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Experimental absence seizures: potential role of γ-hydroxybutyric acid and $GABA_B$ receptors

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Summary. We have investigated whether the pathogenesis of spontaneous generalized non-convulsive seizures in rats with genetic absence epilepsy is due to an increase in the brain levels of γ-hydroxybutyric acid (GHB) or in the rate of its synthesis. Concentrations of GHB or of its precursor γ-butyrolactone (GBL) were measured with a new GC/MS technique which allows the simultaneous assessment of GHB and GBL. The rate of GHB synthesis was estimated from the increase in GHB levels after inhibition of its catabolism with valproate. The results of this study do not indicate significant differences in GHB or GBL levels, or in their rates of synthesis in rats showing spike-and-wave discharges (SWD) as compared to rats without SWD. Binding data indicate that GHB, but not GBL, has a selective, although weak affinity for $GABA_B$ receptors ($IC_{50} = 150\,\mu M$). Similar IC_{50} values were observed in membranes prepared from rats showing SWD and from control rats. The average GHB brain levels of 2.12 ± 0.23 nmol/g measured in the cortex and of 4.28 ± 0.90 nmol/g in the thalamus are much lower than the concentrations necessary to occupy a major part of the $GABA_B$ receptors. It is unlikely that local accumulations of GHB reach concentrations 30–70-fold higher than the average brain levels. After injection of 3.5 mmol/kg GBL, a dose sufficient to induce SWD, brain concentrations reach 240 ± 31 nmol/g (Snead, 1991) and GHB could thus stimulate the $GABA_B$ receptor.

Like the selective and potent $GABA_B$ receptor agonist R(−)-baclofen, GHB causes a dose-related decrease in cerebellar cGMP. This decrease and the increase in SWD caused by R(−)-baclofen were completely blocked by the selective and potent $GABA_B$ receptor antagonist CGP 35348, whereas only the increase in the duration of SWD induced by GHB was totally antagonized by CGP 35348. The decrease in cerebellar cGMP levels elicited by GHB was only partially antagonized by CGP 35348.

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These findings suggest that all effects of R(−)-baclofen are mediated by the $GABA_B$ receptor, whereas only the induction of SWD by GHB is dependent on $GABA_B$ receptor mediation, the decrease in cGMP being only partially so. Taken together with the observations of Marescaux et al. (1992), these results indicate that $GABA_B$ receptors are of primary importance in experimental absence epilepsy and that $GABA_B$ receptor antagonists may represent a new class of anti-absence drugs.

1. Introduction

Primary generalized epilepsy of the absence type is a childhood-onset seizure disorder of unknown etiology characterized behaviourally by brief staring spells and arrest of motor activity, and electrically by generalized 3 Hz spike-and-wave discharges (SWD) in the electroencephalogram (EEG) (Godschalk et al., 1976, 1977; Mirsky et al., 1986). Three Hz SWD are associated with enhanced GABA-mediated synaptic inhibition and absence epilepsy could conceivably represent generalized inhibitory seizures due to an excess, rather than to a deficit of GABA-mediated transmission (Fariello and Golden, 1987; Fromm and Kohli, 1972; Gloor and Fariello, 1988). Evidence for this premise is based on the fact that direct $GABA_A$ and $GABA_B$ receptor agonists, GABA uptake inhibitors and 4-aminobutyrate: 2-oxoglutarate aminotransferase (EC 2.6.1.19; GABA-T) inhibitors augment the number and duration of discharges (King, 1979; Marescaux et al., 1984; Micheletti et al., 1985; Smith and Bierkamper, 1990; Snead, 1990; Vergnes et al., 1984). The GABA metabolite and/or putative neurotransmitter (Vayer et al., 1987) γ-hydroxybutyric acid (GHB) or its lactonized prodrug γ-butyrolactone (GBL), also induces 4–6 Hz SWD accompanied by arrest of motor activity, with staring, facial myoclonus and vibrissal twitches, which mimic the events of absence seizures in rats (Snead et al., 1976; Snead, 1988). As the changes in EEG observed after administration of GHB are not followed by convulsions, GHB-induced seizures have been proposed as an animal model of petit mal epilepsy (Godschalk et al., 1976, 1977).

Because of the structural resemblance of GHB to GABA, GHB has also been described as a "GABA agonist" (Meldrum, 1981), suggesting that the epileptiform discharges caused by GHB may be due to its GABAergic activity. In agreement with this hypothesis, Pericic et al. (1978) have shown that GHB, like $GABA_A$ agonists, does not alter GABA levels, but produces a marked and dose-related reduction in the rate of GABA synthesis, indicating strong interactions between GHB and GABA-mediated inhibition. In contrast to the action of muscimol, this effect is not secondary to a direct effect of GHB on $GABA_A$ receptors (Enna and Snyder, 1975). Thus, GHB modulates GABA neurotransmission and induces absence-like seizures by way of a mechanism which is not mediated through $GABA_A$ receptors.

Since exogeneous GHB is capable of inducing absence seizures, the question naturally arises whether GHB-mediated mechanisms might play a role in the genesis of petit mal epilepsy. One possibility of testing the GHB hypothesis of petit mal epilepsy is to assess biochemical parameters related to GHB activity in the brain (e.g. GHB levels, its rate of synthesis, GHB binding or second messengers) in animals with absence seizures as compared to non-epileptic animals.

Recently, a genetic model of spontaneous generalized non-convulsive seizures has been described (Vergnes et al., 1982), which satisfies most of the criteria proposed for a useful animal model of petit mal epilepsy (Mirsky et al., 1986). Spontaneous and recurrent SWD were originally seen in the EEG of some Wistar rats (Vergnes et al., 1982). By successive inbreeding of such rats, a strain in which spontaneous SWD can be recorded in 100% of the animals has been selected and named the Genetic Absence Epilepsy Rats from Strasbourg (GAERS) (Vergnes et al., 1987). Concurrently, another strain of rats was selected which never displayed SWD (controls). Both the electrographic characteristics and pharmacological response of these SWD are reminiscent of petit mal epilepsy in man (Vergnes et al., 1982; Micheletti et al., 1985). The GAERS strain thus affords a reproducible and pharmacologically specific model for the study of biochemical mechanisms involved in spontaneous generalized non-convulsive seizures (Engel et al., 1990).

The aim of the present study was to examine the involvement of GHB and GBL in such seizures by measuring the endogenous concentration of both in hippocampus, thalamus and frontal cortex in GAERS and to compare them with the levels in seizure-free rats. The increase in GHB induced by valproate, an index of its rate of synthesis, was also examined in both strains. Levels of GHB and GBL were assessed by a new capillary gas chromatography-mass spectrometry method with selected-ion monitoring (GC-MS) which allows simultaneous measurement of GHB and GBL with the necessary sensitivity. As the SWD induced by GHB are antagonized by the selective $GABA_B$ receptor antagonist CGP 35348 (Marescaux et al., 1992), the interactions of GHB and of its prodrug GBL with 12 neurotransmitter receptