, transports thyroid hormone (T4 and T3) in a sodium- and proton-independent manner with a K_m of about 1 μM. Neither aromatic amino acids nor lactate are transported. Northern blot analysis of human tissues has shown MCT8 to be widely expressed, but most strongly in liver and heart. An interesting feature of MCT8 is that its predicted N-terminal sequence is extended by a 75-amino acid sequence that contains the PEST motif indicative of rapid degradation [63]. The significance of this is currently unknown. MCT9 (*SLC16A9*, Accession No. BN000144) was identified by searching the human genomic and EST databases [24] and, in collaboration with Theo Visser we have used the same technique to identify four new MCT family members which we will refer to as MCT11 (*SLC16A11*, NM_153357), MCT12 (*SLC16A12*, ENSG00000152779), MCT13 (*SLC16A13*, BN000145) and MCT14 (*SLC16A14*, BN000146). These are included in Table 1 but no information is available on their properties or function.

Physiological implications of MCT family members

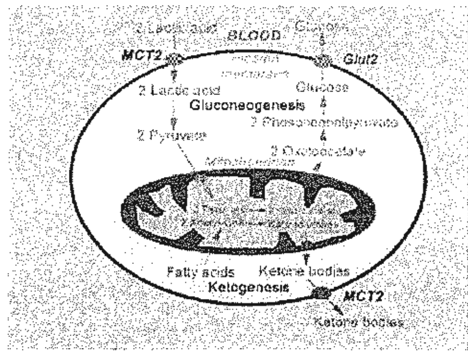
Rapid transport of lactic acid transport across the plasma membrane is of fundamental importance to all mammalian cells under hypoxic conditions [24] when they become glycolytic. Lactic acid is exported by MCT1 (Fig. 2A) to prevent a fall in cytosolic pH (pH_i) and inhibition of continued glycolysis [55]. However, for those cells that rely on glycolysis for their normal energy metabolism MCT4 appears to be the major isoform. The high K_m of MCT4 for pyruvate ensures that this metabolite is not lost from the cell but is reduced to lactic acid, regenerating NAD^+ and so allowing glycolysis to continue. The high K_m of MCT4 for L-lactate is intriguing and may explain why lactic acid accumulates in muscle during exercise, causing the decrease in pH_i that is responsible for fatigue. This probably represents an important physiological mechanism whereby the fatigue prevents exercise continuing to the point at which lactic acid would overload the buffering capacity of the blood and cause damaging systemic acidosis [29, 40]. In other tissues, lactic acid is transported into the cell to supply gluconeogenesis and lipogenesis (liver and kidney: Fig. 2B) or, together with ketone bodies, for oxidation as a major respiratory fuel (heart, skeletal muscle, brain: Fig. 2A) [24]. MCT1 fulfils this role in skeletal muscle and heart, whilst in liver, kidney and brain either MCT1 or MCT2 may be used, the latter providing a higher-affinity lactate uptake mechanism [24]. There is one tissue with no detectable MCT activity; the β-cells of the Islets of Langerhans in the endocrine pancreas [72]. This, together with low levels of lactate dehydrogenase, ensures that pyruvate is fully oxidised via the citric acid cycle, rather than being converted to lactate, so ensuring ATP production and thus insulin secretion accurately reflects glucose levels.

In many tissues more than one MCT isoform is found, which would appear to correlate with either the influx or efflux of lactic acid into different cell types. Two well-documented examples are illustrated in Fig. 2C. In skeletal muscle, the white fibres are glycolytic and contain primarily MCT4 that catalyses the efflux of lactic acid. This is then taken up and oxidised by the red fibres that express primarily MCT1 (and perhaps a little MCT2 in some species). Similarly, glial cells in the brain, which contain MCT1 and MCT4, can export lactic acid to be oxidised by the neurons that contain MCT1 and MCT2. MCT2 is especially concentrated in the post synaptic density which is rich in mitochondria and thus likely to be a major site of lactate oxidation, whilst the endothelial cells of the blood vessels contain only MCT1 [3, 4, 51].

A General role in most tissues



B Role in liver and kidney



C Compartmentation of lactate metabolism in muscle and brain

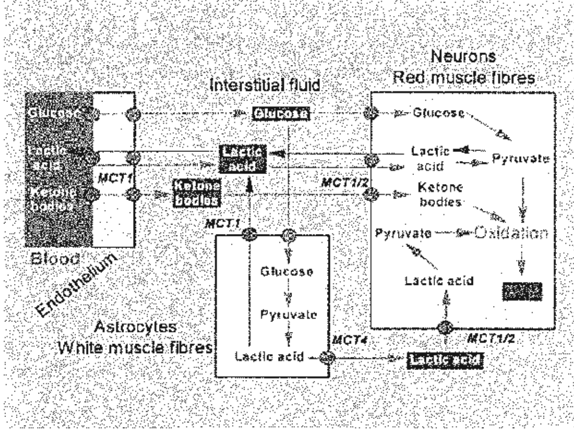


Fig. 2A–C MCT isoforms play a critical role in the metabolism of all cells. A Most cells oxidise glucose to CO_2 and water to produce energy, but some, including white skeletal muscle, red blood cells and tumour cells, are primarily glycolytic, converting glucose to two lactic acids that must leave the cell. All cells must rely on glycolysis when oxygen supplies are low (hypoxia/ischaemia). Some tissues like the heart, red muscle, spermatozoa and brain can oxidise lactic acid. B The liver and kidney import lactic acid for gluconeogenesis. C In some tissues, intercellular cellular compartmentation of lactate metabolism occurs and this is reflected in the distribution of different MCT isoforms. Thus in both the brain and mixed muscle fibres, some cells (neurons and red fibres) oxidise the lactic acid produced by other cells (glia and white fibres). MCTs are also responsible for the transport of ketone bodies into and out of cells that produce them (liver) or utilize them (red muscle, heart and brain)

There is also evidence that lactate metabolism in the retina is subject to a complex interplay between the retinal pigment epithelium (RPE), photoreceptor cells (which oxidise lactate), other neurons and glial cells (Müller cells) which export lactate through MCT4. MCT3 is located exclusively on the basolateral surface of the RPE and is responsible for lactate efflux into the choroidal blood supply, whereas MCT1 is exclusively located on the apical surface of the RPE [20, 49, 50, 70]. MCT1 has been proposed to play an additional role in regulating the volume of the sub-retinal space by also transporting water with the lactate/protons [71]. An accumulation of lactate within the subretinal space would cause osmotic swelling, resulting in the retina becoming detached from the RPE, which could be prevented by an ability of MCT1 (in association with MCT3) to rapidly transport both lactic acid and water across the RPE and into the blood.

Although L-lactate is quantitatively by far the most important substrate of MCT1-MCT4, these isoforms also catalyse the transport of other metabolically important monocarboxylates including the ketone bodies acetoacetate and β-hydroxybutyrate, and the branched-chain keto-acids such as α-ketoisocaproate formed from transamination of amino acids [24, 55]. In the colon, MCT1 may also be important for the uptake of short-chain fatty acids such as acetate and butyrate [12], although these are capable of entering most cells by free diffusion [55]. It has also been proposed that the MCTs may play an important role in communicating information on the redox state between cells [55]. The ratio of lactate to pyruvate reflects the cytosolic $NADH/NAD^+$ ratio through equilibration of the lactate dehydrogenase reaction. MCTs enable these metabolites to be transported between tissues and this may provide some harmonization of cytosolic redox potential. Similar arguments may apply to the mitochondrial $NADH/NAD^+$ ratio through equilibration of β-hydroxybutyrate dehydrogenase and the transport of β-hydroxybutyrate and acetoacetate between tissues.

Regulation of MCT isoforms

In skeletal muscle, MCT1 is up-regulated in response to chronic stimulation or exercise in rats and humans (see [6, 8, 21, 29]). Conversely MCTs are down-regulated in response to denervation of muscle or spinal injury [29]. MCT1 expression also increases in the heart following surgical ligation of a major branch of the left coronary artery, presumably reflecting the greater energy demands of the remaining functional hypertrophied left ventricle [28]. In adipose tissue, heart and skeletal muscle MCT1 protein expression has been reported to be reduced in streptozotocin (STZ)-induced diabetes [23, 15], although others have not observed the effect in skeletal muscle [61]. A decrease in MCT4 expression has been observed in skeletal muscle from STZ-induced diabetic rats [15] and obese rats, in which MCT1 expression is also decreased [60]. There is also evidence for changes in MCT isoform expression during development in heart and muscle [25], the inner ear [48] and brain [37], whilst the transition to malignancy is accompanied by changes in the level of MCT1 expression in the colon [36] and brain [17]. In these two tissues there is evidence for up-regulation of MCT1 expression mediated by butyrate and ketone bodies respectively [12, 38]. It has also been shown that MCT4 expression is up-regulated in skeletal muscle of a patient with a mitochondrial myopathy [1].

Parallel measurements of MCT1 and MCT2 mRNA and protein in several tissues suggest that both transcriptional and post-transcriptional mechanisms may be involved in regulating their expression at the plasma membrane [6, 8, 27, 28]. In addition, there is evidence for a novel intracellular pool of MCT1 associated with cisternae close to the t-tubules in hypertrophied left ventricles with increased expression of MCT1. This may indicate that de-novo synthesis and translocation to the sarcolemma occurs via this pool and act as a potential regulatory site [28]. Interestingly, MCT1 possesses two acidic clusters and an LL-motif in the C-terminus; these motifs are believed to be important in endosomal-lysosomal targeting of glucose uniporter-4 (GLUT-4) [64]. Another potential mechanism for post-transcriptional regulation is at the level of translation and this usually involves specific sequences and secondary structure in the 5′- or, frequently, the 3′-UTR with which initiation factors and regulatory factors interact to enhance or repress translation [59]. Thus it may be significant that the 3′-UTR of MCT1 is very long (some 1.2 kb longer than either MCT4 or MCT2 in rats) [24]. There are no convincing experimental data to support regulation of any MCT isoform by phosphorylation.

Biochemical and structural aspects of the MCT family

Hydropathy plots predict 10–12 α-helical TMDs for MCT family members, with the N- and C-termini located within the cytoplasm as illustrated in Fig. 1. This has been confirmed experimentally for MCT1 in erythrocytes [57]. The greatest sequence variation between isoforms is found in the long C-terminus and the large intracellular loop TMD 6–7 that varies substantially in length from 105 residues in MCT5 to only 29 residues in MCT4. The predicted phylogeny of MCT family members is shown in Fig. 3 as a radial tree. Theoretical predictions and experimental evidence indicate that no MCT family member is glycosylated [11, 24, 70]. Two highly conserved sequences can be identified as characteristic of the MCT family (including its non-mammalian members); these are **[D/E]G**[G/S][**W/F**][**G/A**]**W** which traverses the lead into TMD1 and **YF**x**K**[**R/K**][**R/L**]x**LA**x[**G/A**]x**A**x**AG** which leads into TMD5 [24] (residues in bold face are totally conserved whilst consensus residues are indicated in normal type). Site-directed mutagenesis has shown that the conservative change of Asp_{302} to Glu in TMD 8 of rat MCT1 leads to total loss of lactate transport activity [62], whilst conver-

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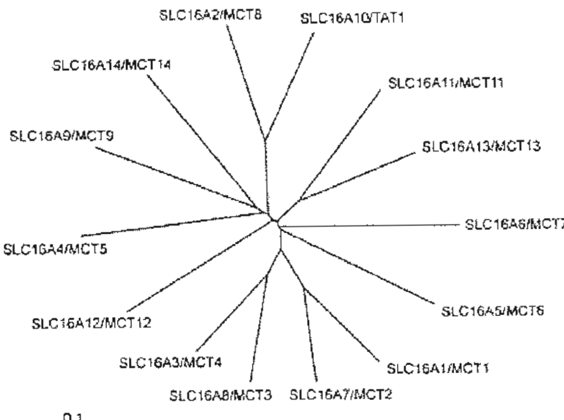

Fig. 3 The predicted phylogeny of MCT family members. Sequences were aligned using the Clustal W algorithm and are displayed as a *radial tree*. The *bar* indicates the number of substitutions per residue with 0.1 corresponding to a distance of 10 substitutions per 100 residues. The calculated, pairwise, percentage identity of MCT2, MCT3, MCT4, MCT5, MCT6, MCT7, MCT8, MCT9, TAT1, MCT11, MCT12, MCT13 and MCT14 to MCT1 are 58, 38, 42, 22, 27, 26, 22, 23, 22, 27, 28, 31, and 23 respectively; MCT4 is 55% and 44% identical to MCT3 and MCT2 respectively and TAT1 50% identical to MCT8

sion of Phe_{360} in TM 10 to Cys enables MCT1 to transport mevalonate whilst reducing its ability to carry lactate and pyruvate [33]. The DIDS-binding site of MCT1 is in the C-terminal half of the transporter [57] and mutation of either Lys_{290} in the loop between TMDs 7 and 8 or Lys_{413} in the loop between TMDs 11 and 12 to glutamine, prevents irreversible inhibition [42]. An arginine in TM8 (Arg_{313} of human MCT1) is conserved in all the putative MCTs from higher eukaryotes except MCT5, and site-directed mutagenesis of this residue greatly reduces the affinity of MCT1 for lactate [62] whilst the arginine-specific reagent phenylglyoxal inhibits transport [55]. This arginine may act as the positively charged group that binds the COO^- anion much as it does in lactate dehydrogenase [10, 24, 55].

Ancillary proteins are required for MCT expression at the plasma membrane

MCT1 and MCT4 require CD147 [also known as OX-47, extracellular matrix metalloproteinase inducer (EMMPRIN), HT7 or basigin) or the related protein GP70 (Embigen) for proper cell surface expression and function. These are widely distributed cell surface glycoproteins with a single transmembrane span, two immunoglobulin-like domains in the extracellular region and a short C-terminal cytoplasmic tail. In contrast, MCT2 does not interact with CD147 although it does appear to require an, as yet unidentified, ancillary protein to be properly expressed at the cell surface [34]. In the red blood cell, DIDS cross-links MCT1 to GP70 whilst in a variety of cell lines antibodies against CD-147 co-immunoprecipitates MCT1 and MCT4, but not MCT2. Immunofluorescence microscopy has confirmed that CD147 co-localises with MCT1 or MCT4 in other tissues [34, 72]. We have confirmed the close association between CD147 and MCT1/MCT4 within the plasma membrane by co-expressing the two proteins with their C- and N-termini tagged with cyan- and yellow-fluorescent proteins. Fluorescence resonance energy transfer (FRET) between the two proteins could be demonstrated, but only if both were tagged on intracellular domains [67]. The interaction between MCT1/4 and CD147 appears to be essential for their correct expression at the cell surface. Only when CD147 is co-expressed with MCT1 or MCT4 are both proteins correctly targeted to the plasma membrane rather than accumulating in the endoplasmic reticulum/Golgi apparatus [34]. Furthermore, co-injection of *Xenopus* oocytes with MCT1 cRNA and an antisense oligonucleotide to a *Xenopus* homologue of CD147 reduced MCT1 expression and activity [41].

Pathological Implications

The critical importance of lactate transport for key metabolic processes such as glycolysis and gluconeogenesis suggest that any impairment in the activity of an MCT isoform is likely to have far-reaching consequences. These may not be compatible with life unless compensated for by changes in expression of another isoform. MCT1 has been implicated in one rare condition known as cryptic exercise intolerance. Apparently healthy sufferers of this condition suffer severe chest pain and muscle cramping on vigorous exercise, and it has been suggested that this may be due to a defect in MCT which prevents lactate efflux [16]. Measurements of lactate uptake by the erythrocytes of such patients showed a reduction in transport that was attributed to an MCT defect. More recently, RT-PCR of MCT1 from muscle biopsy identified a number of amino acid differences that were not attributable to polymorphisms and therefore could be affecting protein function [44, 45]. Of these, only a lysine-to-glutamate mutation in the large cytoplasmic loop between TMDs 6 and 7 was considered a likely candidate. However, we have expressed the K204E mutant in *Xenopus* oocytes and were unable to demonstrate any difference in its properties from wild type MCT1 (A.P. Halestrap, D. Meredith, unpublished data). Thus it remains unclear whether mutations in MCT1 are responsible for cryptic exercise intolerance or whether other factors (such as the interaction with CD147 or GP70) may be involved. No data are available on mutations in other MCT isoforms, although it is interesting to speculate whether unexplained muscle fatigue might be associated with impaired MCT4 expression.

Many tumour cells exhibit an absolute dependence on glycolysis that may reflect the tendency of rapidly growing tumours to become hypoxic. It is of interest that CD147 is up-regulated in aggressive tumours [5, 46]

whilst the transition from normality to malignancy is accompanied by increased MCT1 expression in the brain [17], although the opposite has been reported in colon carcinomas [36].

MCTs as a site of pharmacological intervention.

There is some evidence that MCTs may play a role in the transport of some drugs across the plasma membrane, such as salicylate and valproic acid [26, 65], atorvastatin [68] and nateglinide [47]. At present, the best MCT inhibitors exert powerful effects on other cellular targets [24, 55]. Furthermore, as outlined above, it is likely that global inhibition of MCT function would have disastrous consequences for the well being of an organism. Thus MCT inhibitors are unlikely to be of pharmacological use, unless isoform specific compounds become available.

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Note added in proof A recent publication has shown that MCT3, like MCT1 and MCT4, probably also requires CD147 for its correct expression at the plasma membrane [Philp NJ, Ochrietor JD, Rudoy C, Muramatsu T, Linser PJ (2003) Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse. Invest Ophthalmol Vis Sci 44:1305–1311]

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PHARMACOKINETICS OF DICLOFENAC SODIUM IN NORMAL MAN

S.M. FARID HASAN, TASNEEM AHMED*, NASIRA TALIB AND FOUZIA HASAN*****
*Jinnah Medical & Dental College, Karachi, *Pharma Professional Services, Karachi*
***Department of Pharmacy, University of the Punjab, Lahore*
****Department of Pharmaceutics, Faculty of Pharmacy*
University of Karachi, Karachi-75270

Diclofenac sodium was administered as 50 mg tablets to four healthy male volunteers in a two-way randomized crossover study in which volunteers were either fasted or were given a standard breakfast immediately prior to dosing. Blood samples were obtained upto 9 hours period and drug concentration were determined by HPLC method.
Besides a significantly delayed (at $p>0.1$) peak in fed state; an increase in absorption rate constant was observed as the only significant (at $p>0.05$) effect of food on biopharmaceutic characteristic of diclofenac sodium. However, the intrinsic absorption of diclofenac sodium is also fast and it is not being the rate limiting factor in the bioavailability of diclofenac sodium, its decrease upto about 18 minutes (0.31 ± 0.05 hr) from 11 minutes (0.19 ± 0.02 hr) produces a null difference as the net effect on bioavailability, particularly when the drug is to be used in multiple dosage regimen.

Keywords: Pharmacokinetics, diclofenac sodium, HPLC method.

INTRODUCTION

The first nonsteroidal anti-inflammatory agent introduced after salicylic acid was phenylbutazone, which made its appearance in 1952. A decade or more later, competitive compounds such as mefenamic acid, ibuprofen, and indomethacin were introduced (Sallmann, 1975).

At this point it was decided to start a project aimed at developing a novel anti-inflammatory drug which should be superior both in activity and tolerability.

Diclofenac sodium has an acidity constant of 4 and a partition coefficient of 13.4. The structural elements include a phenylacetic acid group, a secondary amino group, and a phenyl ring containing chlorine atoms which cause maximum twisting of the ring (Salmann, 1986).

The half-life of diclofenac sodium in plasma varies from 1-3 hours (Adeyeye & Li, 1990; Reynold, 1993; Willis *et al.*, 1979; Degen *et al.*, 1988; Said & Sharaf, 1981 and Landsdorp *et al.*, 1990), with mean peak plasma levels of approximately 0.5 μg/ml and 1.0 μg/ml occurring after about two hours after single doses of 25 mg and 50 mg of enteric-coated tablets respectively (Riess *et al.*, 1978).

There are no data available on the distribution of diclofenac in organs or tissues of man. Animal studies have shown that the highest concentrations of diclofenac are found in bile, liver, and kidneys followed by blood, heart and lungs (Riess *et al.*, 1978).

Diclofenac, like all NSAIDs, is ≥ 99.5% bound to human serum proteins, specifically albumin (Riess *et al.*, 1978 and Chamouard *et al.*, 1985). It accumulates in synovial fluid after oral administration, which may explain the duration of therapeutic effect that is considerably longer that the plasma half-life (Insel, 1991).

Diclofenac has been marketed since 1973 (Todd & Sorkin, 1988 and Sengupta *et al.*, 1985). It has been approved in the United States (Ciba-Geigy, 1988). Experimental and clinical findings obtained to date have indicated that diclofenac sodium was synthesized on well-founded principles (Sallmann, 1986).

In this study, the effect of food on the pharmacokinetic parameters of diclofenac sodium (voltaren 50 mg) was examined in four healthy male volunteers.

MATERIALS AND METHODS

Materials and methods utilized in this study are described elsewhere (Farid *et al.*, 2004).

RESULTS AND DISCUSSION

The plasma concentrations of diclofenac sodium in four healthy human volunteers after a single oral administration of 50 mg dose (voltaren 50 mg) in fasting and fed conditions are given in table-1 (mean ± SEM). The mean levels produced by single dose in both the states were subjected to super position method for simulating levels in multi-dose treatment. Table-2 and table-3 report the generated levels after four doses six hourly in fasting and fed conditions. Thus it is clear from these tables that multi-dose usage of the drug will finally produce identical steady state pattern and thus neither the fasting nor fed condition has any beneficial edge over each other as far as the levels are concerned.

Absorption rate is the only parameter in present work which

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Table 1
Plasma levels of diclofenac sodium in all human volunteers in control state (fasting) and in treatment state (after food) after oral administration of 50 mg dose in the form of enteric-coated tablet

Time (hours)	Control state (fasting) Mean ± SEM (ug/ml)	Treatment state (after food) Mean ± SEM (ug/ml)
1.00	0.1275 ± 0.0775	0.0975 ± 0.0542
1.50	0.4300 ± 0.3239	0.3300 ± 0.2568
2.00	1.0425 ± 0.6855	0.6300 ± 0.2946
2.50	1.0150 ± 0.2216	0.8925 ± 0.5053
3.00	1.0975 ± 0.4389	0.8375 ± 0.2418
3.50	0.6150 ± 0.2141	0.7525 ± 0.3250
4.00	0.3325 ± 0.1048	0.4500 ± 0.1695
4.50	0.2325 ± 0.0671	0.2450 ± 0.0913
5.00	0.1525 ± 0.0413	0.1675 ± 0.0664
5.50	0.1025 ± 0.0217	0.1150 ± 0.0448
6.00	0.0900 ± 0.0187	0.0875 ± 0.0335
7.00	0.0625 ± 0.0111	0.0650 ± 0.0185
8.00	0.0500 ± 0.0091	0.0500 ± 0.0122
9.00	0.0350 ± 0.0050	0.0375 ± 0.0125

is found significantly different at 0.05 level of significance. The absorption half-lives observed in both the states along with the results of ANOVA and t-tests; paired and unpaired are given in table-4.

As has been discussed previously that peak time in fed state is delayed (Farid *et al.*, 2004). However, the absorption rate is significantly (at $p>0.05$) higher in fed state and consequently the half-life is lower. It is thus concluded that the presence of food delays the dissolution which is responsible of longer lag time in fed state, however, once the absorption started it was found faster. The possible reason which may be offered for this rapid absorption is the more blood flow towards, stomach in presence of the food.

Absorption half-life (mean ± SEM) in control as found to be 0.31 ± 0.05 hr where as in treatment it appeared to be 0.19 ± 0.02 hr.

Willis *et al* (1979) examined the pharmacokinetics of diclofenac sodium following intravenous and oral administration, to healthy female volunteers. Individual drug profiles were described by a triexponential function and mean- half-lives of the three exponential phases were 0.05, 0.26 and 1.1 hr. After oral doses of enteric-coated tablets, the lag time between dosing and the appearance of drug in plasma varied between 1.0 and 4.5 hr. Peak plasma diclofenac levels ranged from 1.4 to 3.0 μg/ml. The mean terminal drug half-life in plasma was 1.8 hr after oral doses. Fifty percent of orally dosed diclofenac did not reach the systemic circulation due to first pass metabolism.

Said and Sharaf (1981) gave single oral dose of 10 mg/kg of diclofenac sodium to rabbits and determined serum concentrations by a developed HPLC method. The observed serum levels of rabbits were fitted to a one-compartment open model. The mean values of $t_{1/2}e$, ke, $t_{1/2}a$, ka and t_{max} were 1.98 h, 0.3455h^{-1}, 1.2357 h, 0.5653 h^{-1} and 2.2267 h, respectively.

Thus the value of absorption half-life cited in the above studies were 0.05 hr (Willis, 1979) and 1.2357 hr (Said and Sharaf, 1981) respectively. A large difference in absorption half-lives may be due to physiological variability of subjects participated in the studies.

Distribution half-life (mean ± SEM) in treatment was found to be 0.27 ± 0.05 hr where as in control it appeared to be 0.39 ± 0.06 hr. The distribution half-lives observed in both the states along with the results of ANOVA and t-tests; paired and unpaired are given in table-5.

A previous study (Willis *et al.*, 1979) indicates the distribution half-life of diclofenac sodium in normal state

Table 2
Simulation of plasma levels of diclofenac sodium in an 6 hourly 50 mg dose system upto 4 doses generated through superposition technique by utilizing mean levels obtain after single dose oral administration with tablets in control (fasting) state

Time	First dose	Second dose		Third dose		Fourth dose	
0	0						
1	0.1275						
2	1.0425						
3	1.0975						
4	0.3325						
5	0.1525						
6	0.0900	(+0)	0.0900				
7	0.0625	(+0.1275)	0.19				
8	0.0500	(+1.0425)	1.0925				
9	0.0350	(+1.0975)	1.1325				
10	0	(+0.3325)	0.3325				
11		(+0.1525)	0.1525				
12		(+0.0900)	0.0900	(+0)	0.0900		
13		(+0.0625)	0.0625	(+0.1275)	0.19		
14		(+0.0500)	0.0500	(+1.0425)	1.0925		
15		(+0.0350)	0.0350	(+1.0975)	1.1325		
16		(+0)	0	(+0.3325)	0.3325		
17				(+0.1525)	0.1525		
18				(+0.0900)	0.0900	(+0)	0.0900
19				(+0.0625)	0.0625	(+0.1275)	0.19
20				(+0.0500)	0.0500	(+1.0425)	1.0925
21				(+0.0350)	0.0350	(+1.0975)	1.1325
22				(+0)	0	(+0.3325)	0.3325
23						(+0.1525)	0.1525
24						(+0.0900)	0.0900
25						(+0.0625)	0.0625
26						(+0.0500)	0.0500
27						(+0.0350)	0.0350
28						(+0)	0

after intravenous administration, which is 0.26 hr. This value is not in very close agreement with the value estimated in the present work, however; it is not very much different.

The plasma levels versus time profiles in the post-absorptive phases of all subjects were found to have a value of correlation coefficient 0.9. This shows the linear disposition of diclofenac sodium for the dose administered. The biological half-lives (mean ± SEM) in the two states were found to be 2.72 ± 0.29 hr with food and 2.63 ± 0.19 hr without food. The two values are obviously very close and resembling. This indicates that food given in the treatment state has no effect on drug metabolism or drug filtration

Table 3
Simulation of plasma levels of diclofenac sodium in an 6 hourly 50 mg dose system upto 4 doses generated through superposition technique by utilizing mean levels obtain after single dose oral administration with tablets in treatment (after food) state

Time	First dose	Second dose		Third dose		Fourth dose	
0	0						
1	0.0975						
2	0.6300						
3	0.8375						
4	0.4500						
5	0.1675						
6	0.0875	(+0)	0.0875				
7	0.0650	(+0.0975)	0.1625				
8	0.0500	(+0.6300)	0.68				
9	0.0375	(+0.8375)	0.875				
10	0	(+0.4500)	0.4500				
11		(+0.1675)	0.1675				
12		(+0.0875)	0.0875	(+0)	0.0875		
13		(+0.0650)	0.0650	(+0.0975)	0.1625		
14		(+0.0500)	0.0500	(+0.6300)	0.68		
15		(+0.0375)	0.0375	(+0.8375)	0.875		
16		(+0)	0	(+0.4500)	0.4500		
17				(+0.1675)	0.1675		
18				(+0.0875)	0.0875	(+0)	0.0875
19				(+0.0650)	0.0650	(+0.0975)	0.1625
20				(+0.0500)	0.0500	(+0.6300)	0.68
21				(+0.0375)	0.0375	(+0.8375)	0.875
22				(+0)	0	(+0.4500)	0.4500
23						(+0.1675)	0.1675
24						(+0.0875)	0.0875
25						(+0.0650)	0.0650
26						(+0.0500)	0.0500
27						(+0.0375)	0.0375
28						(+0)	0

through kidneys. The difference in this respect apparently is non-existent.

The elimination half-lives observed in both the states along with the results of ANOVA and t-tests; paired and unpaired are given in Table-6.

Elimination half-lives in the normal states reported by other workers ranges between 1-3 hours (Adeyeye & Li, 1990; Reynolds, 1993; Willis *et al.*, 1979; Degen *et al.*, 1988; Said & Sharaf, 1981 and Landsdorp *et al.*, 1990).

Landsdorp *et al* (1990) studied the pharmacokinetics of rectal diclofenac and its hydroxy metabolites in man. When

Table 4
Absorption half-lives of diclofenac sodium in control and treatment states

Volunteer	Absorption half-lives (hr)	
	Control	Treatment
1	0.2800	0.1400
2	0.4600	0.2500
3	0.2700	0.1600
4	0.2100	0.2000
Mean ± SEM	0.3050 ± 0.0539	0.1875 ± 0.0243

Analysis of variance

S.O.V.	S.S.	DF.	M.S.	F
Treatment	0.0276	1	0.0276	--
Residual	0.0420	6	0.0070	3.9470
Total	0.0696	7	--	--

F-ratio = 4.9329 t-test paired = 2.8308 t-test unpaired = 1.9867

Table 5
Distribution half-lives of diclofenac sodium in control and treatment states

Volunteer	Distribution half-lives (hr)	
	Control	Treatment
1	0.4200	0.1600
2	0.5300	0.3000
3	0.3500	0.2300
4	0.2400	0.3800
Mean ± SEM	0.3850 ± 0.0609	0.2675 ± 0.0471

Analysis of variance

S.O.V.	S.S.	DF.	M.S.	F
Treatment	0.0276	1	0.0276	--
Residual	0.0712	6	0.0119	2.3277
Total	0.0988	7	--	--

F-ratio = 1.6682 t-test paired = 1.2918 t-test unpaired = 1.5257

100 mg diclofenac suppository was given to six human volunteers it was well absorbed from the gastrointestinal tract. The apparent half-lives of diclofenac and 4'-hydroxy diclofenac were respectively 1.3 ± 0.3 h and 4.3 ± 1.0 h. When the $t_{1/2}$ values are derived from the renal excretion rate-time profiles, they are as follows: diclofenac 1.8 ± 0.9 h, 3'-hydroxy and 5-hydroxy diclofenac 2.3 ± 1.0 h and 2.5 + 0.4 h respectively, while those of 4'-hydroxy and 4',5-dihydroxy diclofenac are respectively 3.6 ± 0.5 hand 3.1 ± 1.3 h. Diclofenac is excreted for 13.6 ± 6.5%, its renal clearance (Cl_r) = 3.23 ± 1.03 ml/min. The mean metabolite excreted in the urine is 4'-hydroxy diclofenac (27.2 ± 12% dose, Cl_r = 6.14 ± 4.04 ml/min). The total body clearance of the parent drug and the apparent total body clearance of the main metaboilite are similar 28.0 ± 11.9 1/h, and 27.5 ± 10.9 1/h.

Table 6
Elimination half-lives of diclofenac sodium in control and treatment states

Volunteer	Elimination half-lives (hr)	
	Control	Treatment
1	2.5800	2.0500
2	2.1300	3.0100
3	2.7700	2.4600
4	3.0300	3.3600
Mean ± SEM	2.6275 ± 0.1898	2.7200 ± 0.2901

Analysis of variance

S.O.V.	S.S.	DF.	M.S.	F
Treatment	0.0171	1	0.0171	--
Residual	1.4423	6	0.2404	0.0712
Total	1.4594	7	--	--

F-ratio = 0.4277 t-test paired = -0.2894 t-test unpaired = -0.2668

Table 7
Mean residence time of diclofenac sodium in control and treatment states

Volunteer	Mean residence time (hr)	
	Control	Treatment
1	3.7300	3.9500
2	3.9500	4.8200
3	3.2800	3.3200
4	2.4600	2.5300
Mean ± SEM	3.3550 ± 0.3293	3.6550 ± 0.4849

Analysis of variance

S.O.V.	S.S.	DF.	M.S.	F
Treatment	0.1800	1	0.1800	--
Residual	4.1234	6	0.6872	0.2619
Total	4.3034	7	--	--

F-ratio = 0.4611 t-test paired = -1.5461 t-test unpaired = -0.5118

Our value of elimination half-life is in good agreement with the previous findings. Thus the pharmacokinetic parameters found in Pakistani subjects are in close agreement with the previous findings. The observed pharmaceutical data may be used for describing the pharmacokinetic character of the drug in local population. As far as the effect of food is concerned, more or less similar results are obtained as reported by other workers.

The non-compartmental analysis of the data is also performed in order to detect effect of food which might not be reflected from compartmental analysis. The values of mean residence time (MRT) in control and treatment states (mean ± SEM) were found to be 3.3550 ± 0.3293 hr and

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3.6550 ± 0.4849 hr respectively, apparently resembling values bearing no significant difference. Mascher (1989) found MRT of 5.5 hours after oral administration of a sustained-release form of the drug to humans. This high value may be attributed to the sustained character of the dosage form. However no other values of MRT have been reported in the literature.

The MRTs, values along with the results of ANOVA and t-tests; paired and unpaired are given in table-7.

CONCLUSION

A possible explanation of these results may be that the presence of food in the gastric pouch lowers the gastric emptying and increases the viscosity of the gastric contents, this in turn causes a hindrance in the transfer of drug from stomach to its site of absorption, intestine. Since the therapeutic action of enteric-coated dosage form depends upon its entry into the intestine which is delayed in the presence of food. This results in increasing the time to reach the peak plasma concentration. But once the drug enters in the intestine it is rapidly absorbed. So in order to obtain a quick effect, diclofenac sodium should be taken in empty stomach or at least one hour before meal may be advised if the treatment consists of only one single dose, however; for multi-dose treatment this precaution seem unnecessary.

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CASE REPORT

Anaesthesia for bullectomy

A technique with spontaneous ventilation and extradural blockade

M. A. W. M. HASENBOS AND M. J. M. GIELEN

Summary

A patient with severe bullous emphysema in whom one bulla occupied more than 25% of the hemithorax, with a shift of the mediastinum to the opposite side, was anaesthetised for bullectomy with a combination of intravenous gamma-hydroxybutyric acid and a high thoracic extradural catheter technique. During the operation the patient breathed 100% oxygen through a double-lumen endobronchial tube in the lateral thoracotomy position. Extradural analgesia during the operation was provided by bupivacaine 0.25%, with adrenaline 1 : 200 000 and, postoperatively, with nicomorphine diluted in dextrose 5%. The advantages of this technique are described.

Key words

Anaesthesia; thoracic.
Anaesthesia techniques; epidural, thoracic, total intravenous.

For patients with giant bullae of the lung, who require operations either outside or inside the thorax, anaesthesia can be provided in several ways.[1-3]

The development of anaesthetic techniques which improve the safety of pulmonary resections also increase the number of patients who can be considered candidates for such surgical therapy. These patients frequently have chronic obstructive pulmonary disease (COPD), with an increased risk of postoperative morbidity and mortality from respiratory failure.[4]

An anaesthesia technique for resection of a giant bulla is described, which appears to be safe and to be associated with minimal postoperative pulmonary complications.

Case history

A 64-year-old male (weight 80.5 kg) with a history of emphysema for the last 15 years, was referred to the hospital because of increasing dyspnoea at rest. Physical examination showed a dyspnoeic, cyanotic man with intercostal recession, and an increased anteroposterior diameter of the chest wall. The blood pressure was 160/85 mmHg, the heart rate 80/minute and breathing frequency 15/minute. Breath sounds were decreased over both lungs and there was prolonged exhalation. The electrocardiogram showed sinus rhythm, normal atrioventricular and intraventricular conduction with no specific repolarisation disturbances. The patient was treated with the

M.A.W.M. Hasenbos, MD, Anaesthetist, M.J.M. Gielen, MD, Anaesthetist, Department of Anaesthesia, Catholic University of Nijmegen, Geert Grooteplein Zuid 10, NL-6525 GA Nijmegen, The Netherlands.

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Table 1. Lung function tests: results before and after resection of a large bullous lesion

	Normal value	(SD)	Patient values		
				Postoperative	
			Pre-operative	2 months	4 months
IVC	4850 ml	(550)	2950 ml	2750 ml	3150 ml
FRC	4400 ml	(600)	7100 ml	4850 ml	5100 ml
RV	3000 ml	(100)	6250 ml	4150 ml	3900 ml
TLC	7850 ml	(700)	9200 ml	6900 ml	7050 ml
MVV	111 litre/min		13.5 litre/min	18 litre/min	18 litre/min
Tidal volume	—	—	800 ml	800 ml	900 ml
Breathing frequency	—	—	14/min	14/min	12/min
FEV_1	3700 ml	(500)	450 ml	600 ml	600 ml
FEV_1% of IVC	76	(7)	15	22	19

Normal values used in the Netherlands are published by Tammeling G.J. Selected papers. Royal Netherlands Tuberculosis Association: 1961; **1**: 65.
The European Coal and Steel Community (ECSC) values are higher for VC and FEV_1, to convert them for the population in the Netherlands a factor of 0.88 is used. IVC, inspiratory vital capacity, FRC, functional residual capacity, RV, residual volume, TLC, total lung capacity, MVV, maximum voluntary ventilation FEV_1, forced expiratory volume in 1 second.

following drugs: prednisolone 10 mg/day, salbutamol (Ventolin®) 400 mg qds, thiazinamium (Multergan®) 300 mg tds, digoxin 0.250 mg/day, terbutaline sulphate (Bricanyl®) 5 mg tds and doxycycline (Vibramycine®) 100 mg/day.

Despite this therapy, his lung function tests (Table 1) showed signs of marked obstructive airways disease. His minute volume was 6.75 litres (0.45 litres × 15 breaths) and his maximum voluntary ventilation (MVV) 13.5 litres/minute; thus, this patient had virtually no ventilatory reserve. After 30 days of treatment and no real improvement, it was decided, after discussion with the surgeon and the anaesthetist, that excision of the large bulla would probably improve his condition; a left-sided thoracotomy was therefore planned.

The patient was premedicated with diazepam 10 mg orally, and 0.5 mg atropine and prednisolone 50 mg intramuscularly one hour before the operation. The extradural catheter was inserted at the T_{3-4} interspace, with the patient awake and in the sitting position. The paramedian approach with the hanging drop technique was used to identify the extradural space. The catheter was directed cephalad and advanced 3–4 cm. The patient was placed supine and an initial test dose of 3 ml lignocaine 2% was injected through the catheter.

The block was tested 15 minutes later; the segments T_{3-6} were blocked by this test dose. Then 8 ml bupivacaine 0.25% with adrenaline 1:200 000 was given through the extradural catheter; this resulted in a block of the segments T_{2-10}. Five minutes later, gamma-hydrobutyric acid (gamma-OH) 50 mg/kg and droperidol 5 mg were given intravenously to induce general anaesthesia. Topical anaesthesia of the vocal cords with lignocaine 10% was followed by intubation with a double-lumen bronchial tube (Bronchocath 41 Fr.—left-sided); the patient was placed in the right lateral position and 100% oxygen administered. The patient was still breathing spontaneously and did so throughout the operation, sometimes with manual assistance. No inhalation anaesthetics were added.

The surgeon removed a large bulla which compressed a part of the left lung. Several smaller bullae were also removed. Chest tubes were inserted and the operation was completed within 2 hours.

The patient was extubated in the operating theatre and brought to the intensive care unit. Arterial blood gas analysis values before, during and after the operation are listed in Table 2.

Analgesia was maintained postoperatively for 3 days by extradural injection of nicomorphine (the 3,6-dinicotyl ester of morphine) 5 mg in 10 ml dextrose 5% (Vilan®, Nourypharma, Oss, The Netherlands; preservative free). Extradural nicomorphine was given three times a day (5 mg nicomorphine diluted in 10 ml dextrose 5%) and resulted in effective analgesia of rapid onset and a duration of about eight hours. The patient

Table 2. Arterial blood gas analysis, during spontaneous respiration

	Added O_2 litre/min by nasal catheter	Pa_{O_2} (kPa)	pH	Pa_{CO_2} (kPa)	Base excess
Pre-operative	0	6.5	7.38	6.6	+4.4
	1.5	7.9	7.39	7.0	+5.
During operation (by tube)					
Supine position	100%	40.3	7.31	8.6	+4.5
Lateral position	100%	36.9	7.27	8.7	+1.8
Postoperative	3	7.0	7.34	6.6	+0.5
	2	9.7	7.38	6.4	+2.9
First day after operation	3	10.4	7.39	5.3	+0.1
Second day after operation	2	8.8	7.44	5.5	+4.1
Third day after operation	2	8.9	7.43	6.0	+5.2

Pa_{O_2}, arterial oxygen tension. Pa_{CO_2}, arterial carbon dioxide tension.

continued to do well until the fourteenth day after operation, when he developed pneumonia in the left lung; this was treated with antibiotics.

The chest tubes were removed on the tenth postoperative day. The patient had to be treated by a psychiatrist for mental depression and was discharged 3 months after the operation without requiring oxygen therapy. Subjectively, he was greatly improved, but with less improvement in his lung function tests than we had hoped (Table 1).

Discussion

The criteria for selecting patients most likely to benefit from bullectomy include progressive dyspnoea, inability to maintain a normal walking pace on level ground and bullae occupying at least 25% of one hemithorax.[3] Our patient fulfilled all three criteria.

It is useful for the anaesthetist to differentiate between open and closed bullae. Anatomically, there are two types in which surgery can be useful: open type bullae with wide open connexions to the bronchi (type 1) and closed type bullae (type 2). The latter are divisible into stenotic bullae with a small connexion to the bronchi and bullae with a ball valve mechanism. Type 1 and 2 can be differentiated by the chest X-ray, which shows a difference in the size of the bullae from inspiration to expiration in the type 1 bulla. This is the method we used to confirm the presence of an open bulla in our patient.

The differentiation can also be made with body plethysmography, where a large difference is only found in type 2 bullae, between functional residual capacity (FRC) measured by the gas dilution wash-out technique and the plethysmographic method.

Ball valve bullae, either unilateral, require special anaesthetic precautions since there is a risk of rupturing a bulla, thereby producing a tension pneumothorax during controlled ventilation. This requires avoidance of high inspiratory pressures. Our patient had an open type bulla, which was also confirmed by measuring the FRC, using the gas dilution wash-out technique. As can be seen in Table 1, the FRC after operation was 2250 ml less than before the operation. In the case of a closed bulla, there would have been no difference.

For safety we had chosen a double-lumen tube. If spontaneous ventilation was inadequate or impossible, we could have controlled the ventilation of the relatively good right lung.

It is generally accepted that the use of nitrous oxide is contra-indicated during an operation for bullectomy[5] and that a technique which avoids intermittent positive pressure ventilation (IPPV), and in which spontaneous ventilation is maintained, is preferable.

Three main techniques have been described by which nitrous oxide can be avoided.[3] Inhalation anaesthesia, which carries the disadvantage of cardiac depression and hypotension, a neuroleptic technique which involves a danger of

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awareness in the absence of nitrous oxide, and finally, a technique with ketamine. The prolonged effects with this method might prevent cooperation for the (important) postoperative physiotherapy. The mental disorientation after ketamine makes its choice even more questionable.

Our technique has several advantages: Gamma-OH[6,7] produces narcosis after a latent period of 5–10 minutes. Sleep is stable and adequate and ventilation is not significantly depressed. There is tendency for the pulse to slow and the blood pressure to increase, which is counteracted by droperidol. The quality of the recovery is particularly agreeable for the patient and is not accompanied by unpleasant phenomena.

If gamma-OH is not available, another potent intravenous anaesthetic which does not depress circulation or ventilation could be used.

The use of an extradural catheter at a high thoracic level for analgesia during the operation has several advantages. Most of the afferent nervous input from lungs and airways, enters the central nervous system along the sympathetic nerves to the upper four thoracic segments.[8,9] The afferent blockade can prevent and even cure bronchospasm.[8] Furthermore, bronchial toilet can be accomplished without severe circulatory reactions.[10] During the operation, we inject weak analgesic solutions (bupivacaine 0.25% plus adrenaline 1:200000) into the extradural catheter, to ensure blockade with minimal motor involvement. As can be seen from the arterial carbon dioxide tension ($Pa\text{CO}_2$) values in Table 2, motor impairment cannot be excluded completely. In addition, throughout the operation, end-expiratory CO_2 was monitored by capnometer and did not increase significantly.

Nicomorphine diluted in dextrose 5% was injected extradurally postoperatively; this has been found to provide effective analgesia of a rapid onset without affecting muscle power, or producing ventilatory depression[11,12] in various thoracic operations, despite the high position of the thoracic extradural catheter.[13,14]

We believe that with this technique of balanced intravenous anaesthesia (without intravenous, opiates, but with high thoracic extradural regional block during the operation, and extradural nicomorphine postoperatively), there are likely to be less pulmonary complications compared to balanced intravenous anaesthesia with opiates given intravenously during the operation and intramuscularly postoperatively.[13,14]

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Extracellular Events Induced by γ-Hydroxybutyrate in Striatum: A Microdialysis Study

Viviane Hechler, Serge Gobaille, Jean-Jacques Bourguignon, and Michel Maitre

Centre de Neurochimie du CNRS and INSERM U. 44, Strasbourg, France

Abstract: The modification of dopamine release and accumulation induced by γ-hydroxybutyrate (GHB) was studied using both striatal slices and in vivo microdialysis of caudate-putamen. GHB inhibited dopamine release for ~5–10 min in vitro, and this was associated with an accumulation of dopamine in the tissue. Subsequently, there was an increase in dopamine release. In the microdialysis experiments, low doses of GHB inhibited dopamine release, whereas higher doses strongly increased release; the initial decrease seen in slices could not be detected in vivo. Thus, GHB had a biphasic effect on the release of dopamine: An initial decrease in the release of transmitter was followed by an increase. A time-dependent biphasic effect was observed when GHB was added to brain slices, and a dose-dependent biphasic effect was seen in dialysate after systemic administration of GHB. Naloxone blocked GHB-induced dopamine accumulation and release both in vitro and in vivo. GHB also increased the release of opioid-like substances in the striatum. A specific antagonist of GHB receptors completely blocked both the dopamine response and the release of opioid-like substances. These data suggest that GHB increases dopamine release via specific receptors that may modulate the activity of opioid interneurons.
Key Words: γ-Hydroxybutyrate—Dopamine—Striatum—In vivo microdialysis—Opioid/dopamine receptors. **Hechler V. et al.** Extracellular events induced by γ-hydroxybutyrate in striatum: A microdialysis study. *J. Neurochem.* **56,** 938–944 (1991).

The modification of dopaminergic activity of the nigrostriatal pathway by γ-hydroxybutyrate (GHB) has been the subject of numerous in vitro and in vivo studies (Bustos and Roth, 1972*a,b*; Cheramy et al., 1977). It has been reported that in vivo GHB induces a diminution of impulse flow in dopaminergic pathways (Roth et al., 1973, 1980). Concomitantly with these electrophysiologic changes there is an increase in the level of striatal dopamine, which is closely followed by an increase in the levels of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Gessa et al., 1966, 1968; Spano et al., 1971); the latter changes have been shown to result from GHB-induced activation of tyrosine hydroxylase (Morgenroth et al., 1976).

Over the past several years the existence of an endogenous GHB system in brain has been demonstrated. GHB is synthesized (Cash et al., 1979; Rumigny et al., 1981*a,b*) and released (Maitre et al., 1983*b*; Vayer and Maitre, 1988) in brain, and the presence of specific receptor sites for this compound has been shown in brain (Benavides et al., 1982*a*; Maitre et al., 1983*a*; Hechler et al., 1987), particularly in hippocampus, cortex, and striatum. This GHB system may participate in the regulation of dopaminergic activity in the striatum. To investigate this possibility, we carried out experiments to analyze GHB effects on dopaminergic terminals, both in vitro and in vivo. Naloxone has been reported to block GHB-induced elevation of striatal dopamine levels (Snead and Bearden, 1980) as well as GHB-induced changes in second messenger systems in the hippocampus (Vayer et al., 1987; Vayer and Maitre, 1989). Because opioid systems have been implicated in modifications of dopamine release in striatum (Nicoll et al., 1977; Biggio et al., 1978; Chesselet et al., 1981; Rudolph et al., 1983), we also examined the possibility that the GHB system might interact with the opioid system to modulate dopaminergic activity.

MATERIALS AND METHODS

In vitro experiments

Adult Wistar rats were killed and decapitated. Their brains were removed, and the striatum was rapidly dissected. Tissue

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Address correspondence and reprint requests to Dr. V. Hechler at Centre de Neurochimie du CNRS et U. 44 de l'INSERM, 5, rue Blaise Pascal, 67084 Strasbourg, France.

Abbreviations used: DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, γ-aminobutyric acid; GHB, γ-hydroxybutyrate; HVA, homovanillic acid; NCS-382, 6,7,8,9-tetrahydro-5*H*-benzocycloheptene-5-ol-ylideneacetic acid; TTX, tetrodotoxin.

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slices (300 μm thick) were prepared with a Vibratome (Lancer Instruments) according to the method of Luini et al. (1981) and Maitre et al. (1983*b*). The slices, cut in Krebs–Ringer bicarbonate glucose buffer [122 m*M* NaCl, 3.1 m*M* KCl, 1.3 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4$, 0.4 m*M* KH_2PO_4, 24.4 m*M* $NaHCO_3$, and 10 m*M* glucose, pH 7.4, gassed with O_2/CO_2 (95:5)] maintained at 33°C, were incubated with 50 μ*M* tyrosine for 20 min in the same medium. The slices (~40 mg of tissue, wet weight) were next incubated for various times in the presence of the same mixture containing 1 m*M* GHB. Under these conditions, we have demonstrated an active uptake of GHB by striatal slices (Benavides et al., 1982*b*; Hechler et al., 1985). Release of dopamine was induced by transferring the slices for 3 min to the same Krebs–Ringer bicarbonate glucose buffer, but containing 56 m*M* KCl and 64 m*M* NaCl. The slices were then removed, and the incubation medium was analyzed for dopamine content by HPLC (Kontur et al., 1984).

Parallel experiments were performed to determine the dopamine content of the slices. The tissue was treated in the same manner as for the release experiments except that the slices were removed and frozen (−80°C) after the end of the incubations with 1 m*M* GHB.

The tissue was homogenized in cold 0.1 *M* perchloric acid (10 μl/mg wet weight) containing 1 ng/10 μl of internal standard (3,4-dihydroxycinnamic acid) and centrifuged at 30,000 *g* for 20 min. The supernatants were removed and analyzed for dopamine.

In vivo experiments

Adult male Wistar rats (weighing 250–300 g) under ketamine anesthesia (150 mg/kg i.p.) were unilaterally implanted and mounted in a stereotaxic frame (Narishige). The guide cannula was inserted in the middle of the head of the right caudate-putamen 0.5 mm anterior and 3 mm lateral to bregma with the tip 1.8 mm below the dura (Paxinos and Watson, 1986), so that after implantation the probe protruded 4 mm beyond this guide. The guide cannula was secured to the cranium using dental cement. At the same time, a plastic tube 2 cm high and 1.5 cm in diameter was placed around the guide to protect the inlet–outlet tubing from the probe.

Three days after surgery, the rat was lightly anesthetized with ether, and the microdialysis probe (polycarbonate-polyether, 20,000 dalton cut-off; 4 mm long and 0.52 mm in diameter; Carnegie Medicin) was implanted using the guide cannula. The probe was perfused with a nonbuffered saline solution (147.2 m*M* NaCl, 3.4 m*M* $CaCl_2$, and 4 m*M* KCl) at a rate of 1 μl/min using a 300-mm length of Teflon tubing; the pH of this medium, initially 6.0, was increased to 7.0–7.4 after passage through the dialysis probe in vivo. A liquid swivel connected the probe to a microinjection pump (CMA 100; Carnegie). With this system, the rat was allowed to move freely within a hemispherical bowl (55 cm in diameter). The perfusates were collected in small tubes containing 5 μl of 0.5 *M* perchloric acid, 5 pmol of internal standard (3,4-dihydroxycinnamic acid) for catecholamine analyses, or 10 μl of a 20 μ*M* solution of enkephalinase inhibitor (*N*-carboxymethyl-L-phenylalanyl-L-leucine) for the naloxone radioreceptor assay. Samples were collected at 20-min intervals, and at least seven control samples were taken before drug administrations. GHB was locally applied by a 20-min dialysis of a saline solution containing 1 m*M* GHB.

At the end of each experiment, the site of the dialysis probe was verified histologically after fast-blue cresyl violet staining.

Measurement of levels of dopamine and metabolites

The perfusates or the tissue extracts were assayed for dopamine, DOPAC, and HVA by HPLC with electrochemical detection according to the method of Kontur et al. (1984) with several modifications. The chromatographic system consisted of a 25-cm × 4.6-mm Hypersil C_{18} ODS column (particle size, 5 μm; Biochrom, France). The column was kept at a constant temperature of 30°C with a heating block (Waters model TCM), and the flow rate was 1.2 ml/min (Waters model 501 HPLC pump) with a back pressure of 1,500 psi. The system was linked to a Waters model 460 electrochemical detector with a glassy-carbon electrode. The detector potential was maintained at 0.60 V versus an Ag/AgCl reference electrode. The mobile phase consisted of 0.1 *M* NaH_2PO_4 and 0.1 m*M* disodium EDTA (pH 4.85) in double-distilled water with methanol added to a final concentration of 6%. The system was calibrated by injection of various amounts (0.2 nmol–20 fmol) of standard solutions containing dopamine, DOPAC, HVA, and 5 pmol of internal standard. Twenty-five microliters of each sample was injected onto the column, and peak identification was performed by comparison of retention times with regard to the calibration solutions.

In vitro recovery

To estimate the recovery of the dopamine and metabolites through the membrane, dialysis probes were perfused in vitro at adequate flow rates and placed in physiological Ringer's solution containing dopamine, DOPAC, and HVA at 10^{-6} *M*. The amount of substance in the perfusate was compared with the amount outside the dialysis tube and expressed as percent recovery. The same procedure was used to estimate the amount of GHB perfused into the brain through the probe using [^{3}H]GHB.

Experiments were carried out in parallel to measure the diffusion space of [^{3}H]GHB in the striatum during in vivo dialysis. These results give also the amount of GHB perfused in situ during local application: One millimolar [^{3}H]GHB (80 μCi/mmol) was perfused through the dialysis probe for 20 min (1 μl/min). The animal was killed, and the brain was removed and frozen by immersion in isopentane at −40°C for 1 min. The frozen brains were cut in serial sections of 25 μm with a cryostat microtome at −15°C. Anatomical characterization of sections, autoradiographic exposure, and computerized image analysis (BIOCOM 500 system) were carried out as previously described (Hechler et al., 1987). The results showed that 1 m*M* [^{3}H]GHB diffused in situ in a volume of ~70 μl around the probe with a yield of ~16%.

Measurement of opioid-like substances

This study was performed using a radioreceptor assay with [^{3}H]naloxone according to the method of Pert and Snyder (1973).

Membrane preparation. Adult male Wistar rats were killed by decapitation. Their brains were removed, homogenized in 10 volumes of cold 0.05 *M* Tris-citrate buffer (pH 7.4), and centrifuged at 30,000 *g* for 20 min. The supernatant was removed, and the membranes were frozen at −20°C until use for the radioreceptor assay.

Radioreceptor assay. The microdialysis samples (50 μl) were added to 550 μl of Tris-citrate buffer containing 2 n*M* [^{3}H]naloxone (final concentration). At the same time, displacement assay was performed using various concentrations of nonradioactive naloxone (10^{-9}–10^{-6} *M*) to determine a

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standard curve by nonlinear regression. This permitted the estimation of the amount of naloxone-like substances contained in the microdialysis samples. Nonspecific binding was determined by adding 5×10^{-4} *M* (final concentration) unlabeled naloxone. The samples were incubated for 30 min at 0°C and then centrifuged at 45,000 *g* for 20 min. The membranes were washed twice with ice-cold Tris-citrate buffer, and the radioactivity present was assayed by liquid scintillation spectrometry. Nonlinear regression displacement curves were calculated using the Graphpad program.

Statistics

For release experiments both in vivo and in vitro, a one-way analysis of variance for repeated measures was applied. Results showing significant overall changes were subjected to a Newman-Keuls multiple comparison test to determine significant changes from control or basal values.

RESULTS

Influence of GHB on dopamine release from striatal slices

In the presence of 1 m*M* GHB in the incubation medium, the K^+-induced release of dopamine was reduced during the first 5–10 min after GHB stimulation. Under these conditions, the GHB concentration in tissue is ~150 μM after a 5-min incubation (Hechler et al., 1985). At this concentration of GHB, the K^+-induced release of dopamine was reduced by ~25% compared with the control (Fig. 1). After a 15-min incubation under these conditions, dopamine release began to increase compared with controls. The increase was maximal after 20 min (53% increase). With prolonged incubation (30 min), dopamine release returned to control levels.

The presence of naloxone (5 μM) with GHB (1 m*M*) in the incubation medium did not modify decreased dopamine release but completely abolished the increase of dopamine release (Fig. 1).

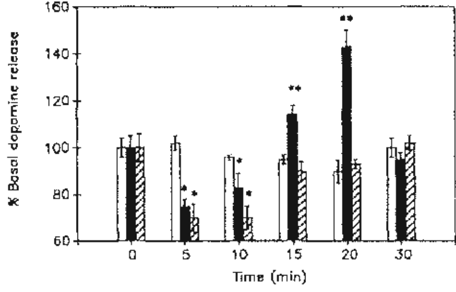

FIG. 1. Effect of naloxone (5 μM; hatched columns) on GHB-induced (solid columns) effects on dopamine release from rat striatal slices. The slices were preincubated in the presence of 50 μM tyrosine in Krebs–Ringer oxygenated medium maintained at 33°C. The slices were then incubated for various times in the presence of 1 m*M* GHB with or without naloxone. Release of dopamine was induced by transfer of the slices to a Krebs–Ringer solution containing 56 m*M* K^+. Control experiments (open columns) were carried out in the same manner but without any drug exposure. Dopamine-induced release in the presence of naloxone alone showed no change compared with control experiments (data not shown). Dopamine content was measured using an HPLC with electrochemical detection system. Data are mean ± SEM (bars) values of three separate experiments. Basal K^+-induced release was 2.43 ± 0.08 $\times 10^{-12}$ mol/min/mg wet weight. Values significantly different by Newman–Keuls multiple comparison test from controls are indicated: $^*p < 0.05$, $^{**}p < 0.01$.

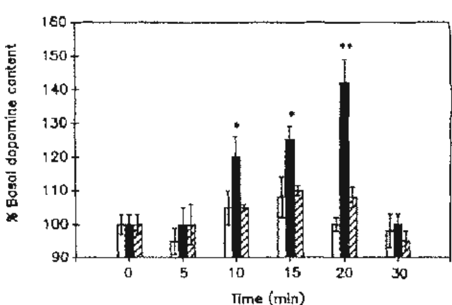

FIG. 2. Effect of naloxone (5 μM; hatched columns) and of GHB (1 m*M*; solid columns) on the accumulation of dopamine in rat striatal slices. The experiments were performed in the same manner as described in Fig. 1 except that the slices were removed and frozen at −80°C after the end of the incubation with 1 m*M* GHB. Dopamine was extracted with 0.1 *M* perchloric acid and injected into an HPLC with electrochemical detection system. Dopamine-induced accumulation in tissue slices by naloxone alone showed no change compared with the control (data not shown). Data are mean ± SEM (bars) values of three separate experiments. Values significantly different by Newman–Keuls multiple comparison test from controls (open columns) are indicated: $^*p < 0.05$, $^{**}p < 0.01$.

Influence of GHB on dopamine level in striatal slices

After a 10-min incubation with 1 m*M* GHB, the level of dopamine increased in the slices (by 20%), with a maximal increase observed after a 20-min incubation (42%). This corresponded to the maximal GHB-induced increase in dopamine release. The tissue level of dopamine subsequently returned to normal. The presence of 5 μM naloxone in the incubation medium completely blocked the accumulation of dopamine in the slices (Fig. 2).

Effects of intraperitoneal administration of GHB on striatal dopamine release

The influence of GHB on dopamine release from striatal terminals was dependent on the dose used. After intraperitoneal administration of 250 mg/kg of GHB, in vivo dialysis of striatum revealed a decrease in dopamine release (−90%) that lasted ~150 min (Fig. 3). Thereafter, the dopamine release slowly increased (70%) and returned to the control values 240 min after the GHB injection. Administration of a higher dose of GHB to the animals (500 mg/kg i.p.) did not inhibit dopamine release. In this latter case, only the increase in dopamine release was seen (530%, ~60 min after administration; Fig. 3).

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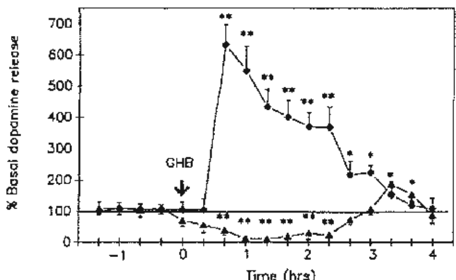

FIG. 3. Effects of GHB, 250 (▲) and 500 (♦) mg/kg i.p., on dopamine efflux (percent basal release/20 min) in dialysates collected from rat striata. Data are mean ± SEM (bars) values of three separate experiments. The overall control (100%) value for dopamine release from striata was 160 ± 26 fmol/20 min (n = 12). Values significantly different by Newman–Keuls multiple comparison test from controls are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$.

Effects of direct striatal application of GHB on dopamine release as measured by in vivo dialysis

As shown in Fig. 4, local application via the dialysis probe of 240 μM GHB (concentration directly measured in tissue by [^{3}H]GHB dialysis) induced an increase in dopamine release (258%) in the first three fractions followed by a return to baseline. Pretreatment of the animals by naloxone (10 mg/kg i.p.) or nalorphine (50 mg/kg i.p.) 20 min before local stimulation by GHB blocked the increase in extracellular dopamine content. The blockade was complete for nalorphine, but with naloxone an increase in dopamine was still observed in the first fraction (96%).

Calcium dependency of GHB-induced release of dopamine

Replacement of Ca^{2+} with Na^+ in the Ringer solution resulted in a fall of dopamine output to ~2–5% of control values after 2 h. This is in accordance with previous results (Imperato and Di Chiara, 1984). During this phase of low dopamine release in the absence of Ca^{2+}, direct local application to the striatum via the dialysis probe of 240 μM GHB (concentration directly measured in situ by [^{3}H]GHB dialysis) did not modify dopamine release. Thus, the presence of Ca^{2+} in the Ringer solution is apparently necessary for the GHB-induced increase in dopamine release (data not shown).

Tetrodotoxin (TTX) sensitivity of GHB-induced release of dopamine

TTX has been shown to block in vivo release of dopamine by inhibiting voltage-dependent Na^+ channels. Figure 5 shows the effects of 1 μM TTX on striatal dopamine release. The contralateral striatum was perfused simultaneously under the same conditions but with normal Ringer solution as a control. TTX reduced dopamine release to undetectable levels after ~20–30 min (Fig. 5). Stimulation of dopamine release by GHB on both sides (240 μM, local application, real concentration in situ) did not induce modification in the striatum receiving TTX as compared with the contralateral striatum perfused with normal Ringer solution, which exhibits a 290% increase in dopamine release following GHB application. Thus, GHB-induced release of dopamine requires the free movement of Na^+ through voltage-dependent fast Na^+ channels.

Local application of GHB in striatum induced release of opioid-like substances

Local GHB application (240 μM) in a similar experiment as previously described induced the release of a significant amount (420%) of naloxone-displacing substances in the five fractions collected between 140 and 240 min, a duration longer than that of dopamine (Fig. 6).

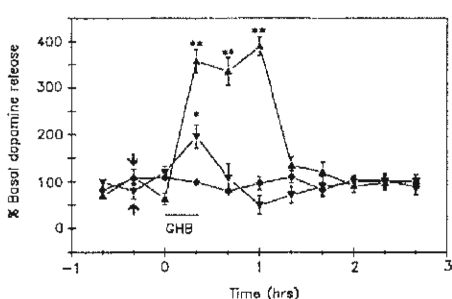

FIG. 4. Effects of naloxone (10 mg/kg i.p.) (▼) and nalorphine (50 mg/kg i.p.) (♦) on GHB (240 μM, local application; ▲)-induced increases in dopamine efflux (percent basal release/20 min) in dialysates from rat striatum. The naloxone or nalorphine (arrow) was injected 20 min before GHB administration (the line indicates the infusion period). Data are mean ± SEM (bars) values of three separate experiments. Control experiments are not shown. Values significantly different by Newman–Keuls multiple comparison test from controls are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$.

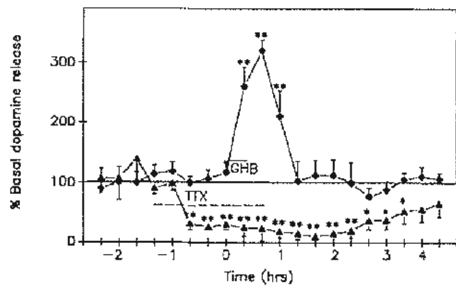

FIG. 5. Effect of TTX (1 μM, local application; ▲) on GHB (240 μM, local application; ♦)-induced increases in dopamine release from rat striatum. TTX was applied for 2 h, and GHB was given 80 min later for 20 min. The dialysis experiments were carried out with bilateral probes implanted in the left and right striata; the right side received TTX plus GHB, and the contralateral side received GHB alone, as a control (the lines indicate the infusion periods). Data are mean ± SEM (bars) values of three separate experiments. Values significantly different by Newman–Keuls multiple comparison test are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$.

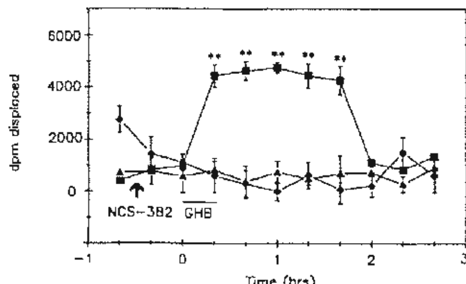

FIG. 6. Effects of NCS-382 (500 mg/kg i.p.) on GHB (240 μM, local application)-induced increases in efflux of opioid-like substances in dialysates collected from rat striatum (1 μl/min, 20 min): GHB alone (■) and plus NCS-382 (♦). NCS-382 (arrow) was injected 30 min before GHB local application (the line indicates the infusion period). The control experiments (saline solution; ▲) were performed in the same manner but without any treatment. Opioid-like substances were quantified using a radioreceptor assay as described in Materials and Methods. Data are mean ± SEM (bars) values of three separate experiments. Values significantly different by Newman–Keuls multiple comparison test are indicated: $^{**}p < 0.01$.

Effect of an antagonist substance of GHB receptors

NCS-382 (6,7,8,9-tetrahydro-5*H*-benzocyclohep-tene-5-ol-ylideneacetic acid, sodium salt), a structural analogue of GHB, is a GHB receptor antagonist (Maitre et al., 1990). Pretreatment of animals 60 min before local stimulation by GHB with NCS-382 (500 mg/kg i.p.) completely inhibited the dopamine response (Fig. 7). To block the GHB-induced increase in level of opioid-like compounds, the time lapse between NCS-382 pretreatment and in situ application of GHB had to be reduced to 30 min (Fig. 6).

DISCUSSION

The effects of GHB on dopamine release and synthesis in the striatum have been the subject of several reports (Spano et al., 1971; Bustos and Roth, 1972*a*,*b*). In vivo experiments have shown that peripheral and/or local administration of GHB rapidly induces an inhibition of the firing rates of dopaminergic cells in the substantia nigra (Roth et al., 1980). This phenomenon is accompanied by a decrease in dopamine release in the caudate-putamen measured in vitro in the presence of 10^{-3} *M* GHB (Bustos and Roth, 1972*b*). A parallel increase in dopamine synthesis has been observed and has been attributed to a stimulation of tyrosine hydroxylase activity (Morgenroth et al., 1976). These effects result in a progressive tissue accumulation of dopamine, as demonstrated in vivo by Gessa et al. (1968). However, using the push–pull cannula technique in vivo, Cheramy et al. (1977) demonstrated a stimulation of dopamine release in the caudate nucleus after peripheral injection of GHB in the cat.

The present results closely correspond to the findings of Roth et al. (1980), who reported a decrease in dopamine cell unit activity of the substantia nigra by 50% after intravenous administration of 150 mg/kg of GHB in rats. This injection was sufficient to increase the endogenous GHB content of caudate-putamen by ~46-fold (Roth et al., 1980), which corresponds to a concentration of ~140 μM.

In parallel with the increased dopamine release, we observed an accumulation of dopamine in the slices. This has also been reported in vivo (Gessa et al., 1966, 1968), and as for increased dopamine release, it appeared after incubation for 10–15 min. Owing to the time required for sample collection with microdialysis and push–pull cannula techniques (Cheramy et al., 1977)—20 min in our experiments and 15 min in the experiments of Cheramy et al. (1977)—only the increase in dopamine release is detected by these techniques.

Thus, it appears that the effects of GHB on dopamine release in striatum are biphasic. At low concentrations of GHB, a decrease in striatal dopaminergic activity is observed with a reduction of release. This is probably the situation observed in the experiments of Imperato and Di Chiara (1984), after injection of γ-butyrolactone, which is generally considered as the prodrug of GHB in brain. In our hands, in vivo dialysis of striatum after 250 mg/kg i.p. of GHB also induced a reduction in dopamine release, followed 3 h later by a small increase. However, intraperitoneal administration of a larger dose (500 mg/kg) induced a large increase in dopamine release after a time lag of ~40 min. This increase is probably due to stimulation of tyrosine hydroxylase (Morgenroth et al., 1976) and dopamine accumulation in the tissue (Gessa et al., 1968). Our in vitro results showed that the biphasic action of GHB is probably due to the slow increase in GHB tissue concentration following uptake from the incubation medium (Hechler et al., 1985). Thus, the time-dependent effect seen in brain slices is probably a dose-de-

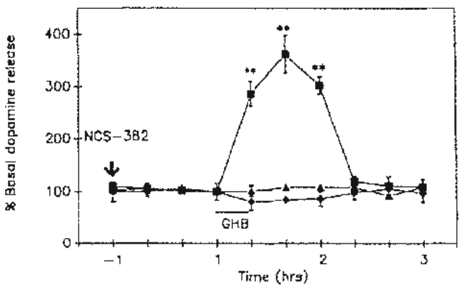

FIG. 7. Effect of NCS-382 (500 mg/kg i.p.) on GHB (240 μM, local administration)-induced increases in dopamine release collected from rat striata: GHB alone (■) and plus NCS-382 (♦). NCS-382 (arrow) was injected 60 min before GHB administration (the line indicates the infusion period). The control experiments (saline solution; ▲) were conducted in the same manner but without any treatment. Data are mean ± SEM (bars) values from three separate experiments. Values significantly different by Newman–Keuls multiple comparison test are indicated: $^{**}p < 0.01$.

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pendent effect due to GHB accumulation in the slices with time.

The characteristics of dopamine release in vivo after GHB stimulation appear to follow the classical parameters of dopamine release (TTX sensitivity, Ca^{2+} dependence) as described in in vivo dialysis experiments by Imperato and Di Chiara (1984). Thus, it seems that a pharmacological dose of GHB increases dopaminergic neurotransmission by stimulating the physiological components of basal release.

To explain the increase in dopamine release induced by GHB, Cheramy et al. (1977) suggest an effect due to GABAergic input, assuming that the nigrostriatal dopaminergic neurons are regulated by GABAergic descending neurons. This hypothesis cannot be completely ruled out, because we have previously demonstrated the in vitro transformation of GHB into γ-aminobutyric acid (GABA) (Vayer et al., 1985). However, the reported effects of low doses of naloxone (Snead and Bearden, 1980), which overcomes GHB dopaminergic effects in vivo, and the results of our present in vitro and in vivo experiments are in favor of an opioid mechanism for controlling dopamine accumulation and increased release. Moreover, in the microdialysis experiments, nalorphine exhibited the same inhibition as naloxone, which was used at too low a dose to support the involvement of a GABAergic effect. GHB also induced the extracellular increase in content of naloxone-displacing compounds in the microdialysis experiments. This increase might represent the effect of GHB stimulation of opioid interneurons, which are known to influence dopamine release and synthesis in the striatum (Biggio et al., 1978; Pollard et al., 1978; Chesselet et al., 1981; Wood and Richards, 1982).

Finally, the GHB effects in vivo on both opioid and dopamine release were blocked by a selective antagonist for the GHB receptor in the striatum (Hechler et al., 1987; Maitre et al., 1990). The shorter NCS-382 pretreatment time required to block GHB-induced release of opioid substances (30 min) seems to indicate that opioid substance release is a phenomenon that precedes dopamine release. However, the time lapse for sample collection in in vivo dialysis make it difficult to know the precise sequence of events in the extracellular space.

Thus, it appears that the GHB endogenous system in brain might exert a regulatory influence on the nigrostriatal dopaminergic pathway. This effect may be mediated by opioid interneurons directly stimulated by GHBergic input, an organization similar to that suggested for the hippocampus that is responsible for GHB-induced EEG changes (Vayer et al., 1988).

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Key Words: divalproex, valproate, valproic acid, history, seizures, headaches, mania

The History of Divalproex in Clinical Neuroscience

By Thomas R. Henry, MD

ABSTRACT ~ The scientific and medical history of valproic acid is relatively long, compared with other frequently used psychopharmacologic agents. Valproic acid was used as an organic solvent in research laboratories for eight decades, until the fortuitous observation of action against pentylenetetrazol-induced convulsions in rodents. Early clinical experience emphasized therapy of absence seizures in primary generalized epilepsies. During two decades of controlled trials in partial-onset and generalized-onset seizures and myoclonus, valproate was established as the prototypical broad-spectrum antiepileptic drug. Anecdotal observations in patients with both epilepsy and migraine headaches who were started on valproate led to prospective, randomized trials that established antimigraine efficacy. Early observations suggested antimanic actions; more than a decade later, controlled clinical trials established significant efficacy of valproate in mania. Antiproliferative effects of valproate were unexpectedly noted during mechanistic studies; two decades later a maintenance adjunctive or chemopreventive role in oncology is being defined. While pharmacokinetic studies appear definitive, completion of comprehensive pharmacodynamic investigations of valproate's biochemical actions and clinical utility is yet to be achieved.
Psychopharmacology Bulletin. *2003;37(Suppl 2): 5–16*

Introduction

The fortuitous discovery of neuropharmacologic properties of the organic solvent 2-propylvaleric acid

Burton first reported the synthesis of valproic acid in 1882.[1] Valproic acid (VPA) is a clear, colorless, fatty acid which is liquid at room and body temperature; it is only slightly soluble in water, but highly soluble in organic solvents. This branched-chain, 8-carbon, aliphatic molecule derived its current generic name from the more descriptive name 2-propylvaleric acid. Other descriptive names, including di-n-propylacetic acid and 2-propylpentanoic acid, are now rarely used.

Dr. Henry is associate professor in the Department of Neurology and director of the Emory Epilepsy Center at Emory University in Atlanta, GA.

To whom correspondence should be addressed: Dr. Thomas R. Henry, The Emory Epilepsy Center, Department of Neurology, Emory University, WMRB#6000, 1639 Pierce Drive, Atlanta, GA 30322; Phone: 404-778-5943; Fax: 404-778-5904; E-mail: thenr01@emory.edu.

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For eight decades valproic acid was used infrequently in laboratory work as a "metabolically inert" solvent for organic compounds. In 1962, Eynard and colleagues were investigating khelline derivatives as potential anticonvulsants, but encountered difficulty in dissolving some derivatives in water or the usual organic solvents. These compounds were instead solubilized using VPA, based on the suggestion of Meunier. Remarkable anticonvulsant activity of all of the solutions was observed, and it was decided that the solvent itself should be checked for possible anticonvulsant activity. Subsequently, these investigators in Carraz' laboratory first established antiseizure effects of VPA, in this instance using the pentylenetetrazol model.[2] The initial human epilepsy trials were reported the following year.[3]

Successful therapy of generalized epilepsies led to approval of VPA in France in 1967, and by the United States Food and Drug Administration (FDA) in 1983. Within another decade, divalproex sodium was synthesized, tested, and marketed as a formulation of VPA that is superior to pure VPA. Divalproex is a stable complex of equimolar quantities of the sodium salt and the acid of VPA. Currently, oral enteric-coated formulations of divalproex, some in extended-release forms, are the most commonly used VPA-based medications for generalized and partial epilepsies, bipolar disorder, migraine, and other disorders. Parenteral and various other VPA-based formulations are also widely available. In the following, "VPA" will refer to any of these VPA-based compounds, with additional descriptions added where distinction may be important.

CLINICAL STUDIES

Epilepsies

The earliest systematic observations of adjunctive VPA use in epilepsy began with open clinical trials.[4-6] These trials included patients with various types of uncontrolled seizures. Seemingly, these trials should have provided strong evidence that VPA is effective in essentially all types of seizures, on retrospective consideration of the results of controlled trials. Nonetheless, based on these uncontrolled trials, only absence (petit mal) seizures of primary generalized epilepsies were initially considered the therapeutic indication for VPA.

Anti-absence effects of VPA in both symptomatic generalized and primary generalized epilepsies were more convincingly established in controlled trials of adjunctive use during the 1970s[7-9] and of VPA monotherapy in comparison against ethosuximide during the 1980s.[10,11] Positive effects on generalized tonic-clonic (GTC) seizures were noted in many of these patients, and by the early 1980s controlled studies showed

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significant VPA efficacy in GTC seizures of generalized epilepsies.[12] Myoclonus often occurs in primary generalized and symptomatic generalized epilepsies, and VPA effects in reducing segmental and massive myoclonus, including infantile spasms, were established by the early 1980s.[9,13-15] Improvements in control of seizures in generalized epilepsies were associated with decreased occurrence of generalized spike-wave discharges on interictal electroencephalography (EEG).[13,16]

Controlled trials in the epilepsies expanded to study VPA effects on partial-onset seizures[12,17] and on epileptic drop attacks (tonic and atonic seizures). Adjunctive and monotherapy trials, with randomized, prospective design, have established VPA efficacy in simple partial, complex partial, and partial-onset GTC seizures.[6,18,19] The results of these studies supported the early characterization of VPA by Chapman and colleagues[20] as a very broad spectrum antiepileptic drug.

The safe and effective use of VPA has been enhanced by anecdotal and population reports of adverse effects, toxicity, kinetics, and serum levels in clinical epileptology. The clinical utility of measuring serum VPA levels to guide dosing was established early on.[21-23] The commonly accepted "therapeutic" serum range of 60-100 μg/ml clearly does not guarantee efficacy and absence of adverse effects, but offers some confidence in initiation of therapy. In particular, this range can serve as an initial target for individualized adjustment of parenteral or oral loading and of early maintenance dosing. In the process of later adjustments of maintenance dosing, serum VPA levels can be used to clarify pharmacokinetic drug interactions and patient compliance. Some "positive" drug interactions may occur independently of peripheral kinetics, however, such as the antiseizure synergism of VPA and lamotrigine first reported by Brodie.[24] Adverse effects and pharmacokinetics of VPA were first studied in epilepsy, as reviewed below, but are equally important in other patient populations.

Migraine

Epilepsy and migraine are among the most commonly occurring of neurologic conditions. Not surprisingly, the earliest anecdotal experiences with VPA in migraine arose in patients with coexisting epilepsy and migraine. Early open VPA trials found benefit in classic, common, and cluster forms of vascular headache, and in chronic daily headaches that may have both migraine and tension components.[25-27] Subsequently, placebo-controlled, randomized, prospective trials established VPA efficacy in acute and chronic migraine therapy,[28-30] and supported FDA approval for migraine in 1996. Head-to-head comparisons of new and older therapies have rarely achieved fully randomized, controlled, and double-blinded trial design, but in one such study, VPA showed equivalent efficacy to propanolol in chron-

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ic migraine prophylaxis.[31] Thus, unlike the fitful and circuitous route of VPA trials in other neurological and psychiatric indications, in migraine therapy VPA was established straightforwardly from anecdotal to objective efficacy in less than a decade.

Bipolar disorder

Lambert initiated VPA therapy for "fits" of manic behavior, using the amide salt of VPA that was developed by Carraz's group for epileptic fits. His first published report in 1975, based on a decade of uncontrolled observations in France, emphasized an observably greater VPA antagonism of manic than of depressed states, and a synergism of clinical effect on comedication with lithium.[32] The next published clinical observations in mania came from Emrich in Germany in 1980,[33] who tried VPA based on γ-aminobutyric acid (GABA)-ergic theories of mania, and reported anti-manic effect sustained over years in a small group of patients.

Mania and depression trials with VPA began another decade later in the US, when other antiepileptic agents were being tried in affective disorders.[34] Early open trials showed antimanic effects more prominently than antidepressant effects.[35,36] FDA approval of VPA for mania in 1995 was based in part on randomized, prospective, blinded, controlled comparisons of VPA with placebo or lithium.[37-39] Shortly after FDA approval, controlled trials addressed the efficacy of oral VPA loading in acute psychotic mania, and the utility of measuring VPA levels in guiding initial dosing targets for chronic therapy.[40,41] Overall, VPA kinetics and adverse effects appeared little different in definitive observations of bipolar disorder than in the epilepsies. Most recently, a controlled trial detected several aspects of attenuated depressive morbidity in bipolar disorder treated chronically with VPA compared with lithium.[42] Comparisons of VPA with other newer antiepileptic drugs in mania and depression can be expected.[43] At this time it would be premature to write a final history of VPA in the affective disorders.

Clinical studies in other cerebral dysfunctions

Uncontrolled observations in the 1970s and 1980s variously suggested that VPA use worsened symptoms of schizophrenia[44] (an observation that has been questioned hy clinical psychiatrists), and exacerbated motor signs in Parkinson's disease[45,46] (an observation that is accepted by most neurologists). Other anecdotal clinical experience suggested decreased aggressive behavior[47] and decreased movements of tardive dyskinesia.[48] By the mid 1980s, placebo-controlled trials showed no VPA benefit in tardive dyskinesia, however.[49-50] Ongoing controlled VPA trials for impulsive aggression may yet support Lambert's uncontrolled observations.[47]

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Antineoplastic effects

Antiproliferative effects of VPA were discovered fortuitously during investigations not designed to study VPA as an antitumor agent. Regan used neoplastically-transformed neuroectodermal cell lines to study teratogenic mechanisms of VPA, and observed cell growth retardation.[51] Subsequent experiments showed additional pro-differentiation effects of VPA.[52] Currently, VPA is viewed by some as an adjunctive therapy in slowing progression of some solid tumors and hematologic malignancies.[53] Based in part on tolerability in chronic use, VPA also may prove useful in long-term use for stabilizing residual tumors or for chemoprevention.

Adverse effects

Adverse effects of VPA were fully described in clinical observations of epilepsy patients, with most of the definitive information generated before 1990. Overall, early clinical studies demonstrated excellent tolerability in acute and chronic VPA therapy, compared with existing medications.[54] The common adverse effects of VPA, including lethargy, appetite stimulation and weight gain, nausea and upset stomach (which declined markedly in occurrence with use of enteric-coated preparations taken with meals), so-called "alopecia" (representing increased hair fragility, and not actual degeneration of hair follicles), and dose-related tremor, were recognized before FDA approval.[18,55-58] Open studies of parenterally administered VPA showed some minor headache occurrence, but little other difference in adverse effect profile from oral administration.[59]

Most of the rare and uncommon adverse effects, such as reversible dose-related thrombocytopenia,[60] idiopathic hepatitis/hepatic failure,[61,62] hemorrhagic pancreatitis,[63,64] and acute-chronic stupor/encephalopathy with or without associated hyperammonemia,[65-67] also were described within the first decade of widespread clinical use. The first reports of spina bifida in infants of mothers taking VPA appeared in letters to various medical journals, and by the late 1980s, the association between first-trimester VPA exposure and neural tube defects was widely accepted.[68-70] While women with epilepsy have a higher incidence of reproductive dysfunction and polycystic ovaries than do women in the general population, it has been known for a decade that polycystic ovaries and hyperandrogenism are more common in epileptic women using VPA than other antiepileptic drugs.[71]

Large-scale descriptive studies served in some instances to identify groups at increased risk of rare but potentially severe adverse effects. For example, Dreifuss' leadership in population studies of VPA hepatotoxicity clearly identified higher risk with polytherapy under two years of age.[72] Chemical hepatitis had already been shown to be sensitively detectable with serum transaminase determinations,[73] permitting presymptomatic

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identification of hepatic injury. Alterations in prescribing patterns resulted in markedly attenuated occurrence of fulminant hepatitis. The considerable safety and tolerability of current VPA use must in large measure be attributed to these careful clinical observations and analyses of the 1970s and 1980s.

CLINICAL PHARMACOKINETIC AND PHARMACODYNAMIC STUDIES

VPA absorption, distribution, biotransformation, and elimination

Rapid and essentially complete absorption of orally administered VPA was recognized from its earliest use. The absolute bioavailability of oral VPA appeared close to unity, when dose-level data were compared following parenteral and oral administration.[74] These kinetic studies showed time to peak levels at under two hours following oral administration,[74] but obviously this does not apply to the enteric-coated and extended-release forms now most often used. This high bioavailability was attributed to high membrane permeability and virtual absence of hepatic first-pass extraction. Early animal and human studies showed that serum protein binding exceeded 90%, but that brain entry was rapid.[75,76] Active transport mechanisms for VPA entry into brain were also detected in early kinetic studies, and probably account for the high levels of brain VPA minutes following parenteral VPA administration, even in the face of high serum protein binding.[77] Human cerebrospinal fluid (CSF) studies found brain tissue and CSF concentrations of VPA that usually were much less than 30 % of total serum concentrations, consistent with predicted dependence of brain VPA concentration on unbound plasma fractions.[78,80]

Hepatic metabolism accounts for over 95 % of VPA elimination, as was established in multiple investigations of the 1970s and 1980s.[81,82] Variability in hepatic VPA metabolism accounts for the large inter-individual variations in serum level-dose relationships.[83,84] These studies emphasized the effects of concurrent medications on hepatic cytochrome P-450 activity in hydroxylation of VPA.[85] Thus, while chronic VPA use itself does not significantly induce hepatic P-450 enzyme expression, other drugs which do so were shown during the 1970s to markedly increase VPA elimination.[86] Hepatic β-oxidation is also quantitatively important in VPA elimination, and in this pathway VPA competes with endogenous lipids and branched-chain amino acids.[87,88] Glucuronidation and other hepatic and extrahepatic metabolic pathways add further complexity to the elimination kinetics of VPA.[84,87] Competition for glucuronidation may account for the fact that chronic VPA use approximately doubles the serum half-life of lamotrigine, which has been known for many years.[89] Overall, serum half-lives of VPA have long been known to be shorter in individuals receiving

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polytherapy with P-450 enzyme-inducers, in children, slightly longer in the otherwise healthy elderly, and longer in clinically significant hepatic failure.[85,90-93] Thus, the "old" pharmacokinetic studies support the current clinical use of VPA, with few remaining questions in any areas that might affect clinical practice.

Clinical studies of VPA pharmacodynamics

Positron emission tomographic (PET) studies were the earliest brain mapping techniques used to study VPA kinetics and dynamics.[94-98] In theory, the serial PET imaging of carbon-11-labeled VPA might provide dynamic somatic-cerebral distribution maps of VPA in humans. A preliminary report of such studies emphasized the synthesis of highly purified [C-11]valproate and successful detection in cerebral PET studies.[94] Unfortunately, further review of the data revealed that kinetic modeling had not reliably determined the radiotracer input function, so that VPA-specific distribution parameters could not be calculated.[99] Pharmacodynamic PET studies of VPA were more revealing, however. Acute VPA use did not cause altered density of cerebral $GABA_A$ receptor complexes, based on pre- and post-VPA imaging with the $GABA_A$-central benzodiazepine receptor marker [C-11]flumazenil, in primary generalized epilepsy patients.[98] Introduction of VPA caused global cerebral declines in glucose metabolism and blood flow, on comparing pre- and post-VPA imaging using PET with [F-18]2-fluoro-2-deoxyglucose and [O-15]water in healthy subjects.[97] These changes suggest overall reduction in cerebral synaptic activities during VPA use.

Magnetic resonance spectroscopy (MRS) studies provided further information on human VPA pharmacodynamics.[100-104] Attempts to map VPA spectra with MRS, so as to determine brain VPA distribution in humans, were unsuccessful.[103] Bipolar patients chronically receiving VPA did not show significant alterations in brain N-actylaspartate or myo-inositol concentrations with MRS, compared with unmedicated healthy subjects.[101,104] In a case report of MRS during VPA-induced encephalopathy, however, brain N-actylaspartate and myo-inositol concentrations were reduced; these and other imaging findings were similar to those of hepatic encephalopathies that are unassociated with VPA use.[105] Petroff and colleagues found low-to-normal GABA signal and normal homocarnosine signal in patients using VPA chronically; these MRS studies reflected chronic VPA use in partial and generalized epilepsies versus healthy subjects not using VPA.[102] Homocarnosine is synthesized from GABA and histidine, and is hydrolyzed with carnosinase to release GABA, thus constituting a second biochemical pathway for GABA release that is unique to primates.[106] Thus, theories of GABAergic actions of VPA, based on increased GABA stores, were not supported by human

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imaging studies. While elevated CSF glutamine is highly associated with VPA-induced encephalopathy,[107] future evaluations of severe encephalopathy in patients taking VPA may rely on MRS findings.

THE HISTORY OF DIVALPROEX IN BASIC NEUROSCIENCE RESEARCH

The history of fundamental VPA research is extensive and beyond the scope of the current review. A few comments must suffice regarding the important but incomplete impact that fundamental research has had on the clinical history of VPA. First, VPA has long been thought to enhance GABA effect. The concept of direct $GABA_A$ receptor agonism by VPA was rejected in early investigations, as were some but not all of various alternative mechanisms for increasing GABAergic inhibition.[20,108-111] To date, no disorder that benefits from VPA therapy has been shown to benefit solely by enhanced GABAergic inhibition. Second, it seems clear that VPA has multiple therapeutic mechanisms in cerebral disorders, probably involving altered cationic ionophore functions (particularly in reducing excessive voltage-sensitive sodium and potassium currents, and T-channel calcium flux), and involving phospholipid-mediated alterations in membrane properties and in intracytoplasmic second messenger systems.[30,112-118] Third, it seems clear that VPA has multiple mechanisms of toxicity, some of which occur independently of therapeutic mechanisms, including altering concentrations of carnitine, folate and protein-lipid components of metabolic and signalling-related enzymes in mitochondrial, microsomal, and peroxisomal processes.[119-122] In the future, molecular neuropharmacology may further advance VPA applications in clinical neuroscience, which remain largely empirical. ✤

DISCLOSURE

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DISCLOSURE OF UNLABELED OR UNAPPROVED USES OF DRUGS

Please note that this review article contains discussions of unlabeled uses of FDA-approved pharmaceutical products. Please refer to the offi-

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cial prescribing information for approved indications, contraindications, and warnings.

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(71) Applicant *(for all designated States except US)*: CORNELL UNIVERSITY [US/US]; 395 Pine Tree Road, Suite 310, CCTEC, Ithaca, NY 14850 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: BYNOE, Margaret, S. [US/US]; 101 Park Lane, Ithaca, NY 14850 (US).

(74) Agents: THISSE, Megan, L. et al.; 290 Linden Oaks, Rochester, NY 14625 (US).

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(54) Title: USE OF ADENOSINE RECEPTOR SIGNALING TO MODULATE PERMEABILITY OF BLOOD-BRAIN BARRIER

(57) Abstract: The present invention relates to a method of increasing blood brain barrier ("BBB") permeability in a subject. This method involves administering to the subject an agent or agents which activate both of the A1 and A2A adenosine receptors. Also disclosed is a method to decrease BBB permeability in a subject. This method includes administering to the subject an agent which inhibits or blocks the A2A adenosine receptor signaling. Compositions relating to the same are also disclosed.

USE OF ADENOSINE RECEPTOR SIGNALING TO MODULATE PERMEABILITY OF BLOOD-BRAIN BARRIER

[0001] This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/383,628, filed September 16, 2010, which is hereby
5 incorporated by reference in its entirety.

[0002] This invention was made with government support under grant numbers K22AI057854 and R01NS063011 awarded by the National Institutes of Health. The Government has certain rights in the invention.

10
FIELD OF THE INVENTION

[0001] The present invention relates to modulation of blood brain barrier permeability.

BACKGROUND OF THE INVENTION

[0002] The barriers to blood entering the central nervous system ("CNS") are
15 herein collectively referred to as the blood brain barrier ("BBB"). The BBB is a tremendously tight-knit layer of endothelial cells that coats 400 miles of capillaries and blood vessels in the brain (Ransohoff et al., "Three or More Routes for Leukocyte Migration Into the Central Nervous System," *Nature Rev. Immun.* 3:569-581 (2003)). The blood-brain barrier (BBB) is comprised of brain endothelial cells, which form the
20 lumen of the brain microvasculature (*see* Abbott et al., "Structure and Function of the Blood-Brain Barrier," *Neurobiol. Dis.* 37:13-25 (2010)). The barrier function is achieved through tight junctions between endothelial cells that regulate the extravasation of molecules and cells into and out of the central nervous system (CNS) (*see* Abbott et al., "Structure and Function of the Blood-Brain Barrier," *Neurobiol. Dis.*
25 37:13-25 (2010)). The nearly impermeable junctions between BBB cells are formed by the interdigitation of about 20 different types of proteins. Molecules must enter a BBB cell through membrane-embedded protein transporters or by slipping directly through its waxy outer membrane. Once inside, foreign compounds must avoid a high concentration of metabolic enzymes and a variety of promiscuous protein pumps
30 primed to eliminate foreign substances. Having avoided these obstacles, foreign molecules must then pass through the inner membrane of a BBB cell to finally reach

the brain. These elaborate defenses allow the BBB to sequester the brain from potential harm, but the BBB also obstructs delivery of neurological drugs to a site of disease in the brain. Researchers in academia and the biotech and pharmaceutical industries are learning to bypass the BBB or allow it to let potential drugs into the brain. They are 5 designing small drugs that can passively diffuse through the BBB or travel on nutrient transporters to get inside the brain. Others are attaching potential therapeutics designed so that the brain will unwittingly engulf them.

[0003] The endothelial cells which form the brain capillaries are different from those found in other tissues in the body (Goldstein et al., "The Blood-Brain Barrier," 10 *Scientific American* 255:74-83(1986); Pardridge, "Receptor-Mediated Peptide Transport Through the Blood-Brain Barrier," *Endocrin. Rev.* 7:314-330(1986)). Brain capillary endothelial cells are joined together by tight intercellular junctions which form a continuous wall against the passive diffusion of molecules from the blood to the brain and other parts of the CNS. These cells are also different in that they have few 15 pinocytic vesicles which in other tissues allow somewhat unselective transport across the capillary wall. Also lacking are continuous gaps or channels running between the cells which would allow unrestricted passage.

[0004] The blood-brain barrier functions to ensure that the environment of the brain is constantly controlled. The levels of various substances in the blood, such as 20 hormones, amino acids, and ions, undergo frequent small fluctuations which can be brought about by activities such as eating and exercise (Goldstein et al., "The Blood-Brain Barrier," *Scientific American* 255:74-83(1986); Pardridge, "Receptor-Mediated Peptide Transport Through the Blood-Brain Barrier," *Endocrin. Rev.* 7:314-330(1986)). If the brain was not protected by the blood brain barrier from these variations in serum 25 composition, the result could be uncontrolled neural activity.

[0005] The isolation of the brain from the bloodstream is not complete. If this were the case, the brain would be unable to function properly due to a lack of nutrients and because of the need to exchange chemicals with the rest of the body. The presence of specific transport systems within the capillary endothelial cells assures that the brain 30 receives, in a controlled manner, all of the compounds required for normal growth and function. In many instances, these transport systems consist of membrane-associated proteins, which selectively bind and transport certain molecules across the barrier membranes. These transporter proteins are known as solute carrier transporters.

[0006] Although the BBB serves to restrict the entry of potentially toxic substances into the CNS, it poses a tremendous hurdle to the delivery of therapeutic drugs into the CNS. It has been estimated that more than 98% of small-molecule drugs less than 500 Da in size do not cross the BBB (*See* Pardridge, "Brain Drug Targeting:
5 the Future of Brain Drug Development," *Cambridge University Press, Cambridge, UK.* (2001) and Pardridge, "The Blood-Brain Barrier: Bottleneck in Brain Drug Development," *NeuroRx* 2:3-14 (2005)). Current approaches aimed at altering the BBB to permit the entry of therapeutics are either too invasive, painful, can result in permanent brain damage or result in loss of drug efficacy (*See* Broadwell et al.,
10 "Morphologic Effect of Dimethyl Sulfoxide on the Blood-Brain Barrier," *Science* 217:164-6 (1982); Hanig et al., "Ethanol Enhancement of Blood-Brain Barrier Permeability to Catecholamines in Chicksm," *Eur. J. Pharmacol.* 18:79-82 (1972); Rapoport, "Advances in Osmotic Opening of the Blood-Brain Barrier to Enhance CNS Chemotherapy," *Expert Opin. Investig. Drugs* 10:1809-18 (2001); Bidros et al., "Novel
15 Drug Delivery Strategies in Neuro-Oncology," *Neurotherapeutics* 6: 539-46 (2009); and Hynynen, "MRI-guided Focused Ultrasound Treatments," *Ultrasonics* 50:221-9 (2010)).

[0007] Current strategies for CNS drug-delivery fall into three broad categories: chemical or physical BBB disruption and drug modification (Pardridge, "The Blood-
20 Brain Barrier: Bottleneck in Brain Drug Development," *NeuroRx* 2:3-14 (2005)). Methods for chemically disrupting the BBB vary. Hypertonic mannitol osmotically shrinks brain endothelial cells, thus increasing BBB permeability and facilitating CNS delivery of chemotherapeutics (Neuwelt et al., "Osmotic Blood-brain Barrier Disruption: A New Means of Increasing Chemotherapeutic Agent Delivery," *Trans Am.*
25 *Neurol. Assoc.* 104:256-260 (1979)). However, it has been demonstrated that this procedure carries the risk of inducing epileptic seizures (Neuwelt et al., "Osmotic Blood-brain Barrier Modification: Clinical Documentation by Enhanced CT Scanning and/or Radionuclide Brain Scanning," *Am. J. Roentgenol.* 141:829-835 (1983); Marchi et al., "Seizure-promoting Effect of Blood-brain Barrier Disruption," *Epilepsia* 48:732-
30 742 (2007)). An analogue of the vasoactive peptide bradykinin was shown to increase permeability of the blood-tumor barrier (Raymond et al., "Pharmacological Modification of Bradykinin Induced Breakdown of the Blood-brain Barrier," *Can. J. Neurol. Sci.* 13:214-220 (1986)), and to some extent the BBB (Borlongan & Emerich,

"Facilitation of Drug Entry into the CNS via Transient Permeation of Blood Brain Barrier: Laboratory and Preliminary Clinical Evidence from Bradykinin Receptor Agonist, Cereport," *Brain Res. Bull.* 60:297-306 (2003)), and was moderately effective in increasing hydrophilic, but not lipophilic, drug delivery to certain CNS gliomas in rat
5 models (Bartus et al., "Permeability of the Blood Brain Barrier by the Bradykinin Agonist, RMP-7: Evidence for a Sensitive, Auto-regulated, Receptor-mediated System," *Immunopharmacology* 33:270-278 (1996); Elliott et al., "Intravenous RMP-7 Selectively Increases Uptake of Carboplatin into Rat Brain Tumors," *Cancer Res* 56:3998-4005 (1996); Matsukado et al., "Enhanced Tumor Uptake of Carboplatin and
10 Survival in Glioma-Bearing Rats by Intracarotid Infusion of Bradykinin Analog, RMP-7," *Neurosurgery* 39:125-133, discussion 133-124 (1996); Emerich et al., "Enhanced Delivery of Carboplatin into Brain Tumours with Intravenous Cereport (RMP-7): Dramatic Differences and Insight Gained from Dosing Parameters," *Br. J. Cancer* 80:964-970 (1999)). However, it failed in clinical trials, due possibly to differences
15 between rat models and human patients (Prados et al., "A randomized, Double-blind, Placebo-controlled, Phase 2 Study of RMP-7 in Combination with Carboplatin Administered Intravenously for the Treatment of Recurrent Malignant Glioma," *Neuro. Oncol.* 5:96-103 (2003)).

[0008] Physical disruption of the barrier is the oldest and most invasive method
20 of by-passing a functional BBB. Direct injections into the brain, especially into the ventricles, have been used for years to deliver therapeutics to the CNS (Cook et al., "Intracerebroventricular Administration of Drugs," *Pharmacotherapy* 29:832-845 (2009)). Recently, high-intensity focused ultrasound technologies have been developed that forcefully push therapeutic compounds past the BBB using compression waves
25 (Bradley, "MR-guided Focused Ultrasound: A Potentially Disruptive Technology," *J. Am. Coll. Radiol.* 6:510-513 (2009)). Still, physically disrupting the BBB remains invasive.

[0009] Drugs that do not cross the BBB can sometimes be modified to allow them to cross. The addition of moieties that increase a drug's lipophilicity can increase
30 the likelihood it will cross the BBB, but these additions also render the drug more capable of entering all cell types (Witt et al., "Peptide Drug Modifications to Enhance Bioavailability and Blood-brain Barrier Permeability," *Peptides* 22:2329-2343 (2001)). It is also often the case that the chemical additions themselves significantly increase the

size of the drug which counteracts the higher lipophilic profile (Witt et al., "Peptide Drug Modifications to Enhance Bioavailability and Blood-brain Barrier Permeability," *Peptides* 22:2329-2343 (2001)). Another approach involves so-called "vector-based" technologies in which the drug is attached to a compound known to enter the CNS
5 through receptor-mediated endocytosis. For example, conjugation of neuronal growth factor (NGF) to a monoclonal antibody to the transferrin receptor, expressed on BECs, greatly increased NGF delivery to rat brains (Granholm et al., "NGF and Anti-transferrin Receptor Antibody Conjugate: Short and Long-term Effects on Survival of Cholinergic Neurons in Intraocular Septal Transplants," *J. Pharmacol. Exp. Ther.*
10 268:448-459 (1994)). Vector-based delivery technologies suffer from two large drawbacks: 1) the BBB transport ability is limited to receptor expression and 2) endocytotic events are limited in BBB endothelium, a hallmark of its physiology.

[0010] There is a monumental need to modulate the BBB to facilitate the entry of therapeutic drugs into the CNS. Determining how to safely and effectively do this
15 could affect a very broad range of neurological diseases, such as Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, neurological manifestations of acquired immune deficiency disorder (AIDS), CNS tumors, and many more. Promising therapies are available to treat some of these disorders, but their potential cannot be fully realized due to the tremendous impediment posed by the BBB. Accordingly,
20 there is need in the art for methods to improve the delivery of compounds into the CNS.

[0011] In addition, patients suffering from edema, brain traumas, stroke and multiple sclerosis exhibit a breakdown of the BBB near the site of primary insults. The level of breakdown can have profound effects on the clinical outcome of these diseases. For instance, the degree of BBB breakdown in patients suffering from multiple
25 sclerosis (MS) is correlated to the severity of the disease. It has been shown using Magnetic Resonance Imaging (MRI) that, when a person is undergoing an MS "attack," the blood-brain barrier has broken down in a section of the brain or spinal cord, allowing white blood cells called T lymphocytes to cross over and destroy the myelin.

[0012] Despite the importance of this barrier, very little is known about the
30 molecular mechanisms controlling the integrity and/or permeability of the BBB. Thus, there remains a considerable need for compositions and methods that facilitate such research and especially for diagnostic and/or therapeutic applications.

[0013] The present invention is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

5 [0014] The present invention relates to a method for increasing blood brain barrier permeability in a subject. This method involves administering to the subject an agent which activates both of A1 and A2A adenosine receptors.

[0015] The present invention also relates to a method for increasing blood brain barrier permeability in a subject. This method involves administering to said subject an 10 A1 adenosine receptor agonist and an A2A adenosine receptor agonist.

[0016] The present invention further relates to a composition. The composition includes an A1 adenosine receptor agonist and an A2A adenosine receptor agonist, and a pharmaceutically acceptable carrier, excipient, or vehicle.

[0017] The present invention also relates to a method for delivering a 15 macromolecular therapeutic agent to the brain of a subject. This method includes administering to the subject an agent which activates both of A1 and A2A adenosine receptors and the macromolecular therapeutic agent.

[0018] The present invention also relates to a method for treating a CNS disease, disorder, or condition in a subject. This method involves administering to the 20 subject at least one agent which activates both of A1 and A2A adenosine receptors and a therapeutic agent.

[0019] The present invention also relates to a method for treating a CNS disease, disorder, or condition in a subject. This method involves administering to the subject an A1 adenosine receptor agonist, an A2A receptor agonist, and a therapeutic 25 agent.

[0020] The present invention further relates to a method of temporarily increasing the permeability of the blood brain barrier of a subject. The method comprises selecting a subject in need of a temporary increase in permeability of the blood brain barrier, providing an agent which activates either the A1 or the A2A 30 adenosine receptor, and administering to the selected subject either the A1 or the A2A adenosine receptor agonist under conditions effective to temporarily increase the permeability of the blood braiu barrier.

[0021] The present invention also relates to a method for decreasing blood brain barrier permeability in a subject. This method involves administering to said patient an agent which blocks or inhibits A2A signaling.

[0022] The present invention also relates to a method of remodeling an actin
5 cytoskeleton of a blood brain barrier endothelial cell. This method involves contacting said endothelial cell with an agent which activates both of A1 and A2A adenosine receptors.

[0023] As shown in the examples that follow, it is demonstrated that signaling through receptors for the purine nucleoside adenosine acts as a potent endogenous
10 modulator of blood-brain barrier permeability. These findings indicate that adenosine receptor ("AR") signaling represents a novel endogenous mechanism for controlling BBB permeability and a potentially useful alternative to existing CNS drug-delivery technologies. Drugs like Lexiscan, the FDA-approved A2A AR agonist, which increases BBB permeability and facilitates CNS entry of macromolecules like dextrans,
15 represent a possible pathway toward future therapeutic applications in humaus. Importantly, the present findings indicate that this technique can be used for CNS delivery of macromolecular therapeutics like antibodies, which traditionally have been limited in their use in treating neurological diseases because they required invasive delivery technologies (Thakker et al., "Intracerebroventricular Amyloid-beta
20 Antibodies Reduce Cerebral Amyloid Angiopathy and Associated Micro-hemorrhages in Aged Tg2576 Mice," *Proc. Natl. Acad. Sci. USA* 106:4501-6 (2009), which is hereby incorporated by reference in its entirety). The results described here represent a novel and promising alteruative to existing CNS drug-delivery paradigms.

[0024] The methods and agents of the present invention provide for an
25 improved treatment of subjects with disorders affecting the blood brain barrier. In addition, the present invention provides improved methods of controlling the blood brain barrier to enhance therapeutic treatment of such patients.

BRIEF DESCRIPTION OF THE DRAWINGS

30 [0025] Figure 1 shows a graph demonstrating $cd73^{-/-}$ mice are resistant to Experimental Autoimmune Encephalomyelitis ("EAE"). EAE was induced, disease

activity was monitored daily, and the mean EAE score was calculated for *cd73*$^{-/-}$ (open diamonds, n=11) and wild type (*cd73*$^{+/+}$) (closed squares, n=13) mice. The results shown are representative of 11 separate experiments.

[0026] Figures 2A-2D show cd73$^{-/-}$ T cells produce elevated levels of IL-1β and IL-17 and mediate EAE susceptibility when transferred to *cd73*$^{+/+}$*tcrα*$^{-/-}$ mice. Figure 2A shows the CD4 and FoxP3 expression measured on splenocytes from naïve and day 13 post-EAE induced *cd73*$^{-/-}$ and wild type mice. Figure 2B shows splenocytes from naïve and day 13 post-MOG immunized wild type mice which were analyzed for CD4 and CD73 cell surface expression by flow cytometry. Figure 2C shows sorted cells from immunized wild type or *cd73*$^{-/-}$ mice which were cultured with $1x10^4$ irradiated splenocytes and 0 or 10μM MOG peptide. Supernatants were taken at 18 hours and run on a cytokine Bio-plex assay. Results represent the fold change in cytokine levels between the 0 and 10μM MOG peptide groups. Samples were pooled from 4 mice and are representative of one out of three similar experiments. Figure 2D shows CD4$^+$ T cells from the spleen and lymph nodes from MOG immunized *cd73*$^{-/-}$ (open diamonds, n=5) or wild type (closed squares, n=5) mice which were adoptively transferred into T cell deficient *cd73*$^{+/+}$*tcrα*$^{-/-}$ mice. EAE was induced and disease progression was monitored daily. Results are representative of two separate experiments.

[0027] Figure 3A-3L show *cd73*$^{-/-}$ mice which display little or no CNS lymphocyte infiltration following EAE induction; donor *cd73*$^{-/-}$ T cells infiltrate the CNS of *cd73*$^{+/+}$*tcrα*$^{-/-}$ recipient mice following EAE induction. Frozen tissue sections from day 13 post-EAE induction wild type (Figures 3A-3C) and *cd73*$^{-/-}$ (Figures 3D-3F) mice were labeled with a CD4 antibody. Figure 3G is a bar graph showing the mean number of CD4$^+$ infiltrating lymphocytes in the brain and spinal cord quantified per field in frozen tissue sections from day 13 post-EAE induction wild type and *cd73*$^{-/-}$ mice. Eight anatomically similar fields per brain and 4 fields per spinal cord per

mouse were analyzed at 10x magnification (n=5 mice/group). Error bars represent the standard error of the mean. Figures 3H-3L show frozen tissue sections of hippocampus (Figures 3H, 3I, and 3K) and cerebellum (Figures 3J and 3L) labeled with a CD4 antibody from EAE-induced $tcr\alpha^{-/-}$ mice that received $CD4^{+}$ cells from wild type
5 (Figures 3H-J) or $cd73^{-/-}$ (Figures 3K-3L) mice at day 12 (Figure 3K), 18 (Figures 3H and 3L), or 22 (Figures 3I and 3J) post-EAE induction. Immunoreactivity was detected with HRP anti-rat Ig plus AEC (red) against a hemotoxylin stained nuclear background (blue). Arrows indicate sites of lymphocyte infiltration. Scale bars represent 500μm.

[0028] Figures 4A-4K show $cd73^{-/-}$ mice which display little or no CNS
10 lymphocyte infiltratiou following EAE induction; $cd73^{-/-}$ T cells infiltrate the CNS after transfer to $cd73^{+/+}tcr\alpha^{-/-}$ mice and EAE induction. Frozen tissue sections from day 13 post-EAE induction wild type (Figures 4A-4C) and $cd73^{-/-}$ (Figures 4D-4F) mice were labeled with a CD45 antibody. Frozen tissue sections of hippocampus (Figures 4G, 4H, and 4J) aud cerebellum (Figures 4I and 4K) labeled with a CD45
15 antibody from EAE-induced $tcr\alpha^{-/-}$ mice that received $CD4^{+}$ cells from wild type (Figure 4G-4I) or $cd73^{-/-}$ (Figures 4J-4K) mice at day 12 (Figure 4J), day 18 (Figures 4G and 4K), or day 22 (Figures 4H and 4I) post EAE induction. Immunoreactivity was detected with HRP anti-rat Ig plus AEC (red) against a hemotoxylin stained nuclear background (blue). Arrows indicate sites of lymphocyte infiltration. Scale bars
20 represent 500mm.

[0029] Figures 5A-5C show myelin specific T cells do not efficiently enter the brain of $cd73^{-/-}$ mice following EAE induction. $V\beta11^{+}$ T cells from MOG_{35-55} immunized trausgenic 2d2 mice, which express TCRs specific for MOG_{35-55}, were isolated from the spleen and lymph nodes and adoptively transferred into wild type or
25 $cd73^{-/-}$ mice with concomitant EAE induction. At days 1, 3, 8, and 15 post transfer and EAE induction, spleens (Figure 5A), lymph nodes (Figure 5B), and brains (Figure 5C) were removed and cells were harvested. Cells were analyzed for CD45 and Vβ11 expression by flow cytometry. The data represent the relative fold change (RFC) in the percentage of $V\beta11^{+}$ cells in the $CD45^{+}$ population for each organ on each given day.

Values were normalized to the percentage of cells found in each organ at 1 day post transfer/EAE induction, with 1.0 equaling the baseline value.

[0030] Figures 6A-6D show adoptively transferred $CD73^+$ T cells from wild type mice can confer EAE susceptibility to *cd73*$^{-/-}$ mice. Figure 6A shows $CD4^+$ T cells from the spleen and lymph nodes of MOG immunized wild type mice were enriched and adoptively transferred into wild type (closed squares, n=5) or *cd73*$^{-/-}$ (open diamonds, n=5) mice followed by concomitant EAE induction. Results are shown from one of two independent experiments. Figure 6B shows T cells from the spleen aud lymph nodes of previously immuuized wild type and *cd73*$^{-/-}$ mice were sorted based on CD4 and CD73 expression and adoptively transferred into *cd73*$^{-/-}$ mice followed by concomitant EAE induction (n=5/each group). Closed squares represent donor cells from wild type mice that express CD73; open squares represent donor cells from wild type mice that lack CD73 expression; open diamonds represent donor cells from *cd73*$^{-/-}$ mice. Figure 6C-6D show frozen tissue sections of the CNS choroid plexus from naïve wild type (Figure 6C, left) and *cd73*$^{-/-}$ (Figure 6C, right) mice and wild type mice day 12 post-EAE induction (Figure 6D) were stained with a CD73 (Figure 6C) or CD45 (Figure 6D) specific antibody. Immunoreactivity was detected with HRP anti-rat Ig plus AEC (red) against a hemotoxylin stained nuclear background (blue). Brackets indicate CD73 staiuiug. Arrows indicate CD45 lymphocyte staining. Scale bars represeut 500μm.

[0031] Figures 7A-7D show adenosine receptor blockade protects mice from EAE development. Figure 7A shows mean EAE scores where EAE was induced, disease activity was monitored daily, and the mean EAE score was calculated in wild type (squares) and *cd73*$^{-/-}$ (diamonds) mice given either drinking water (closed shape) alone or driuking water supplemented with 0.6g/ml of the broad spectrum adenosine receptor antagonist caffeine (open shape). Results are from one experiment (n=5 mice per group). Figure 7B shows adenosine receptor mRNA expression levels relative to the GAPDH housekeeping gene in the Z310 murine choroid plexus cell line. Samples were run in triplicate; error bars represent the standard error of the mean. Figure 7C shows results after mice were treated with the A2A adenosine receptor antagonist

SCH58261 at 2mg/kg (1mg/kg s.c. and 1mg/kg i.p.) in 45% DMSO (closed squares, n=4 mice/group) or 45% DMSO alone (open squares, n=5 mice/group) 1 day prior to and daily up to day 30 following EAE induction. These results are representative of two experiments. Figure 7D shows the mean number of $CD4^+$ infiltrating lymphocytes
5 in the brain and spinal cord quantified per field in frozen tissue sections from day 15 post-EAE induction in SCH58261- and DMSO-treated mice are shown. Eight anatomically similar fields per brain and 4 fields per spinal cord per mouse were analyzed at 10x magnification (n=4 mice). Error bars represent the standard error of the mean.

10 **[0032]** Figure 8 shows the A2A adenosine receptor antagonist SCH58261 prevents ICAM-1 upregulation on the choroid plexus following EAE induction. Mice were treated with the A2A adenosine receptor antagonist SCH58261 2mg/kg (1mg/kg given *s.c.* and 1mg/kg given *i.p.*) in DMSO (n=4 mice/group) or DMSO alone (n=5 mice/group) 1 day prior to and daily up to day 30 following EAE induction. These
15 results are from one experiment. Frozen tissue sections from day 15 post-EAE induction in SCH58261 and DMSO treated mice were examined for ICAM-1 expression at the choroid plexus. WT treated DMSO (left) or SCH58261 (right) and stained for ICAM-1 (red staining, white arrows) and DAPI (blue, nuclei) at 40x magnification. Images are from 4 separate mice.

20 **[0033]** Figures 9A-9B demonstrate that $CD73^{-/-}$ mice, which lack extracellular adenosine and thus cannot adequately signal through adenosine receptors, were treated with NECA, resulting in an almost five fold increase in dye migration vs. the PBS control (Figure 9A). WT mice treated with NECA also show an increase over control mice (Figure 9B). Pertussis was used as a positive control, as it is known to induce
25 blood brain barrier leakiness in the mouse EAE model.

[0034] Figure 10 shows adenosine receptor expression on the human endothelial cell line hCMEC/D3.

[0035] Figure 11 shows results after hCMEC/D3 cells were seeded onto transwell membranes and allowed to grow to confluencey; $2x10^6$ Jurkat cells were
30 added to the upper chamber with or without NECA (general adenosine receptor [AR] agonist), CCPA (A1 AR agonist), CGS 21860 (A2A AR agonist), or DMSO vehicle; and migrated cells were counted after 24 hours.

[0036] Figure 12 shows results after transwell membranes were seeded with Z310 cells and allowed to grow to confluencey; $2x10^6$ Jurkat cells were added to the upper chamber with or with out NECA (n=1, general AR agonist), CCPA (n=1, A1 AR agonist), CGS 21860 (n=1, A2A AR agonist), or DMSO vehicle(n=1); and migrated
5 cells were counted after 24 hours.

[0037] Figure 13 shows results after hCMEC/D3 cells were grown to confluencey on 24 well plates; cells were treated with or without various concentrations of NECA (general AR agonist), CCPA (A1 AR agonist), CGS 21860 (A2A AR agonist), DMSO vehicle, or Forksolin (induces cAMP); lysis buffer was added after 15
10 minutes and the cells were frozen at -80C to stop the reaction; and cAMP levels were assayed using a cAMP Screen kit (Applied Biosystems, Foster City, CA).

[0038] Figure 14 shows results of female A1 adenosine receptor knockout (A1ARKO, n=5) and wild type (WT, n=5) mice that were immunized with CFA/MOG_{35-55} + PTX on 12-2-08 and scored daily for 41 days.

15 **[0039]** Figures 15A-15B show brains of wild type mice fed caffeine and brains from $CD73^{-/-}$ mice fed caffeine, as measured by FITC-Dextran extravasation through the brain endothelium.

[0040] Figure 16 shows results in graph form of FITC-Dextran extravasation across the blood brain barrier of wild type mice treated with adenosine receptor agonist,
20 NECA, while SCH58261, the adenosine receptor antagonist inhibit FITC-Dextran extravasation.

[0041] Figure 17 shows results of Evans Blue dye extravasation across the blood brain barrier, as measured by a BioTex spectrophotometer at 620nm, after mice were treated with adenosine receptor agonist NECA.

25 **[0042]** Figure 18 shows results in graphical form that demonstrate PEGylated adenosine deaminase ("PEG-ADA") treatment inhibits the development of EAE in wild-type mice. EAE was induced, disease activity was monitored daily, and mean EAE score was calculated in wild-type mice given either control PBS vehicle alone or 15 units/kg body weight of PEG-ADA i.p. every 4 days. Closed squares represent
30 wild-type mice given PBS vehicle (n=3); open squares respresent wild-type mice given PEG-ADA (n=3). These results are from one experiment. These results demonstrate

that adenosine deaminase treatment and adenosine receptor blockade protect wild type mice against EAE induction.

[0043] Figures 19A-19B are bar graphs of results showing dose-dependent increases in 10,000 Da (Figure 19A) and 70,000 Da (Figure 19B) dextrans into WT mouse brain 3 h after *i.v.* administration of NECA or vehicle (DMSO/PBS) as measured by fluorimetry (10-15 animals/group). Inset in Figure 19A is a splined scatter plot of data points. Experiments were performed at least twice. Significant differences (Student's T-test) from vehicle are indicated (*) where $P \leq 0.05$. Data are mean ± s.e.m. These results demonstrate that *i.v.*-administered NECA increases BBB permeability to high molecular weight dextrans.

[0044] Figures 20A-20B show experimental results in graphical form of NECA-mediated increase in BBB permeability. Figure 20A left panel shows extravasation of 10,000 Da FITC-dextran into WT mouse brain when co-administered with NECA or vehicle (DMSO/PBS). Gray bars = vehicle, black bars = NECA. Figure 20A right panel is a splined scatter plot with scaled time on the x-axis, which shows an extravasation time-course of 10 kDa FITC-dextran into WT mouse brain when co-administered *i.v.* with NECA (0.08 mg/kg) or vehicle, as measured by fluorimetry (10-15 animals/group). Figure 20B left panel shows the results of extravasation of 70,000 Da Texas Red-dextran into WT mouse brain tissue when injected at indicated times after NECA or vehicle administration. Gray bars = vehicle, black bars = NECA. Figure 20B right panel is a splined scatter plot with scaled time on the x-axis, which shows extravasation time-course of 10 kDa Texas Red-dextran, administered *i.v.* 90 minutes prior to harvest times (as displayed), into WT mouse brain tissue after *i.v.* pre-treatment (time = 0) with NECA (0.08 mg/kg) or vehicle,as measured by fluorimetry (3-5 animals/group). Experiments were performed at least twice. Significant differences (Student's T-test) from vehicle are indicated (*) where $P \leq 0.05$. Data are mean ± s.e.m. Insets in Figures 20A and 20B, left panels, are splined scatter plots with scaled time on the x-axis; diamonds = vehicle, squares = NECA. These results demonstrate that NECA treatment increases BBB permeability in a temporally discrete and reversible manner.

[0045] Figures 21A-21J illustrate results that show that increased BBB permeability depends on selective agonism of A1 and A2A adenosine receptors. Figure 21A is a bar graph showing relative expression of adenosine receptor subtypes on

cultured mouse brain endothelial cells ("BEC")(bEnd.3). Figure 21B shows images of immunofluorescent staining and Figure 21C shows images of fluorescence in situ hybridization of CD31 (endothelial cell marker; green) and A1 (left column; red) and A2A (right column; red) ARs near the cortical area of the brain in naïve mice (scale bar
5 = 20μm). Figure 21D shows an image of western blot analysis of A1 AR (left panel) and A2A AR (right panel) expression in isolated primary BECs from naïve mice. β-actin expression is shown as a loading control. Figures 21E and 21F are bar graphs showing levels of 10,000 Da FITC-dextran in WT and A1 AR (Figure 21E) and A2A AR (Figure 21F) knock-out mouse brain 3 h after *i.v.* administration of NECA or
10 vehicle (DMSO/PBS), as measured by fluorimetry. Gray bars = vehicle, black bars = NECA. Figure 21G is a bar graph showing decreased levels of dextran in brains of A1 and A2A AR knock-out mouse brain 3 h after *i.v.* administration of NECA (0.08 mg/kg) or vehicle compared with WT mice, as measured by fluorimetry. No significant increase in dextran levels were detected in brains of A1 knock-out mice that
15 were pre-treated with the selective A2A antagonist SCH 58261 (5-8 animals/group). Also shown are data demonstrating dose-dependent entry of 10,000 Da FITC-dextran into WT brain tissue 3 h after *i.v.* co-administration of the specific A2A AR agonist CGS 21860 (bar graphs of Figure 21H) or the specific A1 AR agonist CCPA (bar graphs of Figure 21I), as measured by fluorimetry. Figure 21J shows bar graphs
20 illustrating levels of 10,000 Da FITC-dextran in WT mouse brain tissue 3 h after *i.v.* administration of vehicle, NECA, CCPA, CGS 21680 and in combination. n = 3-4 mice/treatment group. Experiments were repeated at least twice. Significant differences (Student's T-test) from vehicle are indicated (*) where $P \leq 0.05$. Data are mean ± s.e.m.

25 **[0046]** Figures 22A-22F show results in graphical form demonstrating that the A2A agonist Lexiscan increases BBB permeability to 10,000 Da dextrans. Figure 22A shows results in graphical form that demonstrate Lexiscan administration increases BBB permeability in mice. Data bars before the axis break represent groups that received 3 Lexiscan injections. The bar after the axis break represents a group that
30 received a single Lexiscan injection. For the groups receiving 3 injections, perfusion occurred 15 min after the initial injection. The group that received a single injection was perfused 5 min after injection (10-15 animals/group). Vehicle treated mice (V) were perfused 15 min after injection. Figure 22B shows Lexiscan increases BBB

permeability in rats. Animals received 3 injections of Lexiscan, 5 min apart, and were perfused 15 min after the initial injection (3-4 animals/group). Figure 22C shows the results in graphical form of BBB permeability in rats to FITC-dextran administered simultaneously with 1 μg of Lexiscan at 5 minutes. As a control reference, animals
5 received 1 injection of NECA, and were perfused 15 min after injection. Vehicle treated mice (V) were perfused 15 min after injection. Statistics indicate significant differences from vehicle (*) or from 0.01 μg Lexiscan (**), $P \leq 0.05$ by Student's T-test. Data are mean ± s.e.m. Figure 22D is a graph showing the time-course of BBB permeability after Lexiscan treatment in mice. Lexiscan (0.05 mg/kg) was
10 administered at Time 0 (10-14 animals/group). Figure 22E is a graph showing the time-course of BBB permeability after Lexiscan treatment in rats. Lexiscan (0.0005 mg/kg) was administered at Time 0 (3-4 animals/group). Figure 22F shows *i.p.* administered SCH 58261 decreases BBB permeability to 10,000 Da FITC-dextran in mice. All experiments were repeated at least twice. Significant differences (Student's
15 T-test) from vehicle are indicated (*) where $P \leq 0.05$. Data are mean ± s.e.m.

[0047] Figures 23A-23H show results demonstrating that *i.v.*-administered antibody to β-amyloid antibody crosses BBB and labels β-amyloid plaques in transgenic mouse brains after NECA administration. Figures 23A-23D are immunofluorescent microscopic images near the hippocamppi of transgenic AD
20 (APP/PSEN) mice. Mice were treated with either NECA (0.08 mg/kg) (Figures 23A and 23C) or vehicle (Figures 23B and 23D) and antibody to β-amyloid (6E10) was administered *i.v.* (top panels: Figures 23A and 23B). For mice that did not receive *i.v.* 6E10 antibody (lower panels: Figures 23C and 23D), 6E10 was used as a primary antibody to control for the presence of plaques and was applied ex vivoduring
25 immunostaining. Figure 23A shows the same immunofluorescent microscopic images of hippocamppi of transgenic AD (APP/PSEN) as shown in Figures 23A-23D, as well as those of WT mice treated with *i.v.*-administered antibody to β-amyloid (Covance 6E10) or not and with 0.8 μg *i.v.* NECA (left panels) or vehicle (right panels). In Figures 23A-23E, blue = DAPI and red = Cy5-antibody labeling 6E10-labeled β-
30 amyloid plaques (scale bar = 50 μm). Figures 23F and 23G are immunofluorescent microscopic images of the hippocampal and cortical regions from the brains of transgenic AD mice showing an overview (Figure 23F) and close-up (Figure 23G) of

β–amyloid plaque locations relative to blood vessels (endothelial cells = CD31 stained green; β-amyloid plaques = 6E10 stained red; nuclei = DAPI stained blue; scale bars = 50μm). Figure 23H is a bar graph showing quantification of 6E10-labeled amyloid plaques per mouse brain section in transgenic AD mice treated with NECA or vehicle
5 alone.

[0048] Figures 24A-24Y show results deominstrating that adenosine receptor signaling results in changes in the paracellular but not transcellular pathway on BECs. Figure 24A is a bar graph showing relative genetic expression of adenosine receptor subtypes on cultured mouse BECs (Bend.3). Figure 24B shows western blot analysis
10 of A1 (left panel) and A2A (right panel) AR expression in cultured mouse BECs (Bend.3). Figure 24C is a graph showing results that demonstrate that AR activation decreases TEER iu mouse BEC monolayers. Decreased transendothelial electrical resistance was observed after addition of NECA (1 μM) or Lexiscan (1 μM) treatment. Significant differences (Student's T-test) from vehicle for Lexiscan (#) and NECA (*)
15 are indicated where $P \leq 0.05$. Data are mean ± s.e.m. Figures 24D-24G are images of Bend.3 cells that were incubated with fluorescently labeled albumin and either media alone (Figure 24D), vehicle (Figure 24E), NECA (1 μM) (Figure 24F), or Lexiscan (1 μM) (Figure 24G) for 30 minutes. Albumin uptake was visualized by fluorescence microscopy (albumin = red; DAPI stained nuclei = blue). Scale bar = 50 μm. Figure
20 24H is a bar graph showiug albumin uptake results. Albumin uptake is displayed as relative values compared to the media alone control (set to 100%). Data are mean ± s.e.m (n = 5 fields/group). Figures 24I -24P are images showing results that actinomyosin stress fiber formation correlates with AR activation in cultured BECs. Phalloidin staining of Bend.3 cells is shown and reveals increased actinomyosin stress
25 fiber formation following treatment with CCPA (1 μM) (Figures 24M and 24N) or Lexiscan (1 μM) (Figures 24O and 24P) when compared with media (Figures 24I and 24J) or vehicle alone (Figures 24K and 24L). Left panels = 3 min treatment; right panels = 30 min treatment. Scale bar = 50 μm. Figures 24Q-24Y are images showing results that demonstrate that AR activation induces changes in tight junction adhesion
30 molecules in cultured BECs. ZO-1 (Figures 24Q-24S), Claudin-5 (Figures 24T-24V), and Occludin (Figures 24W-24Y) staining of Bend.3 cells is shown following 1 hr treatment with DMSO (left column), NECA (1 μM, middle column), and Lexiscan (1

μM, right column). Adhesion molecules = pink/red; DAPI stained nuclei = blue. Arrow heads indicate examples of deserete changes in expression (scale bar = 20 μm).

[0049] Figure 25 is a schematic showing a model of adenosine receptor signaling and modulation of BBB permeability. (i) Basal conditions favor a tight barrier. (ii) Activation of the A1 or A2A AR results in increased BBB permeability. (iii) Activation of both A1 and A2A ARs results in even more permeability than observed after activation of either receptor alone. (iv) A2A receptor antagonism decreases BBB permeability.

DETAILED DESCRIPTION OF THE INVENTION

[0050] Adenosine is a cellular signal of metabolic distress being produced in hypoxic, ischaemic, or inflammatory conditions. Its primary undertaking is to reduce tissue injury and promote repair by different receptor-mediated mechanisms, including the increase of oxygen supply/demand ratio, preconditioning, anti-inflammatory effects and stimulation of angiogenesis (Jacobson et al., "Adenosine Receptors as Therapeutic Targets," *Nat. Rev. Drug Discov.* 5:247-264(2006), which is hereby incorporated by reference in its entirety).

[0051] The biological effects of adenosine are ultimately dictated by the different pattern of receptor distribution and/or affinity of the four known adenosine receptor ("AR") subtypes in specific cell types. Four AR subtypes are expressed in mammals: A1, A2A, A2B and A3 (Sebastiao et al., "Adenosine Receptors and the Central Nervous System," *Handb. Exp. Pharmacol.* 471-534 (2009), which is hereby incorporated by reference in its entirety). Adenosine receptors are now known to be integral membrane proteins which bind extracellular adenosine, thereby initiating a transmembrane signal via specific guanine nucleotide binding proteins known as G-proteins to modulate a variety of second messenger systems, including adenylyl cyclase, potassium channels, calcium channels and phospholipase C. See Stiles, "Adenosine Receptors and Beyond: Molecular Mechanisms of Physiological Regulation," *Clin. Res.* 38(1):10-18 (1990); Stiles, "Adenosine Receptors," *J. Biol Chem.* 267: 6451-6454 (1992), which are hereby incorporated by reference in their entirety.

[0052] The first clues to adenosine's involvement in CNS barrier permeability came from the recent findings demonstrating that extracellular adenosine, produced by the catalytic action of CD73 (a 5'-ectonucleotidase) from adenosine monophosphate (AMP), promotes leukocyte entry into the CNS in experimental autoimmune encephalomyelitis (EAE) (Mills et al., "CD73 is Required for Efficient Entry of Lymphocytes Into the Central Nervous System During Experimental Autoimmune Encephalomyelitis," *Proc. Natl. Acad. Sci. U.S.A.* 105: 9325-30 (2008), which is hereby incorporated by reference in its entirety). These studies demonstrated that mice lacking CD73 (Thompson et al., "Crucial Role for Ecto-5'-nucleotidase (CD73) in Vascular Leakage During Hypoxia," *J. Exp. Med.* 200:1395-405 (2004), which is hereby incorporated by reference inits entirety), which are unable to produce extracellular adenosine, are protected from EAE and that blockade of the A_{2A} adenosine receptor (AR) blocks T cell entry into the CNS (Mills et al., "CD73 is Required for Efficient Entry of Lymphocytes Into the Central Nervous System During Experimental Autoimmuue Eucephalomyelitis," *Proc. Natl. Acad. Sci. U.S.A.* 105: 9325-30 (2008), which is hereby incorporated by reference in its entirety). Furthermore, in a pilot experiment, it was observed that after intravenous (*i.v.*) injection of fluorescein isothiocyanate (FITC)-labeled 10,000 Da dextran, $CD73^{-/-}$ mice had much less FITC-dextran in their brains compared to WT mice; treatment with the broad spectrum AR agonist 5'-N-ethylcarboxamido adenosine (NECA) resulted in a dramatic increase in FITC-dextran extravasation in these mice compare to WT mice (data not shown). These observations led to the hypothesis that modulation of adenosine receptor signaling at BECs might modulate BBB permeability to facilitate the entry of molecules into the CNS. As is demonstrated in the Examples that follow, AR signaling represents a novel, endogenous modulator of BBB signaling.

[0053] As suprisingly shown here, the activation of the A1 and the A2A adenosine receptors increases the BBB permeability of a subject. In particular, adenosine, acting through the A1 or A2A receptors, can modulate BBB permeability to either facilitate or restrict the entry of molecules into the CNS. These changes in BBB permeability are dose-dependent and temporally discrete. Given that adenosine has a relatively short half-life, ~ <10 seconds (Klabunde, "Dipyridamole Inhibition of Adenosine Metabolism in Human Blood," *Eur. J. Pharmacol.* 93:21-6 (1983), which is hereby incorporated by reference in its entirety), its role as a physiologic modulator is

probably limited to the local environment in which it is produced. Indeed, the expression of CD39 and CD73 with adenosine receptors on brain endothelial cells indicates these cells have the ability to respond to extracellular ATP, a well-established damage signal (Davalos et al., "ATP Mediates Rapid Microglial Response to Local Brain Injury *in vivo*," *Nat. Neurosci.* 8:752-8 (2005) and Haynes et al., "The P2Y12 Receptor Regulates Microglial Activation by Extracellular Nucleotides," *Nat. Neurosci.* 9:1512-9 (2006), which are hereby incorporated by reference in their entirety). Adenosine receptor signaling at BBB endothelial cells is a key event in the "sensing" of damage that would necessitate changes in barrier permeability, and BBB permeability (mediated through A1 and A2A ARs) operates as a door where activation opens the door, antagonism closes the door and local adenosine concentration is the key. The absence of elevated levels of extracellular adenosine favors a tight and restrictive barrier. As shown schematically in Figure 25, activation of either the A1 or A2A AR temporarily increases BBB permeability, while activation of both receptors results in an additive effect of increased BBB permeability. It is shown here that BBB permeability mediated through A1 and A2A ARs operates as a door where activation opens the door and local adenosine concentration is the key.

[0054] One aspect of the present invention is directed to a method for increasing blood brain barrier permeability in a subject. This method involves administering to the subject an agent which activates both of A1 and A2A adenosine receptors.

[0055] It will be understood by those of skill in the art that the barrier between the blood and central nervous system is made up of the endothelial cells of the blood capillaries (blood-brain barrier ("BBB")) and by the epithelial cells of the choroid plexus ("CP") that separate the blood from the cerebrospinal fluid ("CSF") of the central nervous system ("CNS"). Together these structures function as the CNS barrier.

[0056] In one embodiment, the methods of the present invention for increasing BBB permeability, increase the permeability of the CP. In another embodiment, the methods of the present invention for increasing the permeability of the BBB, increase the permeability of the CNS barrier.

[0057] In one embodiment, the method further involves selecting a subject in need of increased BBB permeability, providing a therapeutic, and administering to the

selected subject the therapeutic and an agent which activates both of A1 and A2A adenosine receptors under conditions effective for the therapeutic to cross the blood brain barrier.

[0058] A suitable subject in need of increased permeability of the BBB according to the present invention includes any subject that is in need of a therapeutic to cross the BBB to treat or prevent a disease, disorder, or condition of the CNS or that which manifests within the CNS (e.g., HIV-associated neurological disorders).

[0059] It will be understood that a therapeutically effective amount of the agents according to the present invention is administered. The terms "effective amount" and "therapeutically effective amount," as used herein, refer to the amount of a compound or combination that, when administered to an individual, is effective to treat, prevent, delay, or reduce the severity of a condition from which the patient is suffering. In particular, a therapeutically effective amount in accordance with the present invention is an amount sufficient to treat, prevent, delay onset of, or otherwise ameliorate at least one side-effect associated with the treatment of a disease and/or disorder.

[0060] Suitable A1 and/or A2A adenosine receptor activators according to the present invention include agonists that are selective for the A1 adenosine receptor, agonists that are selective for the A2A adenosine receptor, agonists that activate both the A1 and the A2A adenosine receptors, broad spectrum adenosine activators or agouists, and combinations thereof. According to certain embodiments of the present invention a combination of the A1-selective agonist, A2A-selective agonist, an agonist that activates both the A1 and the A2A adenosine receptors, and/or broad spectrum adenosine activators or agonists are administered. These agents may be administered simultaneously, in the same or different pharmaceutical formulation, or sequentially. The timing of the sequential administration can be determined by a skilled practitioner. In certain embodiments, the agonists are combined in a single unit dosage form.

[0061] Suitable A2A adenosine receptor activators are A2A agonists, which are well known in the art (Press et al., "Therapeutic Potential of Adenosine Receptor Antagonists and Agonists," *Expert Opin. Ther. Patents* 17(8): 979-991 (2007), which is hereby incorporated by reference in its entirety). Examples of A2A adenosine receptor agonists include those described in U.S. Patent No. 6,232,297 and in U.S. Published Patent Application No. 2003/0186926 A1 to Lindin et al., 2005/0054605 A1 to Zablocki

et al., and U.S. Pnblished Patent Application Nos. 2006/0040888 Al, 2006/0040889 Al, 2006/0100169 Al, and 2008/0064653 A1 to Li et al., which are hereby incorporated by reference in their entirety. Such compounds may be synthesized as described in: U.S. Patent Nos. 5,140,015 and 5,278,150 to Olsson et al.; U.S. Patent No. 5,593,975 to
5 Cristalli ; U.S. Patent No. 4,956,345 Miyasaka et al.; Hutchinson et al., "CGS 21680C, an A2 Selective Adenosine Receptor Agonist with Preferential Hypotensive Activity," *J. Pharmacol. Exp. Ther.*, 251: 47-55 (1989); Olsson et al, "N6-Substituted N-alkyladenosine-5'-uronamides: Bifunctional Ligands Having Recognition Groups for A1 and A2 Adenosine Receptors," *J. Med. Chem.*, 29: 1683-1689 (1986); Bridges et
10 al., "N6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl]adenosine and its Uronamide Derivatives: Novel Adenosine Agonists With Both High Affinity and High Selectivity for the Adenosine A2 Receptor," *J. Med. Chem.* 31: 1282 (1988); Hutchinson et al., *J. Med. Chem.*, 33:1919 (1990); Ukeeda et al., "2-Alkoxyadenosines: Potent and Selective Agonists at the Coronary Artery A2 Adenosine Receptor," *J. Med.*
15 *Chem.* 34: 1334 (1991); Francis et al., "Highly Selective Adenosine A2 Receptor Agonists in a Series of N-alkylated 2-aminoadenosines," *J. Med. Chem.* 34: 2570-2579 (1991); Yoneyama et al, "Vasodepressor Mechanisms of 2-(1-octynyl)-adenosine (YT-146), a Selective Adenosine A2 Receptor Agonist, Involve the Opening of Glibenclamide-sensitive K+ Channels," *Eur. J. Pharmacol.* 213(2):199-204 (1992);
20 Peet et al., "Conformationally Restrained, Chiral (phenylisopropyl)amino-substituted pyrazolo[3,4-d]pyrimidines and Purines with Selectivity for Adenosine A1 and A2 Receptors," *J. Med. Chem.*, 35: 3263-3269 (1992); and Cristalli et al.,"2-Alkynyl Derivatives of Adenosine and Adenosine-5'-N-ethyluronamide as Selective Agonists at A2 Adenosine Receptors, " *J. Med. Chem.* 35(13): 2363-2368 (1992), which are hereby
25 incorporated by reference in their entirety. Additional examples of adenosine A2A receptor agonists are disclosed in U.S. Patent Application Publication 2004/0809916, which is hereby incorporated by reference in its entirety. Particularly suitable A2A adenosine receptor agonists inclnde 4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2yl]amino]ethyl] benzenepropanoic acid ("CGS
30 21680"), and Lexiscan, or combinations thereof. These adenosine A2A receptor agonists are intended to be illustrative and not limiting.

[0062] Suitable A1 adenosine receptor activators are A1 adenosine receptor agonists. A1 adenosine receptor agonists are known to those of skill in the art and

include, for example, those described in U.S. Patent Application Publication No. 2005/0054605 A1 to Zablocki et al. and Press et al., "Therapeutic Potential of Adenosine Receptor Antagonists and Agonists," *Expert Opin. Ther. Patents* 17(8): 979-991 (2007), which are hereby incorporated by reference in their entireties. Suitable A1
5 adenosine receptor agonists also include, for example, 2-chloro-N^6-cyclopentyladenosine ("CCPA"), 8-cyclopentyl-1,3-dipropylxanthine ("DPCPX"), R-phenylisopropyl-adenosine, N6-Cyclopentyladenosine, and N(6)-cyclohexyladenosine, or combinations thereof.

[0063] In one embodiment, the agent which activates both the A1 and the A2A
10 adenosine receptors is an agonist of both the A1 and the A2A adenosine receptors. Suitable agonists that activate both the A1 and the A2A adenosine receptors are known to those of skill in the art, and include, for example, AMP 579. In still further embodiments, the agonist of both the A1 and the A2A adenosine receptors may be a broad spectrum adenosine receptor agonist. Suitable broad spectrum adenosine
15 receptor agonists will be known to those of skill in the art and include, for example, NECA, adenosine, adenosine derivatives, or combinations thereof.

[0064] According to one embodiment of the present invention, activating both the A1 and A2A adenosine receptors is synergistic as compared to the level of BBB permeability when activating either the A1 adenosine receptor or A2A adenosine
20 receptor alone. In this context, if the effect of activating the two receptors together (at a given concentration) is greater than the sum of the effects when each receptor is activated individually (at the same concentration), then the activation of both the A1 and the A2A receptors is considered to be synergistic.

[0065] In a further embodiment, activation of both the A1 and the A2A
25 adenosine receptors increases BBB permeability by 2, 3, 4, 5, 6, 7, 8, 9, or 10 fold, or any range encompassed therein. In one embodiment, activating both the A1 adenosine receptor and the A2A adenosine receptor increases the BBB permeability 7-9 fold.

[0066] According to certain embodiments of the present invention, the activation of both the A1 and the A2A receptors is additive. In this context, if the
30 effect of activating the two receptors together (at a given concentration) is equivalent to the sum of the effects when each receptor is activated individually (at the same concentration), then the activation of both the A1 and the A2A receptors together is considered to be additive.

[0067] In one embodiment according to the present invention, the increase in BBB permeability lasts up to 18 hours. In further embodiments, the increase in BBB permeability lasts up to about 17 hours, 16 hours, 15 hours, 14 hours, 13 hours, 12 hours, 11 hours, 10 hours, 9 hours, 8 hours, 6 hours, 4 hours, 3 hours, 2 hours, 1 hour, 30 minutes, 15 minutes, 10 minutes, or 5 minutes.

[0068] Another aspect of the present invention relates to increasing blood brain barrier permeability in a subject. This method includes administering to the subject an A1 adenosine receptor agonist and an A2A adenosine receptor agonist.

[0069] In one embodiment, the A1 adenosine receptor agonist and/or the A2A adenosine receptor agonist are selective agonists. As used herein, "selective" means having an activation preference for a specific receptor over other receptors which can be quantified based upon whole cell, tissue, or organism assays which demonstrate receptor activity.

[0070] Suitable A1-selective receptor agonists according to the present invention include 2-chloro-N^6-cyclopentyladenosine ("CCPA"), N6-Cyclopentyladenosine, N(6)-cyclohexyladenosine, 8-cyclopentyl-1,3-dipropylxanthine ("DPCPX"), R-phenylisopropyl-adenosine, or combinations thereof.

[0071] Suitable A2A-selective receptor agonists according to the present invention include Lexiscan (also known as Regadenoson), CGS 21680, ATL-146e, YT-146 (2-(1-octynyl)adenosine), DPMA (N6-(2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl)adenosine), or combinations thereof.

[0072] In one embodiment, the A1 adenosine receptor agonist and the A2A adenosine receptor agonist may be administered simultaneously. In another embodiment according to the present invention, the A1 adenosine receptor agonist and the A2A adenosine receptor agonist may be administered sequentially.

[0073] In certain embodiments, the A1 adenosine receptor agonist and the A2A adenosine receptor agonist are formulated in a single unit dosage form. Dosage and formulations according to the present invention are described in further detail below.

[0074] In one embodiment, this method further includes the administration of a therapeutic agent. The therapeutic agent may be administered together with one or both of the A1 adenosine receptor agonist and the A2A adenosine receptor agonist, or may be administered following administration of the A1 adenosine receptor agonist and/or the A2A adenosine receptor agonist. Suitable therapeutic agents are described in

further detail below. In certain embodiments, the agonists may be administered up to 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5, hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, or 18 hours before the therapeutic agent.

5 **[0075]** Another aspect of the present invention relates to a composition. The composition includes an A1 adenosine receptor agonist, an A2A adenosine receptor agonist, and a pharmaceutically acceptable carrier, excipient, or vehicle.

[0076] In one embodiment according to this aspect of the present invention, the A1 adenosine receptor agonist and/or the A2A adenosine receptor agonist are selective 10 agonists.

[0077] The compounds, compositions, or agents of the present invention can be administered locally or systemically. In particular the compounds, compositions, or agents of the present invention can be administered orally, parenterally, for example, subcutaneously, intravenously, intramuscularly, intraperitoneally, by intranasal 15 instillation, or by application to mucous membranes, such as, that of the nose, throat, and bronchial tubes. They may be administered alone or with suitable pharmaceutical carriers, and can be in solid or liquid form such as, tablets, capsules, powders, solutions, suspensions, or emulsions.

[0078] The active compounds or agents of the present invention may be orally 20 administered, for example, with an inert diluent, or with an assimilable edible carrier, or they may be enclosed in hard or soft shell capsules, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, these active compounds may be incorporated with excipients and used in the form of tablets, capsules, elixirs, suspensions, syrups, and the 25 like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compound in these compositions may, of course, be varied and may conveniently be between about 2% to about 60% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. A convenient unitary dosage formulation 30 contains the active ingredients in amounts from 0.1 mg to 1 g each, for example 5 mg to 500 mg. Typical unit doses may, for example, contain about 0.5 to about 500 mg, or about 1 mg to about 500 mg of an agent according to the present invention.

[0079] The tablets, capsules, and the like may also contain a binder such as gum tragacanth, acacia, corn starch, or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose, or saccharin.
5 When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a fatty oil.

[0080] Various other materials may be present as coatings or to modify the physical form of the dosage unit. For instance, tablets may be coated with shellac, sugar, or both. A syrup may contain, in addition to active ingredient, sucrose as a
10 sweetening agent, methyl and propylparabens as preservatives, a dye, and flavoring such as cherry or orange flavor.

[0081] These active compounds or agents may also be administered parenterally. Solutions or suspensions of these active compounds can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions
15 can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Illustrative oils are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, or mineral oil. In general, water, saline, aqueous dextrose and related sugar solution, and glycols such as, propylene glycol or polyethylene glycol, are preferred liquid carriers, particularly for injectable solutions.
20 Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0082] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be
25 sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol), suitable mixtures thereof,
30 and vegetable oils.

[0083] The compounds or agents of the present invention may also be administered directly to the airways in the form of an aerosol. For use as aerosols, the compounds of the present invention in solution or suspension may be packaged in a

pressurized aerosol container together with suitable propellants, for example, hydrocarbon propellants like propane, butane, or isobutane with conventional adjuvants. The materials of the present invention also may be administered in a non-pressurized form such as in a nebulizer or atomizer.

5 **[0084]** In one embodiment, the composition according to the present invention includes a therapeutic agent. In a further embodiment, the therapeutic is suitable for treating a central nervous system ("CNS") disease, disorder, or condition. Such therapeutic agents are well known in the art and many are common and typically prescribed agents for a relevant disorder. Dosage ranges for such agents are known to 10 one of ordinary skill in the art and are often found in the accompanying prescription information pamphlet (ofter referred to as the "label").

[0085] Disorders of the CNS (which encompass psychiatric/behavioral diseases or disorders) may include, but are not limited to, acquired epileptiform aphasia, acute disseminated encephalomyelitis, adrenoleukodystrophy, agenesis of the corpus 15 callosum, agnosia, aicardi syndrome, Alexander disease, Alpers' disease, alternating hemiplegia, Alzheimer's disease, amyotrophic lateral sclerosis, anencephaly, Angelman syndrome, angiomatosis, anoxia, aphasia, apraxia, arachnoid cysts, arachnoiditis, Arnold-chiari malformation, arteriovenous malformation, Asperger's syndrome, ataxia telangiectasia, attention deficit hyperactivity disorder, autism, auditory processing 20 disorder, autonomic dysfunction, back pain, Batten disease, Behcet's disease, Bell's palsy, benign essential blepharospasm, benign focal amyotrophy, benign intracranial hypertension, bilateral frontoparietal polymicrogyria, binswanger's disease, blepharospasm, Bloch-sulzberger syndrome, brachial plexus injury, brain abscess, brain damage, brain injury, brain tumor, spinal tumor, Brown-séquard syndrome, canavan 25 disease, carpal tunnel syndrome (cts), causalgia, central pain syndrome, central pontine myelinolysis, centronuclear myopathy, cephalic disorder, cerebral aneurysm, cerebral arteriosclerosis, cerebral atrophy, cerebral gigantism, cerebral palsy, charcot-marie-tooth disease, chiari malformation, chorea, chronic inflammatory demyelinating polyneuropathy ("CIDP"), chronic pain, chronic regional pain syndrome, Coffin lowry 30 syndrome, coma (including persistent vegetative state), congenital facial diplegia, corticobasal degeneration, cranial arteritis, craniosynostosis, Creutzfeldt-jakob disease, cumulative trauma disorders, Cushing's syndrome, cytomegalic inclusion body disease ("CIBD"), cytomegalovirus infection, dandy-walker syndrome, Dawson disease, de

morsier's syndrome, Dejerine-klumpke palsy, Dejerine-sottas disease, delayed sleep phase syndrome, dementia, dermatomyositis, developmental dyspraxia, diabetic neuropathy, diffuse sclerosis, dysautonomia, dyscalculia, dysgraphia, dyslexia, dystonia, early infantile epileptic encephalopathy, empty sella syndrome, encephalitis,
5 encephalocele, encephalotrigeminal angiomatosis, encopresis, epilepsy, Erb's palsy, erythromelalgia, essential tremor, Fabry's disease, Fahr's syndrome, fainting, familial spastic paralysis, febrile seizures, fisher syndrome, Friedreich's ataxia, Gaucher's disease, Gerstmann's syndrome, giant cell arteritis, giant cell inclusion disease, globoid cell leukodystrophy, gray matter heterotopia, Guillain-barré syndrome, htlv-1
10 associated myelopathy, Hallervorden-spatz disease, head injury, headache, hemifacial spasm, hereditary spastic paraplegia, heredopathia atactica polyneuritiformis, herpes zoster oticus, herpes zoster, hirayama syndrome, holoprosencephaly, Huntington's disease, hydranencephaly, hydrocephalus, hypercortisolism, hypoxia, immune-mediated encephalomyelitis, inclusion body myositis, incontinentia pigmenti, infantile
15 phytanic acid storage disease, infantile refsum disease, infantile spasms, inflammatory myopathy, intracranial cyst, intracranial hypertension, Joubert syndrome, Kearns-sayre syndrome, Kennedy disease, kinsbourne syndrome, Klippel feil syndrome, Krabbe disease, Kugelberg-welander disease, kuru, lafora disease, Lambert-eaton myasthenic syndrome, Landau-kleffner syndrome, lateral medullary (Wallenberg) syndrome,
20 learning disabilities, leigh's disease, Lennox-gastaut syndrome, Lesch-nyhan syndrome, leukodystrophy, lewy body dementia, lissencephaly, locked-in syndrome, Lou Gehrig's disease, lumbar disc disease, lyme disease - neurological sequelae, machado-joseph disease (spinocerebellar ataxia type 3), macrencephaly, megalencephaly, Melkersson-rosenthal syndrome, Ménière's disease, meningitis, Menkes disease, metachromatic
25 leukodystrophy, microcephaly, migraine, Miller Fisher syndrome, mini-strokes, mitochondrial myopathies, mobius syndrome, monomelic amyotrophy, motor neurone disease, motor skills disorder, moyamoya disease, mucopolysaccharidoses, multi-infarct dementia, multifocal motor neuropathy, multiple sclerosis, multiple system atrophy with postural hypotension, muscular dystrophy, myalgic encephalomyelitis,
30 myasthenia gravis, myelinoclastic diffuse sclerosis, myoclonic encephalopathy of infants, myoclonus, myopathy, myotubular myopathy, myotonia congenita, narcolepsy, neurofibromatosis, neuroleptic malignant syndrome, neurological manifestations of aids, neurological sequelae of lupus, neuromyotonia, neuronal ceroid lipofuscinosis,

neuronal migration disorders, niemann-pick disease, non 24-hour sleep-wake syndrome, nonverbal learning disorder, O'sullivan-mcleod syndrome, occipital neuralgia, occult spinal dysraphism sequence, ohtahara syndrome, olivopontocerebellar atrophy, opsoclonus myoclonus syndrome, optic neuritis, orthostatic hypotension,
5 overuse syndrome, palinopsia, paresthesia, Parkinson's disease, paramyotonia congenita, paraneoplastic diseases, paroxysmal attacks, parry-romberg syndrome (also known as rombergs syndrome), pelizaeus-merzbacher disease, periodic paralyses, peripheral neuropathy, persistent vegetative state, pervasive developmental disorders, photic sneeze reflex, phytanic acid storage disease, pick's disease, pinched nerve,
10 pituitary tumors, pmg, polio, polymicrogyria, polymyositis, porencephaly, post-polio syndrome, postherpetic neuralgia ("PHN"), postinfectious encephalomyelitis, postural hypotension, Prader-willi syndrome, primary lateral sclerosis, prion diseases, progressive hemifacial atrophy (also known as Romberg's syndrome), progressive multifocal leukoencephalopathy, progressive sclerosing poliodystrophy, progressive
15 supranuclear palsy, pseudotumor cerebri, ramsay-hunt syndrome (type I and type II), Rasmussen's encephalitis, reflex sympathetic dystrophy syndrome, refsum disease, repetitive motion disorders, repetitive stress injury, restless legs syndrome, retrovirus-associated myelopathy, rett syndrome, Reye's syndrome, Romberg's syndrome, rabies, Saint Vitus' dance, Sandhoff disease, schizophrenia, Schilder's disease, schizencephaly,
20 sensory integration dysfunction, septo-optic dysplasia, shaken baby syndrome, shingles, Shy-drager syndrome, Sjögren's syndrome, sleep apnea, sleeping sickness, snatiation, Sotos syndrome, spasticity, spina bifida, spinal cord injury, spinal cord tumors, spinal muscular atrophy, spinal stenosis, Steele-richardson-olszewski syndrome, see progressive supranuclear palsy, spinocerebellar ataxia, stiff-person syndrome, stroke,
25 Sturge-weber syndrome, subacute sclerosing panencephalitis, subcortical arteriosclerotic encephalopathy, superficial siderosis, sydenham's chorea, syncope, synesthesia, syringomyelia, tardive dyskinesia, Tay-sachs disease, temporal arteritis, tetanus, tethered spinal cord syndrome, Thomsen disease, thoracic outlet syndrome, tic douloureux, Todd's paralysis, Tourette syndrome, transient ischemic attack,
30 transmissible spongiform encephalopathies, transverse myelitis, traumatic brain injury, tremor, trigeminal neuralgia, tropical spastic paraparesis, trypanosomiasis, tuberous sclerosis, vasculitis including temporal arteritis, Von Hippel-lindau disease ("VHL"), Viliuisk encephalomyelitis ("VE"), Wallenberg's syndrome, Werdnig-hoffman disease,

west syndrome, whiplash, Williams syndrome, Wilson's disease, and Zellweger syndrome. It is thus appreciated that all CNS-related states and disorders could be treated through the BBB route of drug delivery.

[0086] A CNS disease, disorder, or condition according to embodiments of the present invention may be selected from a metabolic disease, a behavioral disorder, a personality disorder, dementia, a cancer, a neurodegenerative disorder, pain, a viral infection, a sleep disorder, a seizure disorder, acid lipase disease, Fabry disease, Wernicke-Korsakoff syndrome, ADHD, anxiety disorder, borderline personality disorder, bipolar disorder, depression, eating disorder, obsessive-compulsive disorder, schizophrenia, Alzheimer's disease, Barth syndrome and Tourette's syndrome, Canavan disease, Hallervorden-Spatz disease, Huntington's disease, Lewy Body disease, Lou Gehrig's disease, Machado-Joseph disease, Parkinson's disease, or Restless Leg syndrome.

[0087] In one embodiment, the CNS disease, disorder, or condition is pain and is selected from neuropathic pain, central pain syndrome, somatic pain, visceral pain, and/or headache.

[0088] Suitable CNS therapeutics according to the present invention include small molecule therapeutic agents. Suitable small molecule therapeutics for treating a disease, disorder, or condition of the CNS include acetaminophen, acetylsalicylic acid, acyltransferase, alprazolam, amantadine, amisulpride, amitriptyline, amphetamine-dextroamphetamine, amsacrine, antipsychotics, antivirals, apomorphine, arimoclomol, aripiprazole, asenapine, aspartoacyclase enzyme, atomoxetine, atypical antipsychotics, azathioprine, baclofen, beclamide, benserazide, benserazide-levodopa, benzodiazepines, benztropine, bevacizumab, bleomycin, brivaracetam, bromocriptine, buprenorphine, bupropion, cabergoline, carbamazepine, carbatrol, carbidopa, carbidopa-levodopa, carboplatin, chlorambucil, chlorpromazine, chlorprothixene, cisplatin, citalopram, clobazam, clomipramine, clonazepam, clozapine, codeine, COX-2 inhibitors, cyclophosphamide, dactinomycin, dexmethylphenidate, dextroamphetaine, diamorphine, diastat, diazepam, diclofenac, donepezil, doxorubicin, droperidol, entacapone, epirubicin, escitalopram, ethosuximide, etoposide, felbamate, fluoxetine, flupenthixol, fluphenazine, fosphenytoin, gabapentin, galantamine, gamma hydroxybutyrate, gefitinib, haloperidol, hydantoins, hydrocordone, hydroxyzine, ibuprofen, ifosfamide, IGF-1, iloperidone, imatinib, imipramine, interferons, irinotecan,

KNS-760704, lacosamide, lamotrigine, levetiracetam, levodopa, levomepromazine, lisdexamfetamine, lisuride, lithium carbonate, lypolytic enzyme, mechlorethamine, mGluR2 agonists, memantine, meperidine, mercaptopurine, mesoridazine, mesuximide, methamphetamine, methylphenidate, minocycline, modafinil, morphine, N-
5 acetylcysteine, naproxen, nelfinavir, neurotrin, nitrazepam, NSAIDs, olanzapine, opiates, oseltamivir, oxaplatin, paliperidone, pantothenate kinase 2, Parkin, paroxetine, pergolide, periciazine, perphenazine, phenacemide, phenelzine, phenobarbitol, phenturide, phenytoin, pimozide, Pink1, piribedil, podophyllotoxin, pramipexole, pregabalin, primidone, prochlorperazine, promazine, promethazine, protriptyline,
10 pyrimidinediones, quetiapine, rasagiline, remacemide, riluzole, risperidone, ritonavir, rituximab, rivastigmine, ropinirole, rotigotine, rufinamide, selective serotonin reuptake inhibitors (SSRIs), selegine, selegiline, sertindole, sertraline, sodium valproate, stiripentol, taxanes, temazepam, temozolomide, tenofovir, tetrabenazine, thiamine, thioridazine, thiothixene, tiagabine, tolcapone, topiramate, topotecan, tramadol,
15 tranylcypromine, trastuzumab, tricyclic antidepressants, trifluoperazine, triflupromazine, trihexyphenidyl, trileptal, valaciclovir, valnoctamide, valproamide, valproic acid, venlafaxine, vesicular stomatitis virus, vigabatrin, vinca alkaloids, zanamivir, ziprasidone, zonisamide, zotepine, zuclopenthixol, or combinations thereof.

[0089] In another embodiment, the composition according to the present
20 invention may include a therapeutic agent suitable for treatment of human immunodeficiency virus ("HIV"). The agent chosen from nucleoside HIV reverse transcriptase inhibitors, non-nucleoside HIV reverse transcriptase inhibitors, HIV protease inhibitors, HIV integrase inhibitors, HIV fusion inhibitors, immune modulators, CCR5 antagonists, and antiinfectives.

25 **[0090]** Pathogens such as HIV seek refuge in the CNS where they can remain for the life of the host. More than 30 million people world-wide are currently infected with HIV and these numbers are likely to increase (*See* United Nations: Report on The Global AIDS Epidemic (2008), which is hereby incorporated by reference in its entirety). Without an effective method of getting anti-HIV drugs into the CNS to target
30 the virus, it seems unlikely that HIV will ever be eradicated.

[0091] Other therapeutic agents or compounds that may be administered according to the present invention may be of any class of drug or pharmaceutical agent which is desirable to cross the BBB. Such therapeutics inclnde, but not limited to,

antibiotics, anti-parasitic agents, antifungal agents, anti-viral agents and anti-tumor agents. When administered with anti-parasitic, anti-bacterial, anti-fungal, anti-tumor, anti-viral agents, and the like, the compounds according to the present invention may be administered by any method and route of administration suitable to the treatment of 5 the disease, typically as pharmaceutical compositions.

[0092] Therapeutic agents can be delivered as a therapeutic or as a prophylactic (e.g., inhibiting or preventing ouset of neurodegenerative diseases). A therapeutic causes eradication or amelioration of the underlying disorder being treated. A prophylactic is administered to a patient at risk of developing a disease or to a patient 10 reporting one or more of the physiological symptoms of such a disease, even though a diagnosis may not have yet been made. Alternatively, prophylactic administration may be applied to avoid the onset of the physiological symptoms of the underlying disorder, particularly if the symptom manifests cyclically. In this latter embodiment, the therapy is prophylactic with respect to the associated physiological symptoms instead of the 15 underlying indication. The actual amount effective for a particular application will depend, *inter alia*, on the condition being treated and the route of administration.

[0093] The therapeutic may be selected from the group consisting of immunosuppressants, anti-inflammatories, anti-proliferatives, anti-migratory agents, anti-fibrotic agents, proapoptotics, calcium channel blockers, anti-neoplastics, 20 antibodies, anti-thrombotic agents, anti-platelet agents, IIbIIIa agents, antiviral agents, anti-cancer agents, chemotherapeutic agents, thrombolytics, vasodilators, antimicrobials or antibiotics, antimitotics, growth factor antagonists, free radical scavengers, biologic agents, radio therapeutic ageuts, radio-opaque agents, radiolabelled agents, anti-coagulants (e.g., heparin and its derivatives), anti- 25 angiogenesis drugs (e.g., Thalidomide), angiogenesis drugs, PDGF-B and/or EGF inhibitors, anti-inflammatories (e.g., psoriasis drugs), riboflavin, tiazofurin, zafurin, anti-platelet agents (e.g., cyclooxygenase inhibitors (e.g., acetylsalicylic acid)), ADP inhibitors (such as clopidogrel and ticlopdipine), hosphodiesterase III inhibitors (such as cilostazol), lycoprotein II/IIIa agents (such as abcix- imab), eptifibatide, and 30 adenosine reuptake inhibitors (such as dipyridmoles, healing and/or promoting agents (e.g., anti-oxidants and nitrogen oxide donors)), antiemetics, antinauseauts, tripdiolide, diterpenes, triterpenes, diterpene epoxides, diterpenoid epoxide, triepoxides, or

tripterygium wifordii hook F(TWHF), SDZ- RAD, RAD, RAD666, or 40-0-(2-hydroxy)ethyl-rapamycin, derivatives, pharmaceutical salts and combinations thereof.

[0094] In certain embodiments, the therapeutic and the adenosine receptor activator agent(s) (or adenosine receptor blockers or inhibitor, as described in further detail below) and/or therapeutics are formulated as a single "compound" formulation. This can be accomplished by any of a number of known methods. For example, the therapeutic agent and the activator agent can be combined in a single pharmaceutically acceptable excipient. In another approach the therapeutic and the adenosine receptor activator (or adenosine receptor blocker or inhibitor) agent can be formulated in separate excipients that are microencapsulated and then combined, or that form separate laminae in a single pill, and so forth.

[0095] In one embodiment, the therapeutic and adenosine receptor activator agent are linked together. In certain embodiments, the therapeutic and the adenosine receptor activator (or adenosine receptor blocker or inhibitor) agent are joined directly together or are joined together by a "tether" or "linker" to form a single compound. Without being bound to a particular theory, it is believed that such joined compounds provide improved specificity/ selectivity.

[0096] A number of chemistries for linking molecules directly or through a linker/tether are well known to those of skill in the art. The specific chemistry employed for attaching the therapeutic(s) and the adenosine receptor activator (or adenosine receptor blocker or inhibitor) agent to form a bifunctional compound depends on the chemical nature of the therapeutic(s) and the "interligand" spacing desired. Various therapeutics and adenosine receptor activator agents typically contain a variety of functional groups (e.g., carboxylic acid (COOH), free amine (—NEE), and the like), that are available for reaction with a suitable functional group on a linker or on the opposing component (i.e., either the therapeutic or adenosine receptor activator) to bind the components together.

[0097] Alternatively, the components can be derivatized to expose or attach additional reactive functional groups. The derivatization may involve attachment of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford Ill.

[0098] A "linker" or "tether", as used herein, is a molecule that is used to join two or more ligands (e.g., therapeutic(s) or adenosine receptor activator) to form a bi-

functional or poly-functional compound. The linker is typically chosen to be capable of forming covalent bonds to all of the components comprising the bi-functional or polyfunctional moiety. Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic
5 carbon linkers, amino acids, nucleic acids, dendrimers, synthetic polymers, peptide linkers, peptide and nucleic acid analogs, carbohydrates, polyethylene glycol and the like. Where one or more of the components are polypeptides, the linker can be joined to the constituent amino acids through their side groups (e.g., through a disulfide linkage to cysteine) or through the alpha carbon amino or carboxyl groups of the
10 terminal amino acids.

[0099] In certain embodiments, a bifunctional linker having one functional group reactive with a group on the first therapeutic and another group reactive with a functional group on the adenosine receptor activator agent can be used to form a bifunctional compound. Alternatively, derivatization may involve chemical treatment
15 of the component(s) (e.g., glycol cleavage of the sugar moiety of a glycoprotein, a carbohydrate, or a nucleic acid, etc.) with periodate to generate free aldehyde groups. The free aldehyde groups can be reacted with free amine or hydrazine groups on a linker to bind the linker to the compound (See, e.g., U.S. Patent No. 4,671,958 to Rodwell et al., which is hereby incorporated by reference in its entirety). Procedures
20 for generation of free sulfhydryl groups on polypeptide, such as antibodies or antibody fragments, are also known (See U.S. Patent No. 4,659,839 to Nicolotti et al., which is hereby incorporated by reference in its entirety).

[0100] Where the therapeutic and the adenosine receptor activator agent are both peptides, a bifunctional compound can be chemically synthesized or
25 recombinantly expressed as a fusion protein comprising both components attached directly to each other or attached through a peptide linker.

[0101] In certain embodiments, lysine, glutamic acid, and polyethylene glycol (PEG) based linkers of different length are used to couple the components. The chemistry for the conjugation of molecules to PEG is well known to those of skill in the
30 art (see, e.g., Veronese, "Peptide and Protein PEGylation: a Review of Problems and Solutions," *Biomaterials* 22: 405-417 (2001); Zalipsky et al., "Attachment of Drugs to Polyethylene Glycols," *Eur. Plym. J.* 19(12):1177-1183 (1983); Olson et al., "Preparation and Characterization of Poly(ethylene glycol)ylated Human Growth

Hormone Antagonist," *Poly(ethylene glycol) Chemistry and Biological Applications* 170-181, Harris & Zalipsky Eds., ACS, Washington, DC (1997); Delgado et al., "The Uses and Properties of PEG-Linked Proteins," *Crit. Rev. Therap. Drug Carrier Sys.* 9: 249-304(1992); Pedley et al., "The Potential for Enhanced Tumour Localisation by
5 Poly(ethylene glycol) Modification of anti-CEA Antibody," *Brit. J. Cancer* 70:1126-1130 (1994); Eyre & Farver, *Textbook of Clinical Oncology* 377-390 (Holleb et al. eds. 1991); Lee et al., "Prolonged Circulating Lives of Single-chain of Fv Proteins Conjugated with Polyethylene Glycol: a Comparison of Conjugation Chemistries and Compounds," *Bioconjug. Chem.* 10: 973-981(1999); Nucci et al., "The Therapeutic
10 Value of Poly(Ethytene Glycol)-Modified Proteins," *Adv. Drug Deliv. Rev.*6: 133-151(1991); Francis et al., "Polyethylene Glycol Modification: Relevance of Improved Methodology to Tumour Targeting," *J. Drug Targeting* 3: 321-340(1996), which are hereby incorporated by reference in their entirety).

[0102] In certain embodiments, conjugation of the therapeutic and the
15 adenosine receptor activator (or adenosine receptor blocker or inhibitor) agent can be achieved by the use of such linking reagents such as glutaraldehyde, EDCI, terephthaloyl chloride, cyanogen bromide, and the like, or by reductive amination. In certain embodiments, components can be linked via a hydroxy acid linker of the kind disclosed in WO-A- 9317713. PEG linkers can also be utilized for the preparation of
20 various PEG tethered drugs (See, e.g., Lee et al., "Reduction of Azides to Primary Amines in Substrates Bearing Labile Ester Functionality: Synthesis of a PEG-Solubilized, "Y"-Shaped Iminodiacetic Acid Reagent for Preparation of Folate-Tethered Drugs," *Organic Lett.*, 1: 179-181(1999), which is hereby incorporated by reference in its entirety). In other embodiments, the adenosine receptor activator (or
25 adenosine receptor blocker or inhibitor) agent) may be PEGylated (e.g., PEGylated adenosine deaminase).

[0103] Another aspect of the present invention relates to a method of delivering a macromolecule therapeutic agent to the brain of a subject. This method involves administering to the subject (a) an agent which activates both of A1 and A2A adenosine
30 receptors and (b) the macromolecular therapeutic.

[0104] In certain embodiments, the macromolecular therapeutic agent may be a bioactive protein or peptide agent. Examples of such bioactive protein or peptides include a cell modulating peptide, a chemotactic peptide, an anticoagulant peptide, an

antithrombotic peptide, an anti-tumor peptide, an anti-infectious peptide, a growth potentiating peptide, and an anti-inflammatory peptide. Examples of proteins include antibodies, enzymes, steroids, growth hormone and growth hormone-releasing hormone, gonadotropin-releasing hormone and its agonist and antagonist analogues,
5 somatostatin and its analogues, gonadotropins, peptide T, thyrocalcitonin, parathyroid hormone, glucagon, vasopressin, oxytocin, angiotensin I and II, bradykinin, kallidin, adrenocorticotropic hormone, thyroid stimulating hormone, insulin, glucagon and the numerous analogues and congeners of the foregoing molecules. In some aspects of the invention, the BBB permeability is modulated by one or more methods herein above to
10 deliver an antibiotic, or an anti-infectious therapeutic capable agent. Such anti-infectious agents reduce the activity of or kills a microorganism.

[0105] The nature of the peptide agent is not limited, other than comprising amino acid residues. The peptide agent can be a synthetic or a naturally occurring peptide, including a variant or derivative of a naturally occurring peptide. The peptide
15 can be a linear peptide, cyclic peptide, constrained peptide, or a peptidomimetic. Methods for making cyclic peptides are well known in the art. For example, cyclization can be achieved in a head-to-tail manner, side chain to the N- or C-terminus residues, as well as cyclizations using linkers. The selectivity and activity of the cyclic peptide depends on the overall ring size of the cyclic peptide which controls its three
20 dimensional structure. Cyclization thus provides a powerful tool for probing progression of disease states, as well as targeting specific self-aggregation states of diseased proteins.

[0106] In some embodiments, the peptide agent specifically binds to a target protein or structure associated with a neurological condition. In accordance with these
25 embodiments, the invention provides agents useful for the selective targeting of a target protein or structure associated with a neurological condition, for diagnosis or therapy. Peptide agents useful in accordance with the present invention are described in, for example, U.S. Patent Application Publication 2009/0238754 to Wegrzyn et al., which is hereby incorporated by reference in its entirety.

30 **[0107]** In other embodiments, the peptide agent specifically binds to a target protein or structure associated with other neurological conditions, such as stroke, cerebrovascular disease, epilepsy, transmissible spongiform encephalopathy (TSE); Aβ-peptide in amyloid plaques of Alzheimer's disease (AD), cerebral amyloid

angiopathy (CAA), and cerebral vascular disease (CVD); α-synuclein deposits in Lewy bodies of Parkinson's disease, tau in neurofibrillary tangles in frontal temporal dementia and Pick's disease; superoxide dismutase in amylotrophic lateral sclerosis; and Huntingtin in Huntington's disease and benign and cancerous brain tumors such as
5 glioblastoma's, pituitary tumors, or meningiomas.

[0108] In some embodiments, the peptide agent undergoes a conformational shift other than the alpha-helical to beta-sheet shift discussed above, such as a beta-sheet to alpha-helical shift, an unstructured to beta-sheet shift, etc. Such peptide agents may undergo such conformational shifts upon interaction with target peptides or
10 structures associated with a neurological condition.

[0109] In other embodiments, the peptide agent is an antibody that specifically binds to a target protein or structure associated with a neurological condition, such as a target protein or structure (such as a specific conformation or state of self-aggregation) associated with an amyloidogenic disease, such as the anti-amyloid antibody 6E10, and
15 NG8. Other anti-amyloid antibodies are known in the art, as are antibodies that specifically bind to proteins or structures associated with other neurological conditions.

[0110] In certain embodiments, the macromolecular therapeutic agent is a monoclonal antibody. Suitable monoclonal antibodies include 6E10, PF-04360365, 131I-chTNT-1/B MAb, 131I-L19SIP, 177Lu-J591, ABT-874, AIN457, alemtuzumab,
20 anti-PDGFR alpha monoclonal antibody IMC-3G3, astatine At 211 monoclonal antibody 81C6, Bapineuzumab, Bevacizumab, cetuximab, cixutumumab, Daclizumab, Hu MiK-beta-1, HuMax-EGFr, iodine I 131 monoclonal antibody 3F8, iodine I 131 monoclonal antibody 81C6, iodine I 131 monoclonal antibody 8H9, iodine I 131 monoclonal antibody TNT-1/B, LMB-7 immunotoxin, MAb-425, MGAWN1, Me1-14
25 F(ab')2, M-T412, Natalizumab, Neuradiab, Nimotuzumab, Ofatumumab, Panitumumab, Ramucirumab, ranibizumab, SDZ MSL-109, Solanezumab, Trastuzumab, Ustekinumab, Zalutumumab, Tanezumab, Aflibercept, MEDI-578, REGN475, Muromonab-CD3, Abiximab, Rituximab, Basiliximab, Palivizumab, Infliximab, Gemtuzumab ozogamicin, Ibritumomab tiuxetau, Adalimumab,
30 Omalizumab, Tositumomab, Tositumomab-I131, Efalizumab, Abciximab, Certolizumab pegol, Eculizumab, AMG-162, Zanolimumab, MDX-010, Anti0MRSA mAb, Pexelizumab, Mepolizumab, Epratuzumab, Anti- RSV mAb, Afelimomab, Catumaxomab, WX-G250, or combinations thereof.

[0111] In certain embodiments, the macromolecular therapeutic agent is a peptide detection agent. For example, peptide detection agents include fluorescent proteins, such as Green Flourescent Protein (GFP), streptavidin, enzymes, enzyme substrates, and other peptide detection agents known in the art.

5 [0112] In other embodiments, the macromolecular therapeutic agent includes peptide macromolecules and small peptides. For example, neurotrophic proteins are useful as peptide agents in the context of the methods described herein. Neurotrophic proteins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), neurotrophin-5 (NT-5), insulin-like

10 growth factors (IGF-I and IGF-II), glial cell line derived neurotrophic factor (GDNF), fibroblast growth factor (FGF), ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), glia-derived nexin (GDN), transforming growth factor (TGF-α and TGF-β), interleukin, platelet-derived growth factor (PDGF) and S100β protein, as well as bioactive derivatives and analogues thereof.

15 [0113] Neuroactive peptides also include the subclasses of hypothalamic-releasing hormones, neurohypophyseal hormones, pituitary peptides, invertebrate peptides, gastrointestinal peptides, those peptides found in the heart, such as atrial naturetic peptide, and other neuroactive peptides. Hypothalamic releasing hormones include, for example, thyrotropin-releasing hormones, gonadotropin-releasing hormone,

20 somatostatins, corticotropin-releasing hormone and growth hormone-releasing hormone. Neurohypophyseal hormones include, for example, compounds such as vasopressin, oxytocin, and neurophysins. Pituitary peptides include, for example, adrenocorticotropic hormone, β-endorphin, α-melanocyte-stimulating hormone, prolactin, luteinizing hormone, growth hormone, and thyrotropin. Suitable invertebrate

25 peptides include, for example, FMRF amide, hydra head activator, proctolin, small cardiac peptides, myomodulins, buccolins, egg-laying hormone and bag cell peptides. Gastrointestinal peptides include, for example, vasoactive intestinal peptide, cholecystokinin, gastrin, neurotensin, methionineenkephalin, leucine-enkephalin, insulin and insulin-like growth factors I and II, glucagon, peptide histidine

30 isoleucineamide, bombesin, motilin and secretins. Examples of other neuroactive peptides include angiotensin II, bradykinin, dynorphin, opiocortins, sleep peptide(s), calcitonin, CGRP (calcitonin gene-related peptide), neuropeptide Y, neuropeptide Yy,

galanin, substance K (neurokinin), physalaemin, Kassinin, uperolein, eledoisin and atrial naturetic peptide.

[0114] In yet further embodiments, the macromolecular therapeutic agent is a protein associated with membranes of synaptic vesicles, such as calcium-binding
5 proteins and other synaptic vesicle proteins. The subclass of calcium-binding proteins includes the cytoskeleton-associated proteins, such as caldesmon, annexins, calelectrin (mammalian), calelectrin (torpedo), calpactin I, calpactin complex, calpactin II, endonexin I, endonexin II, protein II, synexin I; and enzyme modulators, such as p65. Other synaptic vesicle proteins include inhibitors of mobilization (such as synapsin Ia,b
10 and synapsin IIa,b), possible fusion proteins such as synaptophysin, and proteins of unknown function such as p29, VAMP-1,2 (synaptobrevin), VAT1, rab 3A, and rab 3B.

[0115] Macromolecular therapeutic agents also include α-, β- and γ-interferon, epoetin, Fligrastim, Sargramostin, CSF-GM, human-IL, TNF and other biotechnology drugs.

15 **[0116]** Macromolecular therapeutic agents also include peptides, proteins and antibodies obtained using recombinant biotechnology methods.

[0117] Macromolecular therapeutic agents also include "anti-amyloid agents" or "anti-amyloidogenic agents," which directly or indirectly inhibit proteins from aggregating and/or forming amyloid plaques or deposits and/or promotes
20 disaggregation or reduction of amyloid plaques or deposits. Anti-amyloid agents also include agents generally referred to in the art as "amyloid busters" or "plaque busters." These include drugs which are peptidomimetic and interact with amyloid fibrils to slowly dissolve them. "Peptidomimetic" means that a biomolecule mimics the activity of another biologically active peptide molecule. "Amyloid busters" or "plaque busters"
25 also include agents which absorb co-factors necessary for the amyloid fibrils to remain stable.

[0118] Anti-amyloid agents include antibodies and peptide probes, as described in PCT application PCT/US2007/016738 (WO 2008/013859) and U.S. patent application Ser. No. 11/828,953, the entire contents of which are incorporated herein by
30 reference in their entirety. As described therein, a peptide probe for a given target protein specifically binds to that protein, and may preferentially bind to a specific structural form of the target protein. While not wanting to be bound by any theory, it is believed that binding of target protein by a peptide probe will prevent the formation of

higher order assemblies of the target protein, thereby preventing or treating the disease associated with the target protein, and/or preventing further progression of the disease. For example, binding of a peptide probe to a monomer of the target protein will prevent self-aggregation of the target protein. Similarly, binding of a peptide probe to a soluble 5 oligomer or an insoluble aggregate will prevent further aggregation and protofibril and fibril formation, while binding of a peptide probe to a protofibril or fibril will prevent further extension of that structure. In addition to blocking further aggregation, this binding also may shift the equilibrium back to a state more favorable to soluble monomers, further halting the progression of the disease and alleviating disease 10 symptoms.

[0119] In one embodiment, the macromolecular therapeutic agent is a variant of a peptide agent described above, with one or more amino acid substitutions, additions, or deletions, such as one or more conservative amino acid substitutions, additions, or deletions, and/or one or more amino acid substitutions, additions, or deletions that 15 further enhances the permeability of the conjugate across the BBB. For example, amino acid substitutions, additions, or deletions that result in a more hydrophobic amino acid sequence may further enhance the permeability of the conjugate across the BBB.

[0120] In another embodiment, the macromolecular therapeutic agent is about 20 150 kDa in size. In yet another embodiment, the therapeutic is up to about 10,000 Da in size, up to about 70,000 Da in size, or up to about 150 kDa in size. In still further embodiments the therapeutic is between about 10,000 and about 70,000 Da, between about 70,000 Da and 150 kDa, or between about 10,000 Da and about 150 kDa in size.

[0121] In one embodiment, the agent that activates both of the A1 and A2A 25 adenosine receptors is administered before the therapeutic macromolecule. In further embodiments, the agent that activates both of the A1 and A2A adenosine receptors may be administered up to 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5, hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, or 18 hours before the 30 therapeutic macromolecule agent.

[0122] In another embodiment, the agent or agents that activate both of the A1 and A2A adenosine receptors is administered simultaneously with the therapeutic macromolecule.

[0123] Another aspect of the present invention relates to a method for treating a CNS disease, disorder, or condition in a subject. This method involves administering to the subject at least one agent which activates both of the A1 and the A2A adenosine receptors and a therapeutic agent.

5 [0124] Suitable therapeutic agents are described above and may include small molecule therapeutic agents, macromolecular therapeutic agents, or combinations thereof.

[0125] In one embodiment, the agent which activates both of the A1 and the A2A adenosine receptors is an agonist of both the A1 and the A2A adenosine receptors.

10 In further embodiments, the agonist of both the A1 and the A2A adenosine receptors is a broad spectrum adenosine receptor agonist, such as NECA, adenosine, adenosine derivatives, or combinations thereof.

[0126] Another aspect of the present invention relates to a method of treating a CNS disease, disorder or condition in a subject. This method includes administering to

15 the subject (a) an adenosine receptor agonist; (b) an A2A receptor agonist; and (c) a therapeutic agent.

[0127] In one embodiment according to this aspect of the present invention, the the A1 adenosine receptor agonist and/or the A2A adenosine receptor agonist are selective agonists.

20 [0128] Suitable A1-selective adenosine receptor agonist, A2A-selective adenosine receptor agonists, and therapeutic agents (along with their preparation and administration) are noted above.

[0129] In a further embodiment, this method further involves selecting a subject in need of treatment or prevention of a CNS disease, disorder, or condition; providing a

25 therapeutic agent; and administering to the selected subject the therapeutic, an A1 adenosine receptor agonist, and an A2A receptor agonist under conditions effective for the therapeutic to cross the blood brain barrier and treat or prevent the CNS disease, disorder or condition.

[0130] In one embodiment the A1 adenosine receptor agonist and A2A

30 adenosine receptor agonist are formulated in a single unit dosage form.

[0131] In another embodiment the A1 adenosine receptor agonist and A2A adenosine receptor agonist are administered simultaneously.

[0132] In yet a further embodiment the A1 adenosine receptor agonist and A2A adenosine receptor agonist are administered sequentially.

[0133] In still a further embodiment, the method further includes administering a composition that includes an A1 adenosine receptor agonist and A2A adenosine
5 receptor agonist, and a pharmaceutically acceptable carrier, excipient, or vehicle.

[0134] Another aspect of the present invention relates to a method of temporarily increasing the permeability of the blood brain barrier of a subject. This method includes selecting a subject in need of a temporary increase in permeability of the blood brain barrier, providing an agent which activates either the A1 or the A2A
10 adenosine receptor, and administering to the selected subject either the A1 or the A2A adenosine receptor activating agent under conditions effective to temporarily increase the permeability of the blood brain barrier.

[0135] In one embodiment, the A1 or the A2A activating agent is an A1 or A2A agonist. In a further embodiment, the A1 or the A2A adenosine receptor
15 activating agent is an A1-selective or an A2-selective adenosine receptor agonist. Suitable A1 and A2A adenosine receptor agonists are known to those of skill in the art and are described in detail above.

[0136] In a further embodiment of this aspect of the present invention, the method further includes administering a therapeutic agent to the subject. Suitable
20 therapeutic agents are described in detail above.

[0137] In one embodiment, the agent that activates the A1 or the A2A adenosine receptor is administered before the therapeutic agent. In further embodiments, the agent that activates the A1 or the A2A adenosine receptor may be administered up to 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3
25 hours, 4 hours, 5, hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, or 18 hours before the therapeutic agent.

[0138] In another embodiment the agent that activates the A1 or the A2A adenosine receptor and the therapeutic agent are administered simultaneously.

30 **[0139]** Another aspect of the present invention is directed to a method of decreasing BBB permeability in a subject. This method involves administering to the subject or patient an agent which blocks or inhibits A2A adenosine receptor signaling.

[0140] In decreasing BBB permeability, the selected subject can have an inflammatory disease. Such inflammatory diseases include those in which mediators of inflammation pass the blood brain barrier. Such inflammatory diseases include, but are not limited to, inflammation caused by bacterial infection, viral infection, or autoimmune disease. More specifically, such diseases include, but are not limited to, meningitis, multiple sclerosis, neuromyelitis optica, human immunodeficiency virus ("HIV")-1 encephalitis, herpes simplex virus ("HSV") encephalitis, Toxoplams gondii encephalitis, and progressive multifocal leukoencephalopathy.

[0141] Where BBB permeability is decreased, the selected subject may also have a condition mediated by entry of lymphocytes into the brain. Other conditions treatable in this fashion include encephalitis of the brain, Parkinson's disease, epilepsy, neurological manifestations of HIV-AIDS, neurological sequela of lupus, and Huntington's disease, meningitis, multiple sclerosis, neuromyelitis optica, HSV encephalitis, and progressive multifocal leukoencephalopathy.

[0142] This aspect of the present invention can be carried out using the pharmaceutical formulation methods and methods of administration described above.

[0143] Altering adenosine receptor activity in a subject to decrease blood barrier permeability can be accomplished by, but not limited to, deactivating or blocking the A2A adenosine receptor.

[0144] A number of adenosine A2A receptor antagonists are known to those of skill in the art and can be used individually or in conjunction in the methods described herein. Such antagonists include, but are not limited to (-)-R,S)-mefloquine (the active enantiomer of the racemic mixture marketed as Mefloquine™), 3,7-Dimethyl-l-propargylxanthine (DMPX), 3-(3-hydroxypropyl)-7-methyl-8- (m-methoxystyryl)-l-propargylxanthine (MX2), 3-(3-hy- droxypropyl)-8-(3-methoxystyryl)-7-methyl-l-propargylxanthin phosphate disodium salt (MSX-3, a phosphate prodrug of MSX-2), 7-methyl-8-styrylxanthine derivatives, SCH 58261, KW-6002, aminofuryltriazolo-tri-azinylaminoethylphenol (ZM 241385), and 8-chlorostyryl- caffeine, KF17837, VR2006, istradefylline, the VERNALIS drugs such as VER 6489, VER 6623, VER 6947, VER 7130, VER 7146, VER 7448, VER 7835, VER 8177VER-11135, VER-6409, VER 6440, VER 6489, VER 6623, VER 6947, VER 7130, VER 7146, VER 7448, VER 7835, VER 8177, pyrazolo [4,3-e]l,2,4-triazolo[l,5-c]pyrimidines, and 5-amino-imidazolo-[4,3-e]-l,2,4-triazolo[l,5-c]pyrimidines, and the like (U.S. Patent

Application Publication No. 2006/0128708 to Li et al., which is hereby incorporated by reference in its entirety), pyrazolo[4,3-e]-[1 ,2,4]- triazolo[l,5-c]pyrimidines (See e.g., WO 01/92264 to Kase et al., which is hereby incorporated by reference in its entirety), 2,7-disubstituted-5-amino- [l,2,4]triazolo[l,5-c]pyrimidines (See e.g., WO 03/048163 to
5 Kase et al., which is hereby incorporated by reference in its entirety), 2,5-disubstituted-7-amino-[l,2,4]triazolo[l,5-a][l,3,5]triazines (See e.g., Vu et al., "Piperazine Derivatives of [1,2,4]Triazolo[1,5-a][1,3,5]triazine as Potent and Selective Adenosine A2A Receptor Antagonists," *J. Med. Chem.* 47(17):4291-4299 (2004), which is hereby incorporated by reference in its entirety), 9-substituted-2- (substituted-ethyn-l-yl)-
10 adenines (See e.g., U.S. Patent No. 7,217,702 to Beauglehole et al., which is hereby incorporated by reference in its entirety), 7-methyl-8-styrylxanthine derivatives, pyrazolo [4,3-c)l,2,4-triazolo[l,5-c]pyrimidines, and 5-amino-imidazolo-[4,3-c]-l,2,4-triazolo[l,5-c]pyrimi dines (See e.g., U.S. Patent Application Publication No. 2006/0128708 to Li et al., which is hereby incorporated by reference in its entirety).
15 These adenosine A2A receptor antagonists are intended to be illustrative and not limiting.

[0145] Yet a further aspect of the present invention relates to a method for increasing BBB permeability followed by deacreasing BBB permeability. The method involves administration of one or more agents that activate the A1 and A2A adenosine
20 receptors followed by administration of an agent that blocks or inhibits A2A adenosine receptor signaling.

[0146] In one embodiment, the one or more agents that activate the A1 and A2A adenosine receptors is administered simultaneously with a therapeutic agent. In another embodiment, the one or more agents that activate both the A1 and A2A
25 adenosine receptors is administered before a therapeutic agent. In this embodiment, the agent that blocks or inhibits A2A adenosine receptor signaling is administered following administration of the therapeutic agent.

[0147] Yet another aspect of the present invention relates to a method of remodeling an actin cytoskeleton of a BBB endothelial cell. This method involves
30 contacting an endothelial cell with one or more agents that activates both of the A1 and the A2A adenosine receptors.

[0148] The actin cytoskeleton is vital for the maintenance of cell shape. Endothelial barrier permeability can be affected by reorganization of the actin

cytoskeleton. The actin cytoskeleton is organized into three distinct structures: the cortical actin rim, actomyosin stress fibers, and actin cross-linking of the membrane skeleton (Prasain et al., "The Actin Cytoskeleton in Endothelial Cell Phenotypes," *Microvasc. Res.* 77:53-63 (2009), which is hereby incorporated by reference in its
5 entirety). These structures have unique roles in controlling endothelial cell shape.

[0149] According to one embodiment, the actin cytoskeleton remodeling increases space between endothelial cells and increases BBB permeability.

[0150] Suitable A1 and A2A adenosine receptor activators are disclosed above.

[0151] In one embodiment according to this aspect of the present invention, the
10 activation of both of the A1 and A2A adenosine receptors is synergistic with respect to BBB permeability. In yet another embodiment, the activation of both of the A1 and A2A adenosine receptors is additive with respect to BBB permeability.

[0152] While the identification of the A1 and A2A ARs as critical mediators of BBB permeability represents the first step towards a molecular mechanism, much work
15 remains to elucidate the specific downstream players that facilitate cellular changes in the endothelial cells. Adenosine receptors are G-protein coupled receptors, associated with heterotrimeric G-proteins. Several G_{α} subunits have been localized to tight junctions (Denker et al., "Involvement of a Heterotrimeric G Protein Alpha Subunit in Tight Junction Biogenesis," *J. Biol. Chem.* 271:25750-3 (1996), which is hereby
20 incorporated by reference in its entirety). These G_{α} subunits are known to influence the activity of downstream enzymes like RhoA and Rac1 that have been implicated in cytoskeletal remodeling. Indeed, work by other groups suggests that the RhoA and Rac1 small GTPases are responsive to extracellular signaling and mediate changes in the actin cytoskeleton (Schreibelt et al., "Reactive Oxygen Species Alter Brain
25 Endothelial Tight Junction Dynamics Via RhoA, PI3 kinase, and PKB Signaling," *Faseb J.* 21:3666-76 (2007); Jou et al., "Structural and Functional Regulation of Tight Junctions by RhoA and Rac1 Small GTPases," J Cell Biol 142, 101-15 (1998); and Wojciak-Stothard et al., "Regulation of TNF-alpha-induced Reorganization of the Actin Cytoskeleton and Cell-cell Junctions by Rho, Rac, and Cdc42 in Human
30 Endothelial Cells," *J. Cell. Physiol.* 176:150-165 (1998), which are hereby incorporated by reference in their entireties). Additionally, there is evidence that adenosine affects actin through the Rho GTPase (Sohail et al., "Adenosine Induces Loss of Actin Stress Fibers and Inhibits Contraction in Hepatic Stellate Cells via Rho Inhibition,"

Hepatology 49:185-94 (2009), which is hereby incorporated by reference in its entirety). Importantly, inflammation caused by canonical damage signals like TNF-α and thrombin increases BBB permeability by altering tight junctions through cytoskeletal reorganization (Wojciak-Stothard et al., "Regulation of TNF-alpha-
5 Induced Reorganization of the Actin Cytoskeleton and Cell-Cell Junctions by Rho, Rac, and Cdc42 in Human Endothelial Cells," *J. Cell. Physiol.* 176:150-65 (1998) and Lum et al., "Mechanisms of Increased Endothelial Permeability," *Can. J. Physiol. Pharmacol.* 74:787-800 (1996), which are hereby incorporated by reference in their entireties). Signaling events initiated by activation of A1 and A2A ARs on brain
10 endothelial cells result in actin cytoskeletal remodeling which, by changing cell shape, increases the space between the endothelial cells and allows increased molecular diffusion. Adenosine has been shown to affect other endothelial cell barrier properties in a similar manner (Lu et al., "Adenosine Protected Against Pulmonary Edema Through Transporter- and Receptor A2-mediated Endothelial Barrier Enhancement,"
15 *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 298: L755-67 (2010), which is hereby incorporated by reference in its entirety). However, here actomyosin stress fiber formation in brain endothelial cell monolayers was observed upon A1 or A2A AR activation with specific agonists. Conversely, blockade of these receptors with AR antagonists could act in the opposite fashion and result in increased tightness between
20 the cells. In the absence of active signaling from ARs, this model favors a tight barrier (Figure 25). This strongly correlates AR activation with stress fiber formation. Taken together with the present observations that AR agonists also decrease TEER in BEC monolayers, it indicates that AR modulation, acting through cytoskeletal elements, causes changes in endothelial cell shape that increase barrier permeability.

25

EXAMPLES

[0153] The following examples are provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

Example 1 - Mice

30 **[0154]** $Cd73^{-/-}$ mice have been previously described (Thompson et al., "Crucial Role for Ecto-5'-Nucleotidase (CD73) in Vascular Leakage During Hypoxia," *J. Exp.*

Med. 200:1395-1405 (2004), which is hereby incorporated by reference in its entirety) and have been backcrossed to C57BL/6 for 14 generations. *Cd73*$^{-/-}$ mice have no overt susceptibility to infection and appear normal based on the size and cellular composition of their lymphoid organs and their T and B cell responses in *in vivo* and *in vitro* assays
5 (Thompson et al., "Crucial Role for Ecto-5'-Nucleotidase (CD73) in Vascular Leakage During Hypoxia," *J. Exp. Med.* 200:1395-1405 (2004), which is hereby incorporated by reference in its entirety). C57BL/6 and *tcrα*$^{-/-}$ mice on the C57BL/6 background were purchased from The Jackson Laboratories. Mice were bred and housed under specific pathogen-free conditions at Cornell University or the University of Turku. For
10 adenosine receptor blockade experiments, mice were given drinking water supplemented with 0.6g/L of caffeine (Sigma) or 2mg/kg SCH58261 (1mg/kg *s.c.* and 1mg/kg *i.p.*) in DMSO (45% vol. in PBS) or 45% DMSO alone starting 1 day before EAE induction and continuing throughout the experiment. All procedures performed on mice were approved by the relevant animal review committee.

15

Example 2 - EAE Induction and Scoring

[0155] EAE was induced by subjecting mice to the myelin oligodendrocyte glycoprotein ("MOG") EAE-inducing regimen as described in Swanborg, "Experimental Autoimmune Encephalomyelitis in Rodents as a Model for Human
20 Demyelinatiug Disease," *Clin. Immunol. Immunopathol.* 77:4-13 (1995) and Bynoe et al., "Epicutaneous Immunization with Autoantigenic Peptides Induces T Suppressor Cells that Prevent Experimental Allergic Encephalomyelitis," *Immunity* 19:317-328 (2003), which are hereby incorporated by reference in their entirety. Briefly, a 1:1 emulsion of MOG_{35-55} peptide (3 mg/ml in PBS) (Invitrogen) and complete Freund's
25 adjuvant (CFA, Sigma) was injected subcutaueously (50μl) into each flank. Pertussis toxin (PTX, 20ng) (Biological Laboratories Inc.) was given intravenously (200μl in PBS) at the time of immunization and again 2 days later. Mice were scored daily for EAE based on disease symptom severity; 0=no disease, 0.5 -1=weak/limp tail, 2=limp tail and partial hind limb paralysis, 3=total hind limb paralysis, 4=both hind limb and
30 fore limb paralysis, 5=death. Mice with a score of 4 were euthanized.

Example 3 - T Cell Preparations and Adoptive Transfer

[0156] Mice were primed with MOG_{35-55} peptide in CFA without PTX. After one week, lymphocytes were harvested from spleen and lymph nodes and incubated with ACK buffer (0.15M NH_4Cl, 1 mM $KHCO_3$, 0.1mM EDTA, pH 7.3) to lyse red
5 blood cells. Cells were incubated with antibodies to CD8 (TIB-105), $IA^{b,d,v,p,q,r}$ (212.A1), FcR (2.4-G2), B220 (TIB-164), NK1.1 (HB191) and then BioMag goat anti-mouse IgG, IgM, and goat anti-rat IgG (Qiagen). After negative magnetic enrichment, $CD4^+$ cells were used either directly or further sorted into specific subpopulations. For sorting, cells were stained with antibodies to CD4 (RM4-5) and CD73 (TY/23), and in
10 some experiments CD25 (PC61), and then isolated utilizing a FACSAria (BD Biosciences). Post-sort purity was routinely >99%. For adoptive transfer, total $CD4^+$ or sorted T cells were washed and resuspended in sterile PBS. Recipient mice received $\leq 2.5 \times 10^6$ cells *i.v.* in 200μl of sterile PBS.

15 **Example 4 - Flow Cytometry**

[0157] Cell suspensions were stained with fluorochrome-conjugated antibodies for CD4 (RM4-5), CD73 (TY/23), or FoxP3 (FJK-16s). Intracellular FoxP3 staining was carried out according to the manufacturer's instructions (eBioscience). Stained cells were acquired on a FACSCalibur (BD Biosciences). Data were analyzed with
20 FlowJo software (Tree Star).

Example 5 – T-cell Cytokine Assay

[0158] Sorted T cells from MOG-immunized mice were cultured in the presence of irradiated C57BL/6 splenocytes with 0 or 10μM MOG peptide.
25 Supernatants were collected at 18 hrs and analyzed utilizing the Bio-plex cytokine (Biorad) assay for IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IL-1β, and TNFα.

Example 6 - Immunohistochemistry ("IHC")

[0159] Anesthetized mice were perfused with PBS, and brains, spleens, and
30 spinal cords were isolated and snap frozen in Tissue Tek-OCT medium. Five μm

sections (brains in a sagittal orientation) were affixed to Snpefrost/Plns slides (Fisher), fixed in acetone, and stored at -80°C. For immunostaining, slides were thawed and treated with 0.03% H_2O_2 in PBS to block endogenous peroxidase, blocked with Casein (Vector) in normal goat serum (Zymed), and then incubated with anti-CD45 (YW62.3),
5 anti-CD4 (RM4-5), or anti-ICAM-1 (3E2). Slides were incubated with biotinylated goat anti-rat Ig (Jackson ImmunoResearch) and streptavidin-HRP (Zymed) and developed with an AEC (Red) substrate kit (Zymed) and a hematoxylin counterstain. Cover slips were mounted with Flnoromonnt-G and photographed under light (Zeiss).

10 **Example 7 - Real Time q-PCR**

[0160] Using Trizol (Invitrogen), RNA was isolated from the Z310 choroid plexus cell line (Zheng et al., "Establishment and Characterization of an Immortalized Z310 Choroidal Epithelial Cell Line from Murine Choroid Plexus," *Brain Res.* 958:371-380 (2002), which is hereby incorporated by reference in its entirety). cDNA
15 was synthesized using a Reverse-iT kit (ABGene). Primers (available upon request) specific for ARs were used to determine gene expression levels and standardized to the GAPDH housekeeping gene levels using a SYBR-Green kit (ABGene) run on an ABI 7500 real time PCR system. Melt curve analyses were performed to measure the specificity for each qPCR product.

20

Example 8 - Evaluation of the Role of CD73 in EAE

[0161] Due to the immunomodulatory and immunosuppressive properties of adenosine, the role of CD73 in EAE was evaluated. Based on a report of exacerbated EAE in A1 adenosine receptor (AR)-deficient mice (Tsutsui et al., "A1 Adenosine
25 Receptor Upregulation and Activation Attenuates Neuroinflammation and Demyelination in a Model of Multiple Sclerosis," *J. Neurosci.* 24:1521-1529 (2004), which is hereby incorporated by reference in its entirety), *cd73*$^{-/-}$ mice that are unable to catalyze the production of extracellular adenosine were expected to experience severe EAE. Surprisingly, *cd73*$^{-/-}$ mice were highly resistant to the induction of EAE.
30 However, CD4^{+} T cells from *cd73*$^{-/-}$ mice do possess the capacity to generate an immune response against CNS antigens and cause severe EAE when adoptively

transferred into *cd73*$^{+/+}$ T cell-deficient mice. CD73^{+}CD4^{+} T cells from wild type mice also caused disease when transferred into *cd73*$^{-/-}$ recipients, indicating that CD73 expression, either on lymphocytes or in the CNS, is required for lymphocyte entry into the brain during EAE. Since *cd73*$^{+/+}$ mice were protected from EAE induction by the
5 broad spectrum AR antagonist caffeine and the A2A AR specific antagonist SCH58261, this data indicates that the extracellular adenosine generated by CD73, and not CD73 itself, regulates the entry of lymphocytes into the CNS during EAE. These results are the first to demonstrate a role for CD73 and adenosine in regulating the development of EAE.
10

Example 9 - *Cd73*$^{-/-}$ Mice are Resistant to EAE Induction

[0162] To determine if CD73 plays a role in controlling inflammation during EAE progression, *cd73*$^{-/-}$ and wild type (*cd73*$^{+/+}$) mice were subjected to the myelin oligodendrocyte glycoprotein ("MOG") EAE-inducing regimen (Swanborg,
15 "Experimental Autoimmune Encephalomyelitis in Rodents as a Model for Human Demyelinating Disease," *Clin. Immunol. Immunopathol.* 77:4-13 (1995); Bynoe et al., "Epicutaneous Immunization with Autoantigenic Peptides Induces T Suppressor Cells that Prevent Experimental Allergic Encephalomyelitis," *Immunity* 19:317-328 (2003), which are hereby incorporated by reference in their entirety). Daily monitoring for
20 EAE development revealed that *cd73*$^{-/-}$ mice consistently displayed dramatically reduced disease severity compared to their wild type counterparts (Figure 1). By three weeks after disease induction, *cd73*$^{-/-}$ mice had an average EAE score of only 0.5 (weak tail) compared to 2.0 (limp tail and partial hind limb paralysis) for wild type mice (Figure 1).
25

Example 10 - CD4^{+} T Cells From *cd73*$^{-/-}$ Mice Respond to MOG Immunization

[0163] It was then asked whether the resistance of *cd73*$^{-/-}$ mice to EAE induction could be explained by either an enhanced ability of *cd73*$^{-/-}$ lymphocytes to suppress an immune response or an inability of these lymphocytes to respond to MOG

stimulation. Naturally occurring $CD4^+CD25^+FoxP3^+$ T cells, or Tregs, can regulate actively-induced EAE (Kohm et al., "Cutting Edge: CD4+CD25+ Regulatory T Cells Suppress Antigeu-Specific Autoreactive Immune Responses and Central Nervous System Inflammation During Active Experimental Autoimmune Encephalomyelitis," *J. Immunol.* 169:4712-4716 (2002), which is hereby incorporated by reference in its entirety). As Tregs have recently been shown to express CD73 and some reports suggest that the enzymatic activity of CD73 is needed for Treg function (Kobie et al., "T Regulatory and Primed Uncommitted CD4 T Cells Express CD73, Which Suppresses Effector CD4 T Cells by Converting 5'-Adenosine Monophosphate to Adenosine," *J. Immunol.* 177:6780-6786); Deaglio et al., "Adenosine Generation Catalyzed by CD39 and CD73 Expressed on Regulatory T Cells Mediates Immune Suppression," *J. Exp. Med.* 204:1257-1265 (2007), which are hereby incorporated by reference in their entirety), it was asked whether the number and suppressive activity of Tregs were normal in $cd73^{-/-}$ mice. As shown in Figure 2A, there were no significant differences in the frequencies of $CD4^+FoxP3^+$ Tregs in wild type and $cd73^{-/-}$ mice, either before or after EAE induction. Similarly, the percentage of $CD4^+$ T cells that expressed CD73 changed only modestly after EAE induction in wild type mice (Figure 2B). Additionally, no significant difference was observed in the suppressive capacity of wild type and $cd73^{-/-}$ Tregs to inhibit MOG antigen-specific $CD4^+$ effector T cell proliferation. To determine whether $cd73^{-/-}$ T cells can respond when stimulated with MOG peptide, the capacity of these cells to proliferate and produce cytokines was assessed. $CD4^+$ T cells from MOG-immunized $cd73^{-/-}$ and wild type mice displayed similar degrees of *in vitro* proliferation in response to varying concentrations of MOG peptide. However, $CD4^+$ T cells from MOG-immunized $cd73^{-/-}$ mice secreted higher levels of IL-17 and IL-1β following *in vitro* MOG stimulation, compared to those of wild type $CD73^+CD4^+$ or $CD73^-CD4^+$ T cells (Figure 2C). Elevated levels of IL-17 are associated with MS (Matusevicius et al., "Interleukin-17 mRNA Expression in Blood and CSF Mononuclear Cells is Augmented in Multiple Sclerosis," *Mult. Scler.* 5:101-104 (1999), which is hereby incorporated by reference in its entirety) and EAE development (Komiyama et al., "IL-17 Plays an Important Role in the Development of

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Experimental Autoimmune Encephalomyelitis," *J. Immunol.* 177:566-573 (2006), which is hereby incorporated by reference in its entirety), while high levels of the proinflammatory IL-1β cytokine are a risk factor for MS (de Jong et al., "Production of IL-1beta and IL-1Ra as Risk Factors for Susceptibility and Progression of Relapse-
5 Onset Multiple Sclerosis," *J. Neuroimmunol.* 126:172-179 (2002), which is hereby incorporated by reference in its entirety) and an enhancer of IL-17 production (Sutton et al., "A Crucial Role for Interleukin (IL)-1 in the Induction of IL-17-Producing T Cells That Mediate Autoimmune Encephalomyelitis," *J. Exp. Med.* 203:1685-1691 (2006), which is hereby incorporated by reference in its entirety). No difference in IL-
10 2, IL-4, IL-5, IL-10, IL-13, INF-γ and TNF-α secretion was observed between wild type and $cd73^{-/-}$ T cells following MOG stimulation (Figure 2C). Overall, the results from these assays indicate that $cd73^{-/-}$ T cells can respond well to MOG immunization.

[0164] It was then determined whether T cells from $cd73^{-/-}$ mice possess the ability to cause EAE. To test this, $CD4^+$ T cells from the spleen and lymph nodes of
15 MOG immunized $cd73^{-/-}$ mice were evaluated for their ability to induce EAE after transfer into $tcr\alpha^{-/-}$ ($cd73^{+/+}$) recipient mice. $Tcr\alpha^{-/-}$ mice lack endogenous T cells and cannot develop EAE on their own (Elliott et al., "Mice Lacking Alpha Beta + T Cells are Resistant to the Induction of Experimental Autoimmune Encephalomyelitis," *J. Neuroimmunol.* 70:139-144 (1996), which is hereby incorporated by reference in its
20 entirety). $Cd73^{+/+}tcr\alpha^{-/-}$ recipient mice that received $CD4^+$ T cells from $cd73^{-/-}$ donors developed markedly more severe disease compared to those that received wild type $CD4^+$ T cells (Figure 2D). Wild type and $cd73^{-/-}$ donor $CD4^+$ T cells displayed equal degrees of expansion following transfer into $cd73^{+/+}tcr\alpha^{-/-}$ recipient mice. Thus, $CD4^+$ T cells from $cd73^{-/-}$ mice are not only capable of inducing EAE, but cause
25 more severe EAE than those derived from wild type mice when transferred into $cd73^{+/+}tcr\alpha^{-/-}$ mice. These results are consistent with *in vitro* assays in which $cd73^{-/-}$ $CD4^+$ T cells secreted elevated levels of IL-17 and IL-1β (which are known to exacerbate EAE) in response to MOG stimulation (Figure 2C) and indicate that $cd73^{-/-}$

mice are resistant to MOG-induced EAE because of a lack of CD73 expression in non-hematopoietic cells (most likely lack of expression in the CNS).

Example 11 - *Cd73*$^{-/-}$ Mice Exhibit Little/No Lymphocyte Infiltration into the CNS Following EAE Induction

[0165] EAE is primarily a CD4^{+} T cell mediated disease (Montero et al., "Regulation of Experimental Autoimmune Encephalomyelitis by CD4+, CD25+ and CD8+ T Cells: Analysis Using Depleting Antibodies," *J. Autoimmun.* 23:1-7 (2004), which is hereby incorporated by reference in its entirety) and during EAE progression, lymphocytes must first gain access into the CNS in order to mount their inflammatory response against CNS antigens, resulting in axonal demyelination and paralysis (Brown et al., "Time Course and Distribution of Inflammatory and Neurodegenerative Events Suggest Structural Bases for the Pathogenesis of Experimental Autoimmune Encephalomyelitis," *J. Comp. Neurol.* 502:236-260 (2007), which is hereby incorporated by reference in its entirety). To determine if CNS lymphocyte infiltration is observed following EAE induction in *cd73*$^{-/-}$ mice, brain and spinal cord sections were examined for the presence of CD4^{+} T cells and CD45^{+} cells by immunohistochemistry. *Cd73*$^{-/-}$ mice displayed a dramatically lower frequency of CD4^{+} (Figures 3D-G) and CD45^{+} (Figure 4 [Suppl. Figure 1]) lymphocytes in the brain and spinal cord compared to wild type mice (Figures 3A-C, G) at day 13 post MOG immunization. Additionally, in lymphocyte tracking experiments where MOG-specific T cells from 2d2 TCR transgenic mice (Bettelli et al., "Myelin Oligodendrocyte Glycoprotein-Specific T Cell Receptor Transgenic Mice Develop Spontaneous Autoimmune Optic Neuritis," *J. Exp. Med.* 197:1073-1081 (2003), which is hereby incorporated by reference in its entirety) were transferred into either wild type or *cd73*$^{-/-}$ mice with concomitant EAE induction, the percentage of 2d2 cells in the CNS increased several fold with time in wild type recipient mice, but not at all in *cd73*$^{-/-}$ recipients (Figure 5). Overall, these results indicate that the observed protection against EAE induction in *cd73*$^{-/-}$ mice is associated with considerably reduced CNS lymphocyte infiltration. Nevertheless, CD4^{+} T cells from MOG-immunized *cd73*$^{-/-}$

mice possessed the ability to gain access to the CNS when transferred into *cd73*$^{+/+}$*tcrα*$^{-/-}$ mice that were concomitantly induced to develop EAE (Figures 3K and 3L). In fact, earlier and more extensive CNS CD4^{+} lymphocyte infiltration was observed in *cd73*$^{+/+}$*tcrα*$^{-/-}$ mice that received *cd73*$^{-/-}$ CD4^{+} T cells (Figures 3K,L) than
5 in those that received wild type CD4^{+} T cells (Figures 3H-J). Therefore, these results demonstrate that donor T cells from *cd73*$^{-/-}$ mice have the ability to infiltrate the CNS of *cd73*$^{+/+}$ recipient mice.

Example 12 - CD73 Must be Expressed Either on Lymphocytes or in the CNS for
10 **Efficient EAE Development**

[0166] It was next asked whether CD73 expression on CD4^{+} T cells can compensate for a lack of CD73 expression in the CNS and allow the development of EAE. Therefore, CD4^{+} T cells were adoptively transferred from MOG-immunized wild type mice into *cd73*$^{-/-}$ recipients, concomitantly induced EAE, and compared
15 disease activity with that of similarly treated wild type recipients (Figure 6A). While wild type recipients developed disease following EAE induction as expected, *cd73*$^{-/-}$ recipients also developed prominent EAE with an average disease score of 1.5 by three weeks after disease induction. This was much higher than the 0.5 average score that *cd73*$^{-/-}$ mice normally showed at this same time point (Figure 1). To further define the
20 association of CD4^{+} T cell CD73 expression with EAE susceptibility, sorted CD73^{+}CD4^{+} and CD73^{-}CD4^{+} T cells from immunized wild type mice, or total CD4^{+} (CD73^{-}) T cells from immunized *cd73*$^{-/-}$ mice, were transferred into *cd73*$^{-/-}$ recipients with concomitant EAE induction (Figure 6B). *Cd73*$^{-/-}$ mice that received CD73^{+}CD4^{+} T cells from wild type mice developed EAE with an average score of approximately 1.5
25 at three weeks post induction. Conversely, *cd73*$^{-/-}$ mice that received wild type derived CD73^{-}CD4^{+} T cells did not develop significant EAE. Additionally, CD4^{+} cells from *cd73*$^{-/-}$ donor mice, which have the ability to cause severe EAE in CD73-expressing

tcrα$^{-/-}$ mice (Figure 2D), were also incapable of potentiating EAE in recipient *cd73*$^{-/-}$ mice (Figure 6B). Therefore, although CD73 expression on T cells can partially compensate for a lack of CD73 expression in non-hematopoietic cells, EAE is most efficiently induced when CD73 is expressed in both compartments.

5 **[0167]** The identity of the CD73-expressing non-hematopoietic cells that promote the development of EAE is not known. Vascular endothelial cells at the BBB were considered as likely candidates, as many types of endothelial cells express CD73 (Yamashita et al., "CD73 Expression and Fyn-Dependent Signaling on Murine Lymphocytes," *Eur. J. Immunol.* 28:2981-2990 (1998), which is hereby incorporated
10 by reference in its entirety). However, immunohistochemistry revealed that mouse brain endothelial cells are CD73^{-}. During these experiments, it was observed that CD73 is, however, highly expressed in the brain on the choroid plexus (Figure 6C), which is an entry point into the CNS for lymphocytes during EAE progression (Brown et al., "Time Course and Distribution of Inflammatory and Neurodegenerative Events
15 Suggest Structural Bases for the Pathogenesis of Experimental Autoimmune Encephalomyelitis," *J. Comp. Neurol.* 502:236-260 (2007), which is hereby incorporated by reference in its entirety). Figure 4D shows infiltrating lymphocytes in association with the choroid plexus of wild type mice 12 days post-EAE induction. Minimal CD73 staining was also observed on submeningeal regions of the spinal cord.
20 Taken together, these results indicate that CD73 expression, whether on T cells or in the CNS (perhaps on the choroid plexus), is necessary for efficient EAE development.

Example 13 - Adenosine Receptor Antagonists Protect Mice Against EAE Induction

25 **[0168]** As CD73 catalyzes the breakdown of AMP to adenosine and ARs are expressed in the CNS (Tsutsui et al., "A1 Adenosine Receptor Upregulation and Activation Attenuates Neuroinflammation and Demyelination in a Model of Multiple Sclerosis," *J. Neurosci.* 24:1521-1529 (2004)); Rosi et al., The Influence of Brain Inflammation Upon Neuronal Adenosine A2B Receptors," *J. Neurochem.* 86:220-227
30 (2003), which are hereby incorporated by reference in their entirety), it was next determined if AR signaling is important during EAE progression. Wild type and *cd73*$^{-/-}$ mice were treated with the broad spectrum AR antagonist caffeine (Dall'Igna et

al., "Caffeine as a Neuroprotective Adenosine Receptor Antagonist," *Ann. Pharmacother.* 38:717-718 (2004), which is hereby incorporated by reference in its entirety) at 0.6 g/L in their drinking water, which corresponds to an approximate dose of 4.0 mg/mouse of caffeine per day (Johansson et al., "A1 and A2A Adenosine
5 Receptors and A1 mRNA in Mouse Brain: Effect of Long-Term Caffeine Treatment," *Brain Res.* 762:153-164 (1997), which is hereby incorporated by reference in its entirety), 1 day prior to and throughout the duration of the EAE experiment (Figure 7A). Wild type mice that received caffeine were dramatically protected against EAE development (Figure 7A), comparable to previously published results (Tsutsui et al.,
10 "A1 Adenosine Receptor Upregulation and Activation Attenuates Neuroinflammation and Demyelination in a Model of Multiple Sclerosis," *J. Neurosci.* 24:1521-1529 (2004), which is hereby incorporated by reference in its entirety). As expected, *cd73*$^{-/-}$ mice that received caffeine did not develop EAE (Figure 7A). Since CD73 is highly expressed on the choroid plexus (Figure 6C), it was next determined if ARs are also
15 expressed on the choroid plexus. Utilizing the Z310 murine choroid plexus cell line (Zheng et al., "Establishment and Characterization of an Immortalized Z310 Choroidal Epithelial Cell Line from Murine Choroid Plexus," *Brain Res.* 958:371-380 (2002), which is hereby incorporated by reference in its entirety), only mRNA for the A1 and A2A adenosine receptor subtypes were detected by qPCR (Figure 7B). As *A1AR*$^{-/-}$
20 mice have been previously shown to develop severe EAE following disease induction (Tsutsui et al., "A1 Adenosine Receptor Upregulation and Activation Attenuates Neuroinflammation and Demyelination in a Model of Multiple Sclerosis," *J. Neurosci.* 24:1521-1529 (2004), which is hereby incorporated by reference in its entirety), it was asked if treatment of wild type mice with SCH58261 (Melani et al., "The Selective
25 A2A Receptor Antagonist SCH 58261 Protects From Neurological Deficit, Brain Damage and Activation of p38 MAPK in Rat Focal Cerebral Ischemia," *Brain Res.* 1073-1074:470-480 (2006), which is hereby incorporated by reference in its entirety), an AR antagonist specific for the A2A subtype, could protect against EAE development. Wild type mice were given 1mg/kg of SCH58261 in DMSO or DMSO
30 alone both *i.p.* and *s.c.* (for a total of 2mg/kg) 1 day prior to EAE induction and daily for 30 days throughout the course of the experiment (Figure 7C). Wild type mice that received SCH58261 were dramatically protected against EAE development compared

to wild type mice that received DMSO alone (Figure 7C). Additionally, wild type mice given SCH58261 displayed a significantly lower frequency of $CD4^+$ lymphocytes in the brain and spinal cord compared to DMSO treated wild type mice at day 15 post-EAE induction (Figure 7D). As studies have shown that adhesion molecules (such as
5 ICAM-1, VCAM-1, and MadCAM-1) on the choroid plexus play a role in the pathogenesis of EAE (Engelhardt et al., "Involvement of the Choroid Plexus in Central Nervous System Inflammation," *Microsc. Res. Tech.* 52:112-129 (2001), which is hereby incorporated by reference in its entirety), it was determined if SCH58261 treatment affected adhesion molecule expression on the choroid plexus following EAE
10 induction. Comparison of the choroid plexus from DMSO and SCH58261 treated wild type mice shows that A2A AR blockade prevented the up regulation of ICAM-1 that normally occurs during EAE progression (Figure 8).

[0169] Based on these results, it was concluded that the inability of *cd73*$^{-/-}$ mice to catalyze the generation of extracellular adenosine explains their failure to efficiently
15 develop EAE following MOG immunization and that CD73 expression and A2A AR signaling at the choroid plexus are requirements for EAE progression.

[0170] The goal of this study was to evaluate the role of CD73 in EAE, an animal model for MS. As CD73 catalyzes the formation of extracellular adenosine which is usually immunosuppressive (Bours et al., "Adenosine 5'-Triphosphate and
20 Adenosine as Endogenous Signaling Molecules in Immunity and Inflammation," *Pharmacol. Ther.* 112:358-404 (2006), which is hereby incorporated by reference in its entirety) and *A1AR*$^{-/-}$ mice exhibit severe EAE (Tsutsui et al., "A1 Adenosine Receptor Upregulation and Activation Attenuates Neuroinflammation and Demyelination in a Model of Multiple Sclerosis," *J. Neurosci.* 24:1521-1529 (2004), which is hereby
25 incorporated by reference in its entirety), applicants predicted that *cd73*$^{-/-}$ mice would also develop severe EAE. However, *cd73*$^{-/-}$ mice were highly resistant to EAE induction, a surprising finding considering the plethora of studies demonstrating that *cd73*$^{-/-}$ mice are more prone to inflammation. For example, *cd73*$^{-/-}$ mice are more susceptible to bleomycin-induced lung injury (Volmer et al., "Ecto-5'-Nucleotidase
30 (CD73)-Mediated Adenosine Production is Tissue Protective in a Model of Bleomycin-Induced Lung Injury," *J. Immunol.* 176:4449-4458 (2006), which is hereby

incorporated by reference in its entirety) and are more prone to vascular inflammation and neointima formation (Zernecke et al., "CD73/ecto-5'-Nucleotidase Protects Against Vascular Inflammation and Neointima Formation," *Circulation* 113:2120-2127 (2006), which is hereby incorporated by reference in its entirety). Consistent with these reports, applicants showed that *cd73*$^{-/-}$ T cells produced higher levels of the EAE-associated proinflammatory cytokines IL-1β and IL-17 following MOG stimulation. Furthermore, the adoptive transfer of *cd73*$^{-/-}$ T cells to *tcrα*$^{-/-}$ mice, which lack T cells but express CD73 throughout their periphery, resulted in severe CNS inflammation following MOG immunization, consistent with a role for adenosine as an anti-inflammatory mediator. It is interesting to note that IFN-β treatment, one of the most effective therapies for MS, has been shown to up regulate CD73 expression on endothelial cells both *in vitro* and *in vivo* (Airas et al., "Mechanism of Action of IFN-Beta in the Treatment of Multiple Sclerosis: A Special Reference to CD73 and Adenosine," *Ann. N. Y. Acad. Sci.* 1110:641-648 (2007), which is hereby incorporated by reference in its entirety). Thus, although the mechanism by which IFN-β benefits MS patients is incompletely understood, increased production of adenosine accompanied by its known anti-inflammatory and neuroprotective effects could be a factor.

[0171] Consistent with their resistance to EAE induction, *cd73*$^{-/-}$ mice had a lower frequency of cells infiltrating the CNS during EAE compared to wild type mice. This was also an unexpected finding, as CD73-generated adenosine has previously been shown to restrict the migration of neutrophils across vascular endothelium during hypoxia and of lymphocytes across high endothelial venules of draining lymph nodes (Thompson et al., "Crucial Role for Ecto-5'-Nucleotidase (CD73) in Vascular Leakage During Hypoxia," *J. Exp. Med.* 200:1395-1405 (2004), which is hereby incorporated by reference in its entirety). Applicants' data, in contrast, indicates that CD73, and the extracellular adenosine generated by CD73, are needed for the efficient passage of pathogenic T cells into the CNS. Therefore, the role that CD73 and adenosine play in CNS lymphocyte infiltration during EAE is more profound than their role in modulation of neuroinflammation.

[0172] It is important to know where CD73 must be expressed for T cell migration into the CNS. CD73 is found on subsets of T cells (Yamashita et al., "CD73

Expression and Fyn-Dependent Signaling on Murine Lymphocytes," *Eur. J. Immunol.* 28:2981-2990 (1998), which is hereby incorporated by reference in its entirety) as well as on some epithelial (Strohmeier et al., "Surface Expression, Polarization, and Functional Significance of CD73 in Human Intestinal Epithelia," *J. Clin. Invest.*
5 99:2588-2601 (1997), which is hereby incorporated by reference in its entirety) and endothelial cells (Yamashita et al., "CD73 Expression and Fyn-Dependent Signaling on Murine Lymphocytes," *Eur. J. Immunol.* 28:2981-2990 (1998), which is hereby incorporated by reference in its entirety). The data presented here clearly demonstrates that although $cd73^{-/-}$ T cells respond well to MOG immunization, they cannot enter the
10 CNS unless CD73 is expressed in non-hematopoietic tissues (i.e. $cd73^{+/+}tcr\alpha^{-/-}$ mice which develop EAE after adoptive transfer of $CD4^{+}$ T cells from $cd73^{-/-}$ mice). A lack of CD73 on non-hematopoietic cells can also be compensated for, in part, by CD73 expression on T cells (i.e., $cd73^{-/-}$ mice become susceptible to EAE when $CD73^{+}$, but not $CD73^{-}$, $CD4^{+}$ T cells are adoptively transferred). While BBB endothelial cells as a
15 relevant source of CD73 in the CNS were considered, because CD73 is expressed on human brain endothelial cells (Airas et al., "Mechanism of Action of IFN-Beta in the Treatment of Multiple Sclerosis: A Special Reference to CD73 and Adenosine," *Ann. N. Y. Acad. Sci.* 1110:641-648 (2007), which is hereby incorporated by reference in its entirety), immunohistochemistry revealed that mouse brain endothelial cells are $CD73^{-}$.
20 However, CD73 was found to be highly expressed on choroid plexus epithelial cells, which form the barrier between the blood and the cerebrospinal fluid (CSF) and have a role in regulating lymphocyte immunosurveillance in the CNS (Steffen et al., "CAM-1, VCAM-1, and MAdCAM-1 are Expressed on Choroid Plexus Epithelium but Not Endothelium and Mediate Binding of Lymphocytes In Vitro," *Am. J. Pathol.* 148:1819-
25 1838 (1996), which is hereby incorporated by reference in its entirety). The choroid plexus has also been suggested to be the entry point for T cells during the initiation of EAE progression (Brown et al., "Time Course and Distribution of Inflammatory and Neurodegenerative Events Suggest Structural Bases for the Pathogenesis of Experimental Autoimmune Encephalomyelitis," *J. Comp. Neurol.* 502:236-260 (2007),
30 which is hereby incorporated by reference in its entirety). While the role of lymphocyte-brain endothelial cell interactions via VLA-4/VCAM-1 binding in both

EAE and MS is well-documented (Rice et al., "Anti-Alpha4 Integrin Therapy for Multiple Sclerosis: Mechanisms and Rationale," *Neurology* 64:1336-1342 (2005), which is hereby incorporated by reference in its entirety), perhaps lymphocyte trafficking across the endothelial BBB is more important for disease maintenance and
5 progression than for disease initiation, at least in EAE.

[0173] The next issue is how CD73 facilitates the migration of T cells into the CNS. Earlier work showed that lymphocyte CD73 can promote the binding of human lymphocytes to endothelial cells in an LFA-1-dependent fashion (Airas et al., "CD73 Engagement Promotes Lymphocyte Binding to Endothelial Cells Via a Lymphocyte
10 Function-Associated Antigen-1-dependent Mechanism," *J. Immunol.* 165:5411-5417 (2000), which is hereby incorporated by reference in its entirety). This does not appear to be the function of CD73 in EAE, however, becuase CD73-deficient T cells can enter the CNS and cause severe disease in $cd73^{+/+}tcr\alpha^{-/-}$ mice (Figure 2D). Alternatively, CD73 can function as an enzyme to produce extracellular adenosine, a ligand for cell
15 surface ARs. It is this latter function that is relevant for the current work given that AR blockade with caffeine or SCH58261 can protect mice from EAE. While the broad spectrum AR antagonist caffeine also inhibits certain phosphodiesterases (Choi et al., "Caffeine and Theophylline Analogues: Correlation of Behavioral Effects With Activity as Adenosine Receptor Antagonists and as Phosphodiesterase Inhibitors," *Life*
20 *Sci.* 43:387-398 (1988), which is hereby incorporated by reference in its entirety), its modulation of EAE progression is most likely through its effect on AR signaling (Tsutsui et al., "A1 Adenosine Receptor Upregulation and Activation Attenuates Neuroinflammation and Demyelination in a Model of Multiple Sclerosis," *J. Neurosci.* 24:1521-1529 (2004), which is hereby incorporated by reference in its entirety). This
25 notion is supported by the fact that SCH58261 also prevents EAE progression by specifically inhibiting A2A AR signaling. As CD73 and the A1 and A2A AR subtypes are expressed on the choroid plexus, extracellular adenosine produced by CD73 at the choroid plexus can signal in an autocrine fashion.

[0174] Adenosine signaling most likely regulates the expression of adhesion
30 molecules at the choroid plexus. Studies have shown that the up regulation of the adhesion molecules ICAM-1, VCAM-1, and MadCAM-1 at the choroid plexus are associated with EAE progression (Engelhardt et al., Involvement of the Choroid Plexus

in Central Nervous System Inflammation," *Microsc. Res. Tech.* 52:112-129 (2001), which is hereby incorporated by reference in its entirety). As mice treated with the A2A AR antagonist SCH58261 do not experience increased choroid plexus ICAM-1 expression (Figure 8), as normally occurs following EAE induction (Engelhardt et al.,
5 "Involvement of the Choroid Plexus in Central Nervous System Inflammation," *Microsc. Res. Tech.* 52:112-129 (2001), which is hereby incorporated by reference in its entirety), the present results indicate that A2A AR signaling increases ICAM-1 during EAE progression.

[0175] In snmmary, this data shows that CD73 plays a critical role in the
10 progression of EAE. Mice that lack CD73 are protected from the degenerative symptoms and CNS inflammation that are associated with EAE induction. This is the first study to demonstrate a requirement for CD73 expression and AR signaling for the efficient entry of lymphocytes into the CNS during EAE. The data presented here may mark the first steps of a journey that will lead to new therapies for MS and other
15 neuroinflammatory diseases.

Example 14 - The BBB Can be Modulated Through Activation of the Adenosine Receptors

[0176] The objective of this experiment was to determine if the blood brain
20 barrier could be modulated by activation of adenosine receptors. NECA is a non-selective adenosine receptor agonist, with similar affinities for A1, A2A and A3 adenosine receptors and a low affinity for the A2b adenosine receptor. In order to determine if activation of adenosine receptors would induce extravasation of Evans Blue dye across the blood brain barrier (BBB), mice were treated with: NECA, a non-
25 selective adenosine receptor agonist (n=5, 100μl 0.01nM); SCH58261, an A2A adenosine receptor specific antagonist (n=5, 1mg/kg); pertussis toxin, an agent known to induce BBB leakiness and as such used as a positive control (n=7, 200μl); and, PBS as a vehicle control (n=5, 100μl). CD73$^{-/-}$ mice, which lack the ability to produce extracellular adenosine, were also treated with NECA (n=4, 100μl 0.01nM).
30 Treatments were administered as a single i.v. injection one hour prior to i.v. injection of 200μl 1% Evans Blne dye (2μg total dye injected). Four hours after administration of Evans Blue, mice were anesthetized with a ketamine/xylazine mix and perfused via the

left ventricle with ice cold PBS. Brains were harvested and homogenized in n,n-dimethylformamide (DMF) at 5μl/mg (v:w). Tissue was incubated for 72 hours at room temperature in DMF to extract the dye. Following extraction, the tissue / solvent mixture was centrifuged at 500xg for 30 minutes and 100μl of supernatant was read on
5 a BioTex spectrophotometer at 620nm. Data is expressed as μg Evans Blue / ml DMF.

[0177] Treating mice with the general adenosine receptor agonist NECA can induce migration of dye across the blood brain barrier. This indicates that this barrier can be modulated through activation of the adenosine receptors. In Figure 9A, CD73$^{-/-}$ mice, which lack extracellular adenosine and thus cannot adequately signal through
10 adenosine receptors, were treated with NECA, resulting in an almost five fold increase in dye migration vs. the PBS control. SCH58261 was used as a negative control since applicants have shown that blocking of the A2A adenosine receptor using this antagonist can prevent lymphocyte entry into the brain (Mills et al., "CD73 is Required for Efficient Entry of Lymphocytes into the Central Nervous System During
15 Experimental Autoimmune Encephalomyelitis," *Proc. Natl. Acad. Sci.* 105(27):9325–9330 (2008), which is hereby incorporated by reference in its entirety). In Figure 9B, WT mice treated with NECA also show an increase over control mice. Pertussis is used as a positive control, as it is known to induce blood brain barrier leakiness in the mouse EAE model.

20

Example 15 - The A2A and A2b Adenosine Receptors are Expressed on the Human Endothelial Cell Line hCMEC/D3

[0178] In order to establish an *in vitro* blood brain barrier (BBB), the human brain endothelial cell line hCMEC/D3 (Weksler et al., "Blood-brain Barrier-specific
25 Properties of a Human Adult Brain Endothelial Cell Line," *J. Neurochem.* 19(13):1872-4 (2005); Poller et al., "The Human Brain Endothelial Cell Line hCMEC/D3 as a Human Blood-brain Barrier Model for Drug Transport Studies," *J. Neurochem.* 107(5):1358-1368 (2008), which are hereby incorporated by reference in their entirety) was obtained, which has been previously described as having BBB properties. Here,
30 expression pattern of adenosine receptors on these cells was established.

[0179] hCMEC/D3 cells were grown to confluence, harvested and RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the

manufacturer's instructions. cDNA was synthesized using a Verso cDNA kit (Thermo Scientific, Waltham, MA), and Real Time PCR was performed using Power SYBR Green (Applied Biosystems, Foster City, CA).

[0180] As shown in Figure 10, the A2A and A2b adenosine receptors were found to be expressed on the human endothelial cell line hCMEC/D3.

Example 16 - Adenosine Receptor Stimulation of Brain Endothelial Cells Promotes Lymphocyte Migration Through the BBB

[0181] The blood brain barrier ("BBB") is comprised of endothelial cells. During late stages of EAE, lymphocytes are known to cross the BBB. In order to determine if adenosine receptor stimulation of brain endothelial cells could promote lymphocyte migration through the BBB, an *in vitro* BBB was established. The human brain endothelial cell line hCMEC/D3 (Weksler et al., "Blood-brain Barrier-specific Properties of a Human Adult Brain Endothelial Cell Line," *J. Neurochem.* 19(13):1872-4 (2005); Poller et al., "The Human Brain Endothelial Cell Line hCMEC/D3 as a Human Blood-brain Barrier Model for Drug Transport Studies," *J. Neurochem.* 107(5):1358-1368 (2008), which are hereby incorporated by reference in their entirety) was obtained, which has been previously described as having BBB properties.

[0182] hCMEC/D3 cells were seeded onto Transwell and allowed to grow to confluencey. $2x10^6$ Jurkat cells were added to the upper chamber with or without NECA (general adenosine receptor [AR] agonist), CCPA (A1 AR agonist), CGS 21860 (A2A AR agonist), or DMSO vehicle. After 24 hours, migrated cells in the lower chamber were counted. Values are relative to the number of cells that migrate through non-HCMECD3 seeded transwells.

[0183] As shown in Figure 11, NECA, a broad spectrum adenosine receptor agonist, induced some migration. CGS, the A2A adenosine receptor agonist, promoted lymphocyte migration across the *in vitro* BBB when used at a lower concentration. CCPA, the A1 agonist, induced lymphocyte migration at high levels possibly due to activation of the A2A adenosine receptor, which has a lower affinity for CCPA and thus is only activated at higher levels of CCPA.

Example 17- A2A Adenosine Receptor Activation Promotes Lymphocyte Migration Across the CP

[0184] The choroid plexus ("CP") controls lymphocyte migration into the CNS. The CP expresses the A1 and A2A adenosine receptors. EAE is prevented in mice
5 when A2A adenosine receptor activity is blocked. EAE is enhanced when the A1 adenosine receptor is missing. It was hypothesized that A2A adenosine receptor activation promotes lymphocyte migration across the CP. Z310 cells are a murine choroid plexus cell line.

[0185] To test the hypothesis, Transwell membranes were seeded with Z310
10 cells and allowed to grow to confluencey. $2x10^6$ Jurkat cells were added to the upper chamber with or with out NECA (n=1, general AR agonist), CCPA (n=1, A1 AR agonist), CGS 21860 (n=1, A2A AR agonist), or DMSO vehicle(n=1). After 24 hours, migrated cells in the lower chamber were counted. Values are relative to the number of cells that migrate through non-Z310 seeded transwells and the results are shown in
15 Figure 12.

[0186] As shown in Figure 12, NECA, a broad spectrum adenosine receptor agonist, induced migration. CGS, the A2A adenosine receptor agonist, promoted lymphocyte migration across the CP. CCPA, the A1 agonist, induced lymphocyte migration at high levels possibly due to activation of the A2A adenosine receptor,
20 which has a lower affinity for CCPA and as such is only activated at high levels of CCPA.

Example 18 - Human Brain Endothelial Cells are Sensitive to Adenosine Receptor Induced cAMP Regulation

25 **[0187]** Adenosine receptor activation regulates cAMP levels in cells. In order to determine the sensitivity of human brain endothelial cells to adenosine receptor induced cAMP regulation, human brain endothelial cells were cultured with adenosine receptor agonists at various concentrations, followed by cAMP level analysis, as shown in Figure 13.

30 **[0188]** HCMECD3 cells were grown to confluencey on 24 well plates. As adenosine receptor ("AR") stimulation is known to influence cAMP levels, cells were treated with or without various concentrations of NECA (general AR agonist), CCPA (A1 AR agonist), CGS 21860 (A2A AR agonist), DMSO vehicle, or Forksolin (induces

cAMP). After 15 minutes, lysis buffer was added and the cells were frozen at -80 C to stop the reaction. Duplicate samples were used for each condition. cAMP levels were assayed using a cAMP Screen kit (Applied Biosystems, Foster City, CA).

[0189] As shown in Figure 13, the broad spectrum adenosine receptor agonist NECA increased cAMP levels, verifying that these cells can respond to adenosine receptor signaling. High levels of CCPA, the A1 adenosine receptor agonist, increased cAMP levels, again perhaps due to activation of the A2A adenosine receptor, which has a lower affinity for CCPA and as such is only activated at high levels of CCPA. CGS, the A2A adenosine receptor agonist slightly increased cAMP levels in the human brain endothelial cell line.

Example 19 - Female A1 Adenosine Receptor Knockout Mice Develop More Severe EAE Than Wild Type

[0190] A1 and A2A adenosine receptors are expressed on the choroid plexus. A2A adenosine receptor antagonists protect mice from EAE. Are mice that lack the A1 adenosine receptor prone to development of more severe EAE than wild type controls? To answer this question, disease profiles of wild type and A1 adenosine receptor null mice were compared.

[0191] Female A1 adenosine receptor knockout (A1ARKO, n=5) and wild type (WT, n=5) mice were immunized with CFA/MOG_{35-55} + PTX on 12-2-08 and scored daily for 41 days. As the results in Figure 14 illustrate, A1ARKO mice develop more severe EAE than WT, and also develop disease at a faster rate than WT.

Example 20 - Brains From Wild Type Mice Fed an Adenosine Receptor Antagonist Have Higher Levels of FITC-Dextran Than Brains from $CD73^{-/-}$ Mice Fed an Adenosine Receptor Antagonist

[0192] In order to examine the effects of caffeine, a general adenosine receptor antagonist, on blood brain barrier permeability, mice were fed caffeine for several days and then injected with FITC Dextran, commonly used to assess endothelial permeability.

[0193] More particularly, mice were fed 0.6g/l caffeine (Sigma, St. Louis, MO) in water or regular water ad lib for five days. Mice were injected IP with FITC Dextran (10,000 MW, Molecular Probes, Eugene, OR) and after 30 minutes mice were perfused

with ice cold PBS via the left ventricle. Brains were removed and snap frozen in OCT (Tissue Tek, Torrance, CA) and stored at -80°C until sectioning. Tissue sections (5μm) were stained with hematoxylin for light microscopy and with DAPI for a fluorescent counterstain. The results are shown in Figure 15.

5 **[0194]** As shown in Figure 15A, visualization of brain sections from CD73$^{-/-}$ mice fed caffeine displayed a much less intense green color than wild type mice, indicating less FITC-Dextran extravasation across the blood brain barrier. Brain sections from wild type mice displayed an intensely green background (Figure 15B) that is indicative of more FITC-dextran extravasation across the blood brain barrier.
10 Figure 16 shows the results for wild-type mice in graphical form.

Example 21- Adenosine Receptor Agonist NECA Increases Evans Blue Dye Extravasation Across the Blood Brain Barrier

[0195] The objective of this experiment was to determine if the blood brain
15 barrier could be modulated by activation of adenosine receptors. NECA is a non-selective adenosine receptor agonist, with similar affinities for A1, A2A and A3 adenosine receptors and a low affinity for the A2B adenosine receptor.

[0196] In order to determine if activation of adenosine receptors would induce extravazation of Evans Blue dye across the blood brain barrier (BBB), mice were first
20 treated on day one with NECA, a non-selective adenosine receptor agonist (n=2, 100μl 0.01nM); and, PBS as a vehicle control (n=2, 100μl). On day 2 mice were then immunized with CFA-MOG_{35-55} and pertussis to induce EAE. Then NECA or PBS was administered every other day on day 3, day 5, day 7 and day 9. On day 10, mice were injected intravenously with 200μl 1% Evans Blue dye (2μg total dye injected).
25 Six hours after administration of Evans Blue, mice were anesthetized with a ketamine/xylazine mix and perfused via the left ventricle with ice cold PBS. Brains were harvested and homogenized in n,n-dimethylformamide (DMF) at 5μl/mg (v:w). Tissue was incubated for 72 hours at room temperature in DMF to extract the dye. Following extraction, the tissue / solvent mixture was centrifuged at 500xg for 30
30 minutes and 100μl of supernatant was read on a BioTex spectrophotometer at 620nm. Data is expressed as pg Evans Blue / ml DMF and is shown in Figure 17.

[0197] This experiment demonstrates that treatment of mice with the general adenosine receptor agonist NECA induces migration of Evans Blue dye into the CNS in mice immunized for EAE. This indicates that the blood brain barrier in the EAE model can be modulated through activation of the adenosine receptors. WT EAE mice treated
5 with NECA show an increase in BBB permeability over PBS control EAE mice.

[0198] Figure 18 shows the results in graphical form of an addition experiment that demonstrate PEGylated adenosine deaminase ("PEG-ADA") treatment inhibits the development of EAE in wild-type mice. EAE was induced, disease activity was monitored daily, and mean EAE score was calculated in wild-type mice given either
10 control PBS vehicle alone or 15 units/kg body weight of PEG-ADA i.p. every 4 days. Closed squares represent wild-type mice given PBS vehicle (n=3); open squares respresent wild-type mice given PEG-ADA (n=3). These results demonstrate that adenosine deaminase treatment and adenosine receptor blockade protect wild type mice against EAE induction.

15

Example 22 - Mouse and Rat Models

[0199] C57BL/6 mice from Jackson Laboratories were used as wild types. All mice used were aged 7-9 weeks and weighed between 20-25 g. All rats were female and aged 8 weeks and weighed 200-220 g. Mice and rats were bred and housed under
20 specific pathogen-free conditions. All procedures were carried our in accordance with approved IACUC protocols.

Example 23 - Administration of Drugs and Dextrans

[0200] The adenosine receptor agonists NECA, CCPA, CGS 21860, and SCH
25 58261 (Tocris, Ellisville, MO) were each dissolved in DMSO then diluted in PBS to the desired concentration; in most cases final DMSO concentrations were < 0.5% (vol/vol). Lexiscan (Regadenoson; TRC, Inc., Toronto) was dissolved in PBS. For vehicle controls, DMSO was diluted in PBS to the same concentratiou. Dehydrated dextrans labeled with either FITC or Texas Red (Invitrogen, Carlsbad, CA) were re-suspended in
30 PBS to 10 mg/ml. All experiments involving dextran injection used 1.0 mg dextran in PBS. In experiments where drug and dextran were injected concomitantly, 1.0 mg of dextran was mixed with the drug to the desired concentration in a final volume of 200

μl. In dose-response experiments, drugs and dextrans were injected concomitantly. All injections, except injections of SCH 58261, were retro-orbital *i.v.* with a 27-gauge needle. In the SCH 58261 experiment, SCH 58261 injections, 1 mg/kg, were intraperitoneal and mice were pre-dosed with this concentration daily for 4 days prior
5 to the day of the experiment. An additional injection was administered at the time of the experiment. Vehicle/drug and dextrans were injected on day 5 and tissues were collected 3 h after vehicle/drug administration. In Lexiscan experiments, Lexiscan was administered *i.v.* with 3 injections, 5 min apart and tissues were collected at 15 min unless otherwise indicated.

10

Example 24 - Treatment and Tissue Colleetion

[0201] In dose-response experiments and experiments with the A1 AR and A2A AR knock-out mice, drugs and dextrans were injected concomitantly. After 3 h, the mice were anesthetized with ketamine/xylazine and subjected to a nose cone containing
15 isoflurane. They were perfused with 25-50 ml ice-cold PBS through the left ventricle of the heart then decapitated. Their brains were removed, weighed and frozen for later analysis.

Example 25 - Fluorimetric Analysis of Dextrans in Brains

20 **[0202]** Ice-cold 50 mMTris-Cl (pH 7.6) was added to frozen brains (100 μl per 100 mg brain) and were to thawed on ice. They were homogenized with a dounce homogenizer and centrifuged at 16.1xg in a microfuge for 30 min at room temperature (rt). The supernatants were transferred to new tubes and an equal volume absolute methanol was added. The samples were centrifuged agaiu at 16.1xg for 30 min at rt.
25 Supernataut (200 μl) was transferred to a Corning costar 96 well black polystyrene assay plate (clear bottom). Additionally, a series of standards containing 0.001–10 μg/ml dextran in 50% Tris-Cl / 50% absolute methanol (vol/vol) was added to each plate. Absolute concentratious of dextrans were derived from these standard curves. Fluorimetric analysis was performed on a BioTek (Winooski, VT) Synergy 4. FITC-
30 dextran was detected at 488/519 (excitation/emission) and Texas Red-dextran was detected at 592/618.

Example 26 - Primary Brain Endothelial Cell Isolation

[0203] This method has been adapted from previously described techniques. Song & Patcher, "Culture of murine brain microvascular endothelial cells that maintain expression and cytoskeletal association of tight junction-associated proteins," *In Vitro Cell. Dev. Biol. Anim.* 39:313-320 (2003), which is hereby incorporated by reference in its entirety. Briefly, 12-week-old C57BL/6 mice were euthanized and decapitated. Dissected brains were freed from the cerebellum and large surface vessels were removed by carefully rolling the brains on sterile Whatman paper. The tissue was then homogenized in a Dounce homogenizer in ice-cold DMEM–F12 medium, supplemented with L-glutamine and Pen/Strep, and the resulting homogenate was centrifuged at 3800xg, 4°C for 5 min. After discarding the supernatant, the pellet was resuspended in 18% (w/vol) dextran in PBS solution, vigorously mixed, and centrifuged at 10000 x g, 4°C for 10 min. The foamy myelin layer was carefully removed with the dextran supernatant by gentle aspiration. The pellet was resuspended in pre-warmed (37°C) digestion medium (DMEM supplemented with 1 mg/ml collagenase/dispase, 40 µg/ml DNaseI, and 0.147 µg/ml of the protease inhibitor tosyllysinechloromethylketone) and incubated at 37°C for 75 min with occasional agitation. The suspension was centrifuged at 3800 x g. The supernatant was discarded; the pellet was resuspended in pre-warmed (37°C) PBS and centrifuged at 3800 x g. The pellet was suspended in full medium (DMEM-F12 medium containing 10% plasma-derived serum, L-glutamine, 1% antibiotic-antimycotic, 100 mg/ml heparin, and 100 mg/ml endothelial cell growth supplement). The resulting capillary fragments were plated onto tissue culture dishes coated with murine collagen IV (50µg/ml) at a density corresponding to one brainper 9.5 cm^2. Medium was exchanged after 24 h and 48 h. Puromycin (8 µg/ml) was added to the medium for the first two days. Before analysis, the primary mouse brain endothelial cells were grown until the culture reached complete confluence after 5-7 days *in vitro*.

Example 27 - Cell Culture and qRT-PCR

[0204] The bEnd.3 mouse brain endothelial cell line was obtained from the ATCC (Manassas, VA) and grown in ATCC formulated DMEM supplemented with 10% FBS. Using Trizol (Invitrogen) extraction, RNA was isolated from bEnd.3 cells.

cDNA was synthesized using Multiscribe reverse transcriptase (Applied Biosystems, Carlesbad, CA). Primers (available upon request) specific for adenosine receptors and CD73 were used to determine gene expression levels and standardized to the TBP housekeeping gene levels using KapaSybr Fast (KapaBiosystems, Woburn, MA) run on 5 a BioRad CFX96 real time qPCR system. Melt curve analyses were performed to measure the specificity for each qPCR product.

Example 28 - Adenosine Receptor Western Blotting and Immunofluorescent Analysis

10 **[0205]** Primary mouse brain endothelial cells and Bend.3 cell cultures were grown as described above. Cells were lysed with 1 ml of lysis buffer containing protease inhibitor and condensed with TCA solution up to 200 μl. Samples were run on a 12 % SDS-PAGE and transferred to Nitrocellulose paper. Membranes were blocked with 1% PVP (Polyvinyl Pyrrolidone) and incubated with anti A1 AR (AAR-15 006) and A2A AR (AAR-002) primary antibodies (Alomone Labs, Jerusalem, Israel) overnight. The membranes were washed and then incubated with goat-anti rabbit HRP. Membranes were washed thoroughly and developed with ECL solution and exposed to X-ray film. For adenosine receptor immunostaining, anesthetized mice were perfused with PBS and brains were isolated and snap frozen in Tissue Tek-OCT medium. Five 20 μm sections (brains in a sagittal orientation) were affixed to Supefrost/Plus slides (Fisher), fixed in acetone, aud stored at -80°C. Slides were thawed, washed iu PBS, blocked with Casein (Vector) in normal goat serum (Zymed), and then incubated with anti-CD31(MEC 13.3, BD Biosciences) and anti-A1 AR (A4104, Sigma) or Anti-A2A AR (AAR-002, Alomone Labs). Slides were then incubated with goat anti-rat 25 Igalexafluor488 (Invitrogen) and goat anti-rabbit Ig Texas Red-X (Invitrogen). Sections were mounted with Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA). Images were obtained on a Zeiss Axio Imager M1 fluorescent microscope.

30 **Example 29 - Fluorescence In Situ Hybridization (FISH)**

[0206] For detection of adenosine receptor mRNA in brain endothelium, we performed FISH using FITC-labeled Cd31 and either Biotin-labeled A1 or A2A DNA oligonucleotide probes (Integrated DNA Technologies, probe sequences available upon

request). Anesthetized mice were perfused with PBS and brains were isolated and snap frozen in Tissue Tek-OCT medium. Twelve micron cryosections were mounted on Superfrost/Plus slides (Fisher). After air drying on the slides for 30 minutes, the tissue was fixed in 4% neutral buffered paraformaldehyde (PFA) for 20 minutes and rinsed for 3 minutes in 1x PBS. Next, the tissue was equilibrated briefly in 0.1 M triethanolamine and acetylated for 10 minutes in 0.1 M triethanolamine with 0.25% acetic anhydride. Immediately following acetylation, the sections were dehydrated through an ascending ethanol series, and stored at room temperature. The tissue was rehydrated for 2 x 15 min in PBS, and equilibrated for 15 min in 5x SSC (NaCl 0.75M, Na-Citrate 0.075M). The sections were then prehybridized for 1 h at 42°C in hybridization buffer (50% deionized formamide, 4x SSC, salmon sperm DNA 40 μg/ml, 20% (w/v) dextran sulphate, 1x Denhardt's solution). The probes (300 ng/ml) were denatured for 3 min at 80°C and added to the pre-warmed (42°C) buffer (hybridization mix). The hybridization reaction was carried out at 42°C for 38 h with 250 μl of hybridization mix ou each slide, covered with parafilm. Prehybridization and hybridization were performed in a black box saturated with a 4x SSC – 50% formamide solution to avoid evaporation and photobleaching of FITC. After incubation, the sections were washed for 30 min in 2x SSC (room temperature), 15 min in 2x SSC (65°C), 15 min in 0.2x SSC, 0.1%SDS (65°C), and equilibrated for 5 min in PBS. For detection of the biotin-probes, sections were incubated for 30 min at room temperature with Texas-Red X conjugated streptavidine (Molecular Probes, S6370, 1 μg/ml) in PBS containing 1x Casein (Vector Laboratories). Excess streptavidin was removed by 15 miu in PBS, followed by 15 min in 0.2x SSC, 0.1%SDS (65°C), and 15 min iu PBS washes. Sections were coverslipped with Vectashield mounting medium with DAPI (Vector Laboratories). Images were acquired using a Zeiss Axio Imager M1 fluorescent microscope.

Example 30 - Injection and Anti-β-Amyloid Antibodies and Immunofluorescent Microscopy

[0207] Wild type and transgenic (AD) mice were given 0.80 μg NECA (*i.v.*). After 3 h, 400 μg of antibody to β-amyloid (200 μl of 2 mg/ml; clone 6E10, Covance, Princeton, NJ) was administered *i.v.* and the mice rested for 90 min. Mice were then anesthetized and perfused as described above and their brains were placed in OTC and

flash-frozen for later sectioning. Sagital sections (6 μm) were fixed in acetone for 10 min, then washed in PBS. Sections were blocked with casein for 20 min then incubated with 1:50 dilution of goat anti-mouse IgCy5 (polyclonal, 1 mg/ml, Abcam, Cabridge, MA) for 20 min then washed 3 times in PBS. Sections were then dried and mounted with VectashieldHardset mounting media with DAPI (Vector Laboratories, Burlingame, CA). Images were obtained on a Zeiss Axio Imager M1 fluorescent microscope.

Example 31 - Transendothelial Cell Electrical Resistance (TEER) Assays

[0208] Bend.3 cells were grown in ATCC-formulated DMEM supplemented with 10% FBS on 24-well transwell inserts, 8 μm pore size (BD Falcon, Bedford, MA) until a monolayer was established. TEER was assessed using a Voltohmeter (EVOMX, World Precision Instruments, Sarasota, FL). Background resistance from un-seeded transwells was subtracted from recorded values to determine absolute TEER values. Change in absolute TEER from T0 for each individual transwell was expressed as percentage change and then averaged for each treatment group.

Example 32 - F-actin Staining of Endothelial Cells

[0209] Bend.3 cells were grown (as described above) on circular cover slips in 24-well plates. Cells were treated for 3 or 30 min with 1 μM CCPA, 1 μM Lexiscan, DMSO or media alone. Cover slips were washed with PBS, fixed in 4% paraformaldahyde, washed again in PBS and then permeabilized with 0.5% TritonX-100 in PBS. After washing in PBS/1% BSA, cover slips were blocked with 1% BSA then stained with Phallodin-Alexa 568. Cover slips were washed and mounted on slides with ProlongGold containing DAPI (Invitrogen). Images were obtained on an Olympus BX51 fluorescent microscope.

Example 33 - Albumin Uptake Assay

Bend.3 cells grown on collagen coated coverslips were incubated with albumin-alexafluor 594 (50 mg/ml) (Invitrogen) and either media alone, DMSO vehicle, NECA (1μM), or Lexiscan (1 μM) for 30 minutes. Albumin uptake was visualized (albumin = red) utilizing the Zeiss Axio Imager M1 fluorescent microscope. Total albumin

fluorescence was recorded using Zeiss Axio Vision software, and measured utilizing Image-J software.

Example 34 - Tight Junction Molecule Staining

5 Bend.3 cells grown on collagen coated coverslips were incubated with DMSO vehicle, NECA (1 μM), or Lexiscan (1 μM) for 1 h. Cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.5% Triton-X in PBS. Cells were blocked with PBS/BSA/goat serum and then stained with antibodies (Invitrogen) against either ZO-1 (1A12), Claudin-5 (34-1600), or Occludin (3F10). Following a
10 wash step, cells were incubated with either goat anti-rabbit IgTexas Red-X or goat anti-mouse IgCy5 (Invitrogen). Cover slips were washed and mounted on slides with ProlongGold containing DAPI. Images were obtained on a Zeiss Axio Imager M1 fluorescent microscope.

15 **Example 35 - Analysis Confirms that the Broad Spectrum AR Agonist NECA Increases BBB Permeability to Macromolecules**

[0210] Statistical differences, assessed using the Students T-test, are indicated where $P \leq 0.05$.

[0211] It was established that *i.v.* administration of NECA, which activates all
20 ARs (A1, A2A, A2B, A3), resulted in a dose-dependent increase in extravasation of *i.v.*-administered fluorescently-labeled dextrans into the CNS of mice (Figure 19). Importantly, it was observed that varying the dose of NECA resulted in dose-dependent increases in CNS entry of both 10,000 Da dextrans (Figure 19A) and 70,000 Da dextrans (Figure 19B) compared to treatment with vehicle alone. Maximum entry of
25 dextrans into the CNS was observed with 0.08 mg/kg NECA. Higher concentrations of NECA had no additional effect or show diminished efficacy, possibly due to receptor desensitization (Ferguson et al., "Subtype-Specific Kinetics of Inhibitory Adenosine Receptor Internalization are Determined by Sensitivity to Phosphorylation by G Protein-coupled Receptor Kinases," *Mol. Pharmacol.* 57:546-52 (2000), which is
30 hereby incorporated by reference in its entirety). These results demonstrate that adenosine receptor activation increases BBB permeability.

[0212] It was next determined the duration of BBB permeability after NECA administration and whether the process is reversible. In time-course experiments using

the minimum effective dose of NECA determined by the dose-response experiments (0.08 mg/kg), it was observed that increased barrier permeability following NECA treatment is temporally discrete (Figure 20A), with maximum entry of labeled dextran into the CNS observed between 4-6 h post-treatment. These data represent accumulation of FITC-dextran in the brain over time, since the dextran and NECA were administered at time zero (T_0). To determine how much dextran can enter the brain in a discrete period of time after NECA treatment, in a second experiment, dextran was administered at indicated times after NECA administration (Figure 20B). These data represent dextran entry into the brain 90 min after dextran injection. At 8 h post-NECA treatment (9.5 h collection time), detectable levels of dextran in the brain were decreased from the maximum and by 18 h post-treatment (19.5 h collection time) the levels returned to baseline, as dextrans administered 18 h after NECA treatment were not detectable in the brain at significant levels (Figure 20B). These results demonstrate that *i.v.* NECA administration results in a temporally discrete period of increased barrier permeability that returns to baseline by 8-18 h.

Example 36 - A1 and A2A AR Activation Increases BBB Permeability

[0213] Four AR subtypes are expressed in mammals: A1, A2A, A2B, and A3 (Sebastiao et al., "Adenosine Receptors and the Central Nervous System," *Handb. Exp. Pharmacol.* 471-534 (2009), which is hereby incorporated by reference in its entirety). To determine which ARs might function in barrier permeability, the levels of mRNA expression of each receptor subtype was examined in mouse brain endothelial cells. Expression of A1 and A2A receptors, but not A2B or A3 receptors, was detected in this cell line (Figure 21A). Additionally, expression of CD73 and CD39, the two ecto-enzymes required for the catalysis of extracellular adenosine from ATP (CD39), was observed on cultured mouse brain endothelial cells. As AR activation increases BBB permeability to dextrans in mice, it was next determined if receptors for adenosine are expressed by mouse BECs. Utilizing antibodies and probes against the A1 and A2A ARs, expression of both ARs on CD31 co-stained endothelial cells within the brains of mice by immunofluorescent staining (Figure 21B) and fluorescence in situ hybridization (Figure 21C) was observed. Importantly, both A1 and A2A AR protein expression was detected by Westeru blot analysis on primary endothelial cells isolated

from the brains of mice (Figure 21D). Interestingly, the human brain endothelial cell line hCMEC/D3 also expresses both the A1 and A2A ARs. These data indicate that BECs are capable of directly responding to extracellular adenosine.

[0214] To investigate the functional contribution of A1 and A2A receptors in AR -mediated changes in BBB permeability, this effect was studied in mice lacking these receptors. Importantly, there were no significant differences in the basal levels of BBB permeability to 10,000 Da dextrans between WT, A_1 AR $^{-/-}$ and A2A AR $^{-/-}$ mice (Figures 21E, 21F, 21G). Following *i.v.* administration of NECA, both $A_1^{-/-}$ and $A2A^{-/-}$ mice showed significantly lower levels of *i.v.*-administered dextrans in their brains compared to wild type mice (Figures 21E and 21F). These data indicate that modulation of barrier permeability is, at least in part, mediated by these two AR subtypes. To examine the effect of NECA administration on BBB permeability in mice when neither the A1 nor the A2A AR is available for activation, A1 AR -/- mice were treated with the selective A2A antagonist SCH 58261 before NECA administration. When A2A AR signaling was blocked with this antagonist in mice lacking the A1 AR, no significant increase in BBB permeability was observed (Figure 21G). These data indicate that modulation of BBB permeability is, at least in part, mediated by these two AR subtypes.

[0215] To confirm these results, the specific A1 agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) and the specific A2A agonist 4-[2-[[6-Amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2yl]amino]ethyl] benzenepropanoic acid (CGS 21680) were administered to wild type mice. Both CGS 21680 (Figure 21H) and CCPA (Figure 21I) treatment resulted in increased dextran entry into the CNS and while this increase is substantial compared to vehicle treatment it was significantly lower than that observed after NECA administration. However, when used in combination, CCPA and CGS 21680 recapitulated the effect of increased dextran entry into the CNS that was observed with NECA treatment (Figure 21J). These results confirmed that modulation of adenosine receptors facilitates entry of molecules into the CNS. Taken together, these results indicate that while activation of either the A1 or A2A AR on BECs can facilitateentry of molecules into the CNS, activation of both ARs has an additive effect.

Example 37 - The Selective A2A AR Agonist Lexiscan Increases BBB Permeability

[0216] To explore the possible therapeutic use of AR agonism to facilitate CNS entry of *i.v.*-administered compounds, a commercially-available, FDA-approved AR
5 agonist was tested in the experimental paradigm. The specific A2A AR agonist Lexiscan, which has been successfully used in myocardial perfusion imaging in humans (Iskandrian et al., "Adenosine Versus Regadenoson Comparative Evaluation in Myocardial Perfusion Imaging: Results of the ADVANCE Phase 3 Multicenter International Trial," *J. Nucl. Cardiol.* 14:645-58 (2007), which is hereby incorporated
10 by reference in its entirety), did indeed increase BBB permeability to 10,000 Da dextrans after *i.v.* administration (Figure 22A) in mice. Interestingly, FITC-dextran was detectable in the brain after 5 min following a single Lexiscan injection. Additionally, *i.v.* administration of Lexiscan also increased BBB permeability in rats (Figure 22B). The magnitude of increased BBB permeability after Lexiscan
15 administration was much greater than the magnitude of increased permeability after NECA administration. Also, interestingly, the duration of increased BBB permeability correlates with the half-life of the AR agonist. For example, the time-course of BBB opening and closing after treatment with NECA (half-life ~ 5 h) is much longer than the time-course after treatment with Lexiscan (half-life ~ 3 min; (Astellas Pharma,
20 "Lexiscan: U.S. Physicians Prescribing Information" (2009), which is hereby incorporated by reference in its entirety). In an injection paradigm intended to mimic continuous infusion of the drug, 3 injections of Lexiscan over 15 min resulted in dramatically high levels of labeled-dextran detected in the brains of rats (Figure 22B). To examine the duration of Lexiscan's effects on BBB permeability, we determined
25 CNS dextran entry over time in both mice and rats. Figure 22C shows the results in graphical form of BBB permeability in rates to FITC-dextran administered simultaneously with 1 μg of Lexiscan at 5 minutes. Following a single i.v. injection of Lexiscan, maximum increased BBB permeability was observed after 30 min and returned to baseline by 180 min post-treatment (Figure 22D). Similar results were
30 observed after Lexiscan treatment in rats (Figure 22E). Importantly, the duration of the effects on BBB permeability after Lexiscan treatment is much shorter than after NECA treatment, probably due to the different half-lives of the compounds (NECA ~ 5 h, Lexiscan ~ 3 min; (Astellas Pharma, "Lexiscan: U.S. Physicians Prescribing

Information" (2009), which is hereby incorporated by reference in its entirety). The more than 20-fold increase in labeled-dextran in Figure 22B (compared to single injections, Figure 22E) is explained by a synergistic effect conferred on BBB opening as a result of multiple doses of Lexiscan.

5 **[0217]** These results demonstrate that in addition to the broad AR agonist, NECA, and the specific A1 and A2A AR agonists, CCPA and CGS 21680, used in this study, the FDA-approved A2A agonist Lexiscan increases BBB permeability to macromolecules.

10 **Example 38 - A2A Antagonism Decreases BBB Permeability**

[0218] It was further hypothesized that if agonism of A1 and A2A receptors increases barrier permeability, then AR antagonism might decrease barrier permeability and prevent molecules from entering the CNS. It was previously observed that in WT mice, blockade of the A2A adenosine receptor inhibited leukocyte migration into the
15 CNS (Mills et al., "CD73 is Required for Efficient Entry of Lymphocytes Into the Central Nervous System During Experimental Autoimmune Encephalomyelitis," *Proc Natl Acad Sci USA* 105: 9325-30 (2008), which is hereby incorporated by reference in its entirety). This hypothesis was tested with a specific A2A AR antagonist. Intraperitoneal administration of the A2A AR antagonist 2-(2-Furanyl)-7-(2-
20 phenylethyl)-7H-pyrazolo[4,3-e][1,2, 4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261) resulted in significantly decreased entry of 10,000 Da FITC-dextran into WT mice brains (Figure 22F). This data supports that blocking AR signaling tightens or closes the BBB.

25 **Example 39 - Antibodies to β-amyloid Enter the Brain After NECA Administration**

[0219] The most challenging therapeutic agents to get across the BBB are macromolecules such as antibodies, due to their enormous size (~150 kDa). It was asked whether adenosine receptor modulation with NECA can facilitate the entry of
30 antibodies into the CNS. To test this, a double [amyloid precursor protein (APP)/presenilin (PSEN)] transgenic mouse model of AD [strain B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/J] was used. These mice accumulate similar β-amyloid

(Aβ) plaques that are a hallmark of AD (Jankowsky et al., "Mutant Presenilins Specifically Elevate the Levels of the 42 Residue Beta-amyloid Peptide *in vivo*: Evidence for Augmentation of a 42-specific Gamma Secretase," *Hum. Mol. Genet.* 13:159-170 (2004); Mincur et al., "Genetic Mouse Models of Alzheimer's Disease,"
5 *Neural. Plast.* 12:299-310 (2005), which are hereby incorporated by reference in their entirety).

[0220] The monoclonal antibody 6E10 (Covance) has been shown to significantly reduce Aβ plaque burden in a mouse model of AD when administered by intracerebroventricular injection (Thakker et al., "Intracerebroventricular Amyloid-beta
10 Antibodies Reduce Cerebral Amyloid Angiopathy and Associated Micro-hemorrhages in Aged Tg2576 Mice," *Proc. Natl. Acad. Sci. USA* 106:4501-6 (2009), which is hereby incorporated by reference in its entirety). Three hours after *i.v.* NECA administration, the 6E10 antibody *i.v.* was administered. After 90 min, brains were collected, sectioned and stained with a secondary Cy5-labeled antibody. Binding of 6E10 antibody to Aβ
15 plaques was observed throughout the brains of NECA-treated mice, with a concentration of Aβ plaques in the hippocampal region (Figure 23A and 23E). No binding of i.v. injected 6E10 antibody was observed in AD mice treated with vehicle alone (Figures 23A, 23B, and 23E) or in WT mice treated with NECA or vehicle. Neither NECA nor vehicle treatment alone affected the ability of AD mice to form Aβ
20 plaqnes (Figures 23C and 23D). These results demonstrate that *i.v.* administered antibody to Aβ can cross the BBB following AR agonism and bind CNS Aβ plaques (Figure 23H), most of which are located near blood vessels within the brain (Figures 23F and 23G). These results demonstrate that antibody to β-amyloid administered *i.v.* can cross the BBB after AR agonism.

25

Example 40 - AR Activation Results in Decreased Transendothelial Resistance in Cultured Mouse BEC Monolayers

[0221] To determine how AR signaling mediates changes in BBB permeability, we utilized the pre-established mouse brain endothelial cell-line, Bend.3 (Montesano et
30 al., "Increased Proteolytic Activity is Responsible for the Aberrant Morphogenetic Behavior of Endothelial Cells Expressing the Middle T Oncogene," *Cell* 62:435-445 (1990), which is hereby incorporated by reference in its entirety). While there are four

known AR snbtypes expressed in mammals (A1, A2A, A2B and A3 (Sebastiao et al., "Adenosine Receptors and the Central Nervons System," *Handb. Exp. Pharmacol.* 471-534 (2009), which is hereby incorporated by reference in its entirety), mRNA expression of the A1 and A2A receptors, but not A2B or A3 receptors, was detected in
5 Bend.3 cells (Figure 24A). Additionally, expression of CD73, anecto-enzyme required for the catalysis of extracellular adenosine from ATP, was observed on these cultured mouse BECs (Figure 24A). Importantly, protein expression for the A1 and A2A ARs were detected on Bend.3 cells (Figure 24B), indicating that these cells are capable of directly responding to extracellular adenosine.

10 **[0222]** Decreased transendothelial cell electrical resistance (TEER) in endothelial cell monolayers has been demonstrated to correlate with increased paracellular space between endothelial cells and increased barrier permeability (Wojciak-Stothard et al., "Rho and Rac But not Cdc42 Regulate Endothelial Cell Permeability," *J. Cell. Sci.* 114:1343-1355 (2001); Dewi et al., "In vitro Assessment of
15 Human Endothelial Cell Permeability: Effects of Inflammatory Cytokines and Dengue Virus Infection," *J. Virol. Methods* 121:171-180 (2004), which are hereby incorporated by reference in their entirety). In transwell assays with monolayers of cnltured mouse BECs (starting absolute TEER mean = 182 ohms; median = 187 ohms), we observed decreases in TEER after addition of NECA or Lexiscan,as compared with BECs given
20 vehicle or media alone (Figure 24C). While AR signaling alters TEER in BECs, we did not observe any alterations in the rate of transcytosis in BECs following AR stimulation. For instance, NECA and Lexiscan induced AR signaling did not affect the rate of fluorescently-labeled albumin uptake in BECs, as compared to media and vehicle treated controls (Figures 24D-24H).

25

Example 41 - AR Activation Correlates with Actinomyosin Stress Fiber Formation and Alterations in Tight Junctions in Brain Endothelial Cells

[0223] The actin cytoskeleton is vital for the maintenance of cell shape and for endothelial barrier integrity. Since actomyosin stress fibers are necessary for inducing
30 contraction in cell shape (Hotulainen et al., "Stress Fibers are Generated by Two Distinct Actin Assembly Mechanisms in Motile Cells," *J. Cell. Biol.* 173:383-94 (2006); Prasain et al., "The Actin Cytoskeleton in Endothelial Cell Phenotypes," *Microvasc. Res.* 77:53-63 (2009), which are hereby incorporated by reference in their

entirety), it was hypothesized that adenosine receptor signaling results in actin stress fiber induction.

[0224] To test this, brain endothelial cells ("BECs") were treated with either CCPA (to agonize A1 adenosine receptors) or Lexiscan (to agonize the A2A adenosine receptor) (Figures 24I-24P). The marked induction of actinomycin stress fibers was observed upon A1 and A2A agonist treatment as compared to treatment with vehicle alone, as shown in Figures 24I-24L. This indicates that activation of ARs induces changes in cytoskeletal elements in BECs to increase barrier permeability.

[0225] While AR signaling induces changes in TEER,which is a functional measure ofparacellular permeability, and actinomyosin stress fibers, which regulate cell shape, it is important to determine if AR signaling alters the junctional interactions between BECs. Therefore to determine if AR signaling alters the tight junction of BECs, Bend.3 cells were cultured to confluent monolayers and determined if the expression of ZO-1, claudin-5, or occludin was altered following AR agonist treatment (Figures 24Q-24Y). While confluent Bend.3 cells formed proper tight junctions when grown in media or treated with vehicle (Figures 24Q, 24T, and 24W), AR agonist treatment induced alterations in tight junction protein expression. For example, Bend.3 cells treated with NECA or Lexiscan had severely diminished occludin expression following treatment with discreet alterations in ZO-1 and claudin-5 (Figures 24X and 24Y). Overall, these results indicate BEC permeability can be altered by AR signaling through changes tight junction molecule expressiou.

[0226] As shown schematically in Figure 25, these results demonstrate that activation of either the A1 or A2A AR temporarily increases BBB permeability, while activation of both receptors results in an additive effect of increased BBB permeability. It is shown here that BBB permeability mediated through A1 and A2A ARs operates as a door where activation opens the door and local adenosine concentration is the key.

[0227] Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

WHAT IS CLAIMED:

1. A method for increasing blood brain barrier permeability in a subject, comprising administering to said subject an agent which activates both of A1 and A2A
5 adenosine receptors.

2. The method according to claim 1, wherein the increase in blood brain permeability lasts up to 18 hours.

10 3. The method according to claim 1, wherein the agent which activates both of A_1 and A2A adenosine receptors is an agonist of both A1 and A2A receptors.

4. The method according to claim 3, wherein the agent which activates both of A1 and A2A adenosine receptors is a broad spectrum adenosine receptor
15 agonist.

5. The method according to claim 3, wherein the agonist of both A1 and A2A receptors is AMP 579.

20 6. The method according to claim 4, wherein the agonist of both A1 and A2A receptors is NECA.

7. The method according to claim 3, wherein the activation of both A1 and A2A receptors is synergistic with respect to blood brain barrier permeability.

25

8. The method according to claim 3, wherein the activation of both A1 and A2A receptors is additive with respect to blood brain barrier permeability.

9. A method for increasing blood brain barrier permeability in a subject,
30 comprising administering to said subject an A1 adenosine receptor agonist and an A2A adenosine receptor agonist.

10. The method according to claim 9, wherein the A1 adenosine receptor agonist and an A2A receptor agonist are A1-selective and A2-selective adenosine receptor agonists.

5 11. The method according to claim 9, wherein the A1 adenosine receptor agonist and an A2A receptor agonist are formulated in a single unit dosage form.

12. The method according to claim 9, wherein the A1 adenosine receptor agonist and an A2A receptor agonist are administered simultaneously.

10

13. The method according to claim 9, wherein the A1 adenosine receptor agonist and an A2A receptor agonist are administered sequentially.

14. The method according to claim 10, wherein the A1-selective adenosine
15 receptor agonist is selected from the group consistiug of CCPA, 8-cyclopentyl-1,3-dipropylxanthine, R-phenylisopropyl-adenosine, N6-Cyclopentyladenosine, N(6)-cyclohexyladenosine, and combinations thereof.

15. The method according to claim 10, wherein the A2A-selective adenosine
20 receptor agonist is selected from the group consisting of Lexiscan, CGS 21680, ATL-146e, YT-146 (2-(1-octynyl)adenosine), DPMA (N6-(2-(3,5-dimethoxypheuyl)-2-(2-methylphenyl)ethyl)adenosine), and combinations thereof.

16. A composition comprising an A1 adenosine receptor agonist and an
25 A2A adenosine receptor agonist, and a pharmaceutically acceptable carrier, excipient, or vehicle.

17. The composition according to claim 16, wherein the A1 adenosine receptor agonist and an A2A receptor agonist are A1-selective and A2-selective
30 adenosine receptor agonists.

18. The composition according to claim 16, further comprising a therapeutic agent.

19. The composition according to claim 18, wherein the therapeutic agent is suitable for treating a CNS disease, disorder, or condition.

5 20. The composition according to claim 19, wherein the therapeutic agent is selected from the group consisting of acetaminophen, acetylsalicylic acid, acyltransferase, alprazolam, amantadine, amisulpride, amitriptyline, amphetamine-dextroamphetamine, amsacrine, antipsychotics, antivirals, apomorphine, arimoclomol, aripiprazole, asenapine, aspartoacyclase enzyme, atomoxetine, atypical antipsychotics,
10 azathioprine, baclofen, beclamide, benserazide, benserazide-levodopa, benzodiazepines, benztropine, bevacizumab, bleomycin, brivaracetam, bromocriptine, buprenorphine, bupropion, cabergoline, carbamazepine, carbatrol, carbidopa, carbidopa-levodopa, carboplatin, chlorambucil, chlorpromazine, chlorprothixene, cisplatin, citalopram, clobazam, clomipramine, clonazepam, clozapine, codeine, COX-2
15 inhibitors, cyclophosphamide, dactinomycin, dexmethylphenidate, dextroamphetaine, diamorphine, diastat, diazepam, diclofenac, donepezil, doxorubicin, droperidol, entacapone, epirubicin, escitalopram, ethosuximide, etoposide, felbamate, fluoxetine, flupenthixol, fluphenazine, fosphenytoin, gabapentin, galantamine, gamma hydroxybutyrate, gefitinib, haloperidol, hydantoins, hydrocordone, hydroxyzine,
20 ibuprofen, ifosfamide, IGF-1, iloperidone, imatinib, imipramine, interferons, irinotecan, KNS-760704, lacosamide, lamotrigine, levetiracetam, levodopa, levomepromazine, lisdexamfetamine, lisuride, lithium carbonate, lypolytic enzyme, mechlorethamine, mGluR2 agonists, memantine, meperidine, mercaptopurine, mesoridazine, mesuximide, methamphetamine, methylphenidate, minocycline, modafinil, morphine, N-
25 acetylcysteine, naproxen, nelfinavir, neurotrin, nitrazepam, NSAIDs, olanzapine, opiates, oseltamivir, oxaplatin, paliperidone, pantothenate kinase 2, Parkin, paroxetine, pergolide, periciazine, perphenazine, phenacemide, phenelzine, phenobarbitol, phenturide, phenytoin, pimozide, Pink1, piribedil, podophyllotoxin, pramipexole, pregabalin, primidone, prochlorperazine, promazine, promethazine, protriptyline,
30 pyrimidinediones, quetiapine, rasagiline, remacemide, riluzole, risperidone, ritonavir, rituximab, rivastigmine, ropinirole, rotigotine, rufinamide, selective serotonin reuptake inhibitors (SSRIs), selegine, selegiline, sertindole, sertraline, sodium valproate, stiripentol, taxanes, temazepam, temozolomide, tenofovir, tetrabenazine, thiamine,

thioridazine, thiothixene, tiagabine, tolcapone, topiramate, topotecan, tramadol, tranylcypromine, trastuzumab, tricyclic antidepressants, trifluoperazine, triflupromazine, trihexyphenidyl, trileptal, valaciclovir, valnoctamide, valproamide, valproic acid, venlafaxine, vesicular stomatitis virus, vigabatrin, vinca alkaloids,
5 zanamivir, ziprasidone, zonisamide, zotepine, zuclopenthixol, and combinations thereof.

21. A method for delivering a macromolecular therapeutic agent to the brain of a subject, comprising administering to said subject: (a) an agent which activates both
10 of A1 and A2A adenosine receptors; and (b) the macromolecular therapeutic agent.

22. The method according to claim 21, wherein the agent which activates both of A1 and A2A adenosine receptors is administered before the macromolecular therapeutic agent.

15

23. The method according to claim 21, wherein the agent which activates both of A1 and A2A adenosine receptors is administered simultaneously with the macromolecular therapeutic agent.

20 24. The method according to claim 21, wherein the agent which activates both of A1 and A2A adeuosine receptors is administered up to 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5, hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, or 18 hours before the macromolecular therapeutic agent is administered.

25

25. The method according to claim 21, wherein the macromolecular therapeutic agent is a monoclonal antibody.

26. The method according to claim 25, wherein the macromolecular
30 therapeutic agent is a monoclonal antibody selected from the group consisting of 6E10, PF-04360365, 131I-chTNT-1/B MAb, 131I-L19SIP, 177Lu-J591, ABT-874, AIN457, alemtuzumab, anti-PDGFR alpha monoclonal antibody IMC-3G3, astatine At 211 monoclonal antibody 81C6, Bapineuzumab, Bevacizumab, cetuximab, cixutumumab,

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Daclizumab, Hu MiK-beta-1, HuMax-EGFr, iodine I 131 monoclonal antibody 3F8, iodine I 131 monoclonal antibody 81C6, iodine I 131 monoclonal antibody 8H9, iodine I 131 monoclonal antibody TNT-1/B, LMB-7 immunotoxin, MAb-425, MGAWN1, Mel-14 F(ab')2, M-T412, Natalizumab, Neuradiab, Nimotuzumab, Ofatumumab,
5 Panitumumab, Ramucirumab, ranibizumab, SDZ MSL-109, Solanezumab, Trastuzumab, Ustekinumab, Zalutumumab, Tanezumab, Aflibercept, MEDI-578, REGN475, Muromonab-CD3, Abiximab, Rituximab, Basiliximab, Palivizumab, Infliximab, Gemtuzumab ozogamicin, Ibritumomab tiuxetan, Adalimumab, Omalizumab, Tositumomab, Tositumomab-I131, Efalizumab, Abciximab,
10 Certolizumab pegol, Eculizumab, AMG-162, Zanolimumab, MDX-010, Anti0MRSA mAb, Pexelizumab, Mepolizumab, Epratuzumab, Anti- RSV mAb, Afelimomab, Catumaxomab, WX-G250, and combinations thereof.

27. The method according to any one of claims 21 through 26, wherein the
15 administration of the agent which activates both of A1 and A2A adenosine receptors and the administration of the macromolecular therapeutic agent is systemic administration.

28. The method according to any one of claims 21 through 26, wherein the
20 administration of the agent which activates both of A1 and A2A adenosine receptors or the administration of the macromolecular therapeutic agent is systemic administration

29. A method for treating a CNS disease, disorder, or condition in a subject, comprising administering to said subject (a) at least one agent which activates both of
25 A1 and A2A adenosine receptors; and (b) a therapeutic agent.

30. The method according to claim 29, wherein the agent which activates both of A1 and A2A adenosine receptors is an agonist of both A1 and A2A receptors.

30 31. The method according to claim 30, wherein the agent which activates both of A1 and A2A adenosine receptors is a broad spectrum adenosine receptor agonist.

32. The method according to claim 30, wherein the agonist of both A1 and A2A receptors is AMP 579.

33. The method according to claim 31, wherein the agonist of both A1 and
5 A2A receptors is NECA.

34. The method according to claim 29, wherein the therapeutic agent is a macromolecular therapeutic agent.

10 35. The method according to claim 34, wherein the macromolecular therapeutic agent is a monoclonal antibody.

36. The method according to claim 35, wherein the monoclonal antibody is selected from the group consisting of 6E10, PF-04360365, 131I-chTNT-1/B MAb,
15 131I-L19SIP, 177Lu-J591, ABT-874, AIN457, alemtuzumab, anti-PDGFR alpha monoclonal antibody IMC-3G3, astatine At 211 monoclonal antibody 81C6, Bapineuzumab, Bevacizumab, cetuximab, cixutumumab, Daclizumab, Hu MiK-beta-1, HuMax-EGFr, iodine I 131 monoclonal antibody 3F8, iodine I 131 monoclonal antibody 81C6, iodine I 131 monoclonal antibody 8H9, iodine I 131 monoclonal
20 antibody TNT-1/B, LMB-7 immunotoxin, MAb-425, MGAWN1, Me1-14 F(ab')2, M-T412, Natalizumab, Neuradiab, Nimotuzumab, Ofatumumab, Panitumumab, Ramucirumab, ranibizumab, SDZ MSL-109, Solanezumab, Trastuzumab, Ustekinumab, Zalutumumab, Tanezumab, Aflibercept, MEDI-578, REGN475, Muromonab-CD3, Abiximab, Rituximab, Basiliximab, Palivizumab, Infliximab,
25 Gemmzumab ozogamicin, Ibritumomab tiuxetan, Adalimumab, Omalizumab, Tositumomab, Tositumomab-I131, Efalizumab, Abciximab, Certolizumab pegol, Eculizumab, AMG-162, Zanolimumab, MDX-010, Anti0MRSA mAb, Pexelizumab, Mepolizumab, Epratuzumab, Anti- RSV mAb, Afelimomab, Catumaxomab, WX-G250, and combinations thereof.

30

37. The method according to claim 29, wherein the therapeutic agent is a small molecule therapeutic agent.

38. The method according to claim 37, wherein the small molecule therapeutic agent is selected from the group consisting of acetaminophen, acetylsalicylic acid, acyltransferase, alprazolam, amantadine, amisulpride, amitriptyline, amphetamine-dextroamphetamine, amsacrine, antipsychotics, antivirals, 5 apomorphine, arimoclomol, aripiprazole, asenapine, aspartoacyclase enzyme, atomoxetine, atypical antipsychotics, azathioprine, baclofen, beclamide, benserazide, benserazide-levodopa, benzodiazepines, benztropine, bevacizumab, bleomycin, brivaracetam, bromocriptine, buprenorphine, bupropion, cabergoline, carbamazepine, carbatrol, carbidopa, carbidopa-levodopa, carboplatin, chlorambucil, chlorpromazine, 10 chlorprothixene, cisplatin, citalopram, clobazam, clomipramine, clonazepam, clozapine, codeine, COX-2 inhibitors, cyclophosphamide, dactinomycin, dexmethylphenidate, dextroamphetaine, diamorphine, diastat, diazepam, diclofenac, donepezil, doxorubicin, droperidol, entacapone, epirubicin, escitalopram, ethosuximide, etoposide, felbamate, fluoxetine, flupenthixol, fluphenazine, fosphenytoin, gabapentin, 15 galantamine, gamma hydroxybutyrate, gefitinib, haloperidol, hydantoins, hydrocordone, hydroxyzine, ibuprofen, ifosfamide, IGF-1, iloperidone, imatinib, imipramine, interferons, irinotecan, KNS-760704, lacosamide, lamotrigine, levetiracetam, levodopa, levomepromazine, lisdexamfetamine, lisuride, lithium carbonate, lypolytic enzyme, mechlorethamine, mGluR2 agonists, memantine, 20 meperidine, mercaptopurine, mesoridazine, mesuximide, methamphetamine, methylphenidate, minocycline, modafinil, morphine, N-acetylcysteine, naproxen, nelfinavir, neurotrin, nitrazepam, NSAIDs, olanzapine, opiates, oseltamivir, oxaplatin, paliperidone, pantothenate kinase 2, Parkin, paroxetine, pergolide, periciazine, perphenazine, phenacemide, phenelzine, phenobarbitol, phenturide, phenytoin, 25 pimozide, Pink1, piribedil, podophyllotoxin, pramipexole, pregabalin, primidone, prochlorperazine, promazine, promethazine, protriptyline, pyrimidinediones, quetiapine, rasagiline, remacemide, riluzole, risperidone, ritonavir, rituximab, rivastigmine, ropinirole, rotigotine, rufinamide, selective serotonin reuptake inhibitors (SSRIs), selegine, selegiline, sertindole, sertraline, sodium valproate, stiripentol, 30 taxanes, temazepam, temozolomide, tenofovir, tetrabenazine, thiamine, thioridazine, thiothixene, tiagabine, tolcapone, topiramate, topotecan, tramadol, tranylcypromine, trastuzumab, tricyclic antidepressants, trifluoperazine, triflupromazine, trihexyphenidyl, trileptal, valaciclovir, valnoctamide, valproamide, valproic acid, venlafaxine, vesicular

stomatitis virus, vigabatrin, vinca alkaloids, zanamivir, ziprasidone, zonisamide, zotepine, zuclopenthixol, and combinations thereof.

39. The method according to any one of claims 29 through 38, wherein the CNS disease, disorder, or condition is a metabolic disease, a behavioral disorder, a personality disorder, dementia, a cancer, a neurodegenerative disorder, pain, a viral infection, a sleep disorder, a seizure disorder, acid lipase disease, Fabry disease, Wernicke-Korsakoff syndrome, ADHD, anxiety disorder, borderline personality disorder, bipolar disorder, depression, eating disorder, obsessive-compulsive disorder, schizophrenia, Alzheimer's disease, Barth syndrome and Tourette's syndrome, Canavan disease, Hallervorden-Spatz disease, Huntington's disease, Lewy Body disease, Lou Gehrig's disease, Machado-Joseph disease, Parkinson's disease, or Restless Leg syndrome.

40. The method according to claim 39, wherein the pain is neuropathic pain, central pain syndrome, somatic pain, visceral pain or headache.

41. A method for treating a CNS disease, disorder, or condition in a subject, comprising administering to said subject (a) an A1-selective adenosine receptor agonist; (b) an A2A-selective receptor agonist; and (c) a therapeutic agent.

42. The method according to claim 41, wherein the A1-selective adenosine receptor agonist and an A2A-selective receptor agonist are formulated in a single unit dosage form.

43. The method according to claim 41, wherein the A1-selective adenosine receptor agonist and an A2A-selective receptor agonist are administered simultaneously.

44. The method according to claim 41, wherein the A1-selective adenosine receptor agonist and an A2A-selective receptor agonist are administered sequentially.

45. The method according to claim 41, wherein the A1-selective adenosine receptor agonist is selected from the group consisting of CCPA, 8-cyclopentyl-1,3-dipropylxanthine, R-phenylisopropyl-adenosine, N6-Cyclopentyladenosine, N(6)-cyclohexyladenosine, and combinations thereof.

5

46. The method according to claim 41, wherein the A2A- selective receptor agonist is selected from the group consisting of Lexiscan, CGS 21680, ATL-146e, YT-146 (2-(1-octynyl)adenosine), DPMA (N6-(2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)ethyl)adenosine), and combinations thereof.

10

47. The method according to claim 41, comprising administering to the subject a composition comprising an A1 adenosine receptor agonist and an A2A adenosine receptor agonist, and a pharmaceutically acceptable carrier, excipient, or vehicle.

15

48. The method according to claim 41, wherein the therapeutic agent is a macromolecular therapeutic agent.

49. The method according to claim 48, wherein the macromolecular
20 therapeutic agent is a monoclonal antibody.

50. The method according to claim 49, wherein the monoclonal antibody is selected from the group consisting of 6E10, PF-04360365, 131I-chTNT-1/B MAb, 131I-L19SIP, 177Lu-J591, ABT-874, AIN457, alemtuzumab, anti-PDGFR alpha
25 monoclonal antibody IMC-3G3, astatine At 211 monoclonal antibody 81C6, Bapineuzumab, Bevacizumab, cetuximab, cixutumumab, Daclizumab, Hu MiK-beta-1, HuMax-EGFr, iodine I 131 monoclonal antibody 3F8, iodine I 131 monoclonal antibody 81C6, iodine I 131 monoclonal antibody 8H9, iodine I 131 monoclonal antibody TNT-1/B, LMB-7 immunotoxin, MAb-425, MGAWN1, Me1-14 F(ab')2, M-
30 T412, Natalizumab, Neuradiab, Nimotuzumab, Ofatumumab, Panitumumab, Ramucirumab, ranibizumab, SDZ MSL-109, Solanezumab, Trastuzumab, Ustekinumab, Zalutumumab, Tanezumab, Aflibercept, MEDI-578, REGN475, Muromonab-CD3, Abiximab, Rituximab, Basiliximab, Palivizumab, Infliximab,

Gemtuzumab ozogamicin, Ibritumomab tiuxetan, Adalimumab, Omalizumab, Tositumomab, Tositumomab-I131, Efalizumab, Abciximab, Certolizumab pegol, Eculizumab, AMG-162, Zanolimumab, MDX-010, Anti0MRSA mAb, Pexelizumab, Mepolizumab, Epratuzumab, Anti- RSV mAb, Afelimomab, Catumaxomab, WX-
5 G250, and combinations thereof.

51. The method according to claim 41, wherein the therapeutic agent is a small molecule therapeutic agent.

10 52. The method according to claim 51, wherein the small molecule therapeutic agent is selected from the group consisting of acetaminophen, acetylsalicylic acid, acyltransferase, alprazolam, amantadine, amisulpride, amitriptyline, amphetamine-dextroamphetamine, amsacrine, antipsychotics, antivirals, apomorphine, arimoclomol, aripiprazole, asenapine, aspartoacyclase enzyme,
15 atomoxetine, atypical antipsychotics, azathioprine, baclofen, beclamide, benserazide, benserazide-levodopa, benzodiazepines, benztropine, bevacizumab, bleomycin, brivaracetam, bromocriptine, buprenorphine, bupropion, cabergoline, carbamazepine, carbatrol, carbidopa, carbidopa-levodopa, carboplatin, chlorambucil, chlorpromazine, chlorprothixene, cisplatin, citalopram, clobazam, clomipramine, clonazepam,
20 clozapine, codeine, COX-2 inhibitors, cyclophosphamide, dactinomycin, dexmethylphenidate, dextroamphetaine, diamorphine, diastat, diazepam, diclofenac, donepezil, doxorubicin, droperidol, entacapone, epirubicin, escitalopram, ethosuximide, etoposide, felbamate, fluoxetine, flupenthixol, fluphenazine, fosphenytoin, gabapentin, galantamine, gamma hydroxybutyrate, gefitinib, haloperidol, hydantoins,
25 hydrocordone, hydroxyzine, ibuprofen, ifosfamide, IGF-1, iloperidone, imatinib, imipramine, interferons, irinotecan, KNS-760704, lacosamide, lamotrigine, levetiracetam, levodopa, levomepromazine, lisdexamfetamine, lisuride, lithium carbonate, lypolytic enzyme, mechlorethamine, mGluR2 agonists, memantine, meperidine, mercaptopurine, mesoridazine, mesuximide, methamphetamine,
30 methylphenidate, minocycline, modafinil, morphine, N-acetylcysteine, naproxen, nelfinavir, neurotrin, nitrazepam, NSAIDs, olanzapine, opiates, oseltamivir, oxaplatin, paliperidone, pantothenate kinase 2, Parkin, paroxetine, pergolide, periciazine, perphenazine, phenacemide, phenelzine, phenobarbitol, phenturide, phenytoin,

pimozide, Pink1, piribedil, podophyllotoxin, pramipexole, pregabalin, primidone, prochlorperazine, promazine, promethazine, protriptyline, pyrimidinediones, quetiapine, rasagiline, remacemide, riluzole, risperidone, ritonavir, rituximab, rivastigmine, ropinirole, rotigotine, rufinamide, selective serotonin reuptake inhibitors
5 (SSRIs), selegine, selegiline, sertindole, sertraline, sodium valproate, stiripentol, taxanes, temazepam, temozolomide, tenofovir, tetrabenazine, thiamine, thioridazine, thiothixene, tiagabine, tolcapone, topiramate, topotecan, tramadol, tranylcypromine, trastuzumab, tricyclic antidepressants, trifluoperazine, triflupromazine, trihexyphenidyl, trileptal, valaciclovir, valnoctamide, valproamide, valproic acid, venlafaxine, vesicular
10 stomatitis virus, vigabatrin, vinca alkaloids, zanamivir, ziprasidone, zonisamide, zotepine, zuclopenthixol, and combinations thereof.

53. The method according to any one of claims 41 through 52, wherein the CNS disease, disorder, or condition is a metabolic disease, a behavioral disorder, a
15 personality disorder, dementia, a cancer, a neurodegenerative disorder, pain, a viral infection, a sleep disorder, a seizure disorder, acid lipase disease, Fabry disease, Wernicke-Korsakoff syndrome, ADHD, anxiety disorder, borderline personality disorder, bipolar disorder, depression, eating disorder, obsessive-compulsive disorder, schizophrenia, Alzheimer's disease, Barth syndrome and Tourette's syndrome,
20 Canavan disease, Hallervorden-Spatz disease, Huntington's disease, Lewy Body disease, Lou Gehrig's disease, Machado-Joseph disease, Parkinson's disease, or Restless Leg syndrome.

54. The method according to claim 53, wherein the pain is neuropathic pain,
25 central pain syndrome, somatic pain, visceral pain or headache.

55. A method of temporarily increasing the permeability of the blood brain barrier of a subject comprising:

selecting a subject in need of a temporary increase in permeability of the blood
30 brain barrier;

providing an agent which activates either the A1 or the A2A adenosine receptor; and

administering to the selected subject either the A1 or the A2A adenosine receptor activating agent under conditions effective to temporarily increase the permeability of the blood brain barrier.

5 56. The method according to 55, wherein the A1 or the A2A activating agent is an A1 or A2A adenosine receptor agonist.

57. The method according to 56, wherein the A1 or the A2A agonist is an A1-selective or an A2A-selective adenosine receptor agonist.

10

58. The method according to 55, wherein the method further comprises administering a therapeutic agent to the subject.

59. The method according to 58, wherein the therapeutic agent is suitable
15 for treating a CNS disease, disorder, or condition.

60. The method according to 59, wherein the CNS disease, disorder, or condition is a metabolic disease, a behavioral disorder, a personality disorder, dementia, a cancer, a neurodegenerative disorder, pain, a viral infection, a sleep
20 disorder, a seizure disorder, acid lipase disease, Fabry disease, Wernicke-Korsakoff syndrome, ADHD, anxiety disorder, borderline personality disorder, bipolar disorder, depression, eating disorder, obsessive-compulsive disorder, schizophrenia, Alzheimer's disease, Barth syndrome and Tourette's syndrome, Canavan disease, Hallervorden-Spatz disease, Huntington's disease, Lewy Body disease, Lou Gehrig's disease,
25 Machado-Joseph disease, Parkinson's disease, or Restless Leg syndrome.

61. The method according to claim 58, wherein the therapeutic is selected from the group consisting of acetaminophen, acetylsalicylic acid, acyltransferase, alprazolam, amantadine, amisulpride, amitriptyline, amphetamine-dextroamphetamine,
30 amsacrine, antipsychotics, antivirals, apomorphine, arimoclomol, aripiprazole, asenapine, aspartoacyclase enzyme, atomoxetine, atypical antipsychotics, azathioprine, baclofen, beclamide, benserazide, benserazide-levodopa, benzodiazepines, benztropine, bevacizumab, bleomycin, brivaracetam, bromocriptine, buprenorphine, bupropion,

cabergoline, carbamazepine, carbatrol, carbidopa, carbidopa-levodopa, carboplatin, chlorambucil, chlorpromazine, chlorprothixene, cisplatin, citalopram, clobazam, clomipramine, clonazepam, clozapine, codeine, COX-2 inhibitors, cyclophosphamide, dactinomycin, dexmethylphenidate, dextroamphetaine, diamorphine, diastat, diazepam,
5 diclofenac, donepezil, doxorubicin, droperidol, entacapone, epirubicin, escitalopram, ethosuximide, etoposide, felbamate, fluoxetine, flupenthixol, fluphenazine, fosphenytoin, gabapentin, galantamine, gamma hydroxybutyrate, gefitinib, haloperidol, hydantoins, hydrocordone, hydroxyzine, ibuprofen, ifosfamide, IGF-1, iloperidone, imatinib, imipramine, interferons, irinotecan, KNS-760704, lacosamide, lamotrigine,
10 levetiracetam, levodopa, levomepromazine, lisdexamfetamine, lisuride, lithium carbonate, lypolytic enzyme, mechlorethamine, mGluR2 agonists, memantine, meperidine, mercaptopurine, mesoridazine, mesuximide, methamphetamine, methylphenidate, minocycline, modafinil, morphine, N-acetylcysteine, naproxen, nelfinavir, neurotrin, nitrazepam, NSAIDs, olanzapine, opiates, oseltamivir, oxaplatin,
15 paliperidone, pantothenate kinase 2, Parkin, paroxetine, pergolide, periciazine, perphenazine, phenacemide, phenelzine, phenobarbitol, phenturide, phenytoin, pimozide, Pink1, piribedil, podophyllotoxin, pramipexole, pregabalin, primidone, prochlorperazine, promazine, promethazine, protriptyline, pyrimidinediones, quetiapine, rasagiline, remacemide, riluzole, risperidone, ritonavir, rituximab,
20 rivastigmine, ropinirole, rotigotine, rufinamide, selective serotonin reuptake inhibitors (SSRIs), selegine, selegiline, sertindole, sertraline, sodium valproate, stiripentol, taxanes, temazepam, temozolomide, tenofovir, tetrabenazine, thiamine, thioridazine, thiothixene, tiagabine, tolcapone, topiramate, topotecan, tramadol, tranylcypromine, trastuzumab, tricyclic antidepressants, trifluoperazine, triflupromazine, trihexyphenidyl,
25 trileptal, valaciclovir, valnoctamide, valproamide, valproic acid, venlafaxine, vesicular stomatitis virus, vigabatrin, vinca alkaloids, zanamivir, ziprasidone, zonisamide, zotepine, zuclopenthixol, and combinations thereof.

62. The method according to claim 58, wherein the therapeutic is a
30 macromolecular therapeutic.

63. The method according to claim 62, wherein the therapeutic is a monoclonal antibody.

64. The method according to claim 63, wherein the monoclonal antibody is selected from the group consisting of 6E10, PF-04360365, 131I-chTNT-1/B MAb, 131I-L19SIP, 177Lu-J591, ABT-874, AIN457, alemtuzumab, anti-PDGFR alpha monoclonal antibody IMC-3G3, astatine At 211 monoclonal antibody 81C6, Bapineuzumab, Bevacizumab, cetuximab, cixutumumab, Daclizumab, Hu MiK-beta-1, HuMax-EGFr, iodine I 131 monoclonal antibody 3F8, iodine I 131 monoclonal antibody 81C6, iodine I 131 monoclonal antibody 8H9, iodine I 131 monoclonal antibody TNT-1/B, LMB-7 immunotoxin, MAb-425, MGAWN1, Me1-14 F(ab')2, M-T412, Natalizumab, Neuradiab, Nimotuzumab, Ofatumumab, Panitumumab, Ramucirumab, ranibizumab, SDZ MSL-109, Solanezumab, Trastuzumab, Ustekinumab, Zalutumumab, Tanezumab, Aflibercept, MEDI-578, REGN475, Muromonab-CD3, Abiximab, Rituximab, Basiliximab, Palivizumab, Infliximab, Gemtuzumab ozogamicin, Ibritumomab tiuxetan, Adalimumab, Omalizumab, Tositumomab, Tositumomab-I131, Efalizumab, Abciximab, Certolizumab pegol, Eculizumab, AMG-162, Zanolimumab, MDX-010, Anti0MRSA mAb, Pexelizumab, Mepolizumab, Epratuzumab, Anti- RSV mAb, Afelimomab, Catumaxomab, WX-G250, and combinations thereof.

65. The method according to 58, wherein the A1 or the A2A adenosine receptor activator is administered before the therapeutic agent.

66. The method according to 65, wherein the A1 or the A2A adenosine receptor activator is administered up to 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5, hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, or 18 hours before the therapeutic agent is administered.

67. The method according to 58, wherein the A1 or the A2A adenosine receptor activator and the therapeutic agent are administered simultaneously.

68. A method for decreasing blood brain barrier permeability in a subject comprising administering to said subject an agent which blocks or inhibits A2A signaling.

69. The method according to claim 68, wherein the agent which blocks or inhibits A2A signaling is an A2A selective antagonist.

5 70. The method according to claim 69, wherein the A2A antagonist is SCH 58261.

71. A method of remodeling an actin cytoskeleton of a blood brain barrier endothelial cell, said method comprising contacting said endothelial cell with an agent
10 which activates both of A1 and A2A adenosine receptors.

72. The method according to claim 71, wherein the actin cytoskeleton remodeling increases space between endothelial cells and increases blood brain barrier permeability.

15

73. The method according to claim 71, wherein the agent which activates both of A1 and A2A adenosine receptors is an agonist of both A1 and A2A receptors.

74. The method according to claim 73, wherein the agent which activates
20 both of A1 and A2A adenosine receptors is a broad spectrum adenosine receptor agonist.

75. The method according to claim 73, wherein the agonist of both A1 and A2A receptors is AMP 579.

25

76. The method according to claim 74, wherein the agonist of both A1 and A2A receptors is NECA.

77. The method according to claim 73, wherein the activation of both A1
30 and A2A receptors is synergistic with respect to blood brain barrier permeability.

78. The method according to claim 73, wherein the activation of both A1 and A2A receptors is additive with respect to blood brain barrier permeability.

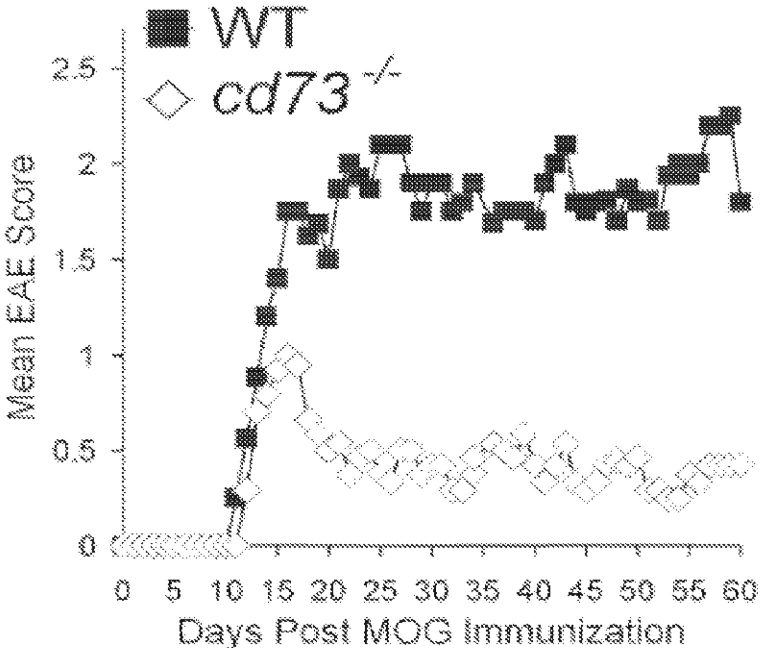

FIG. 1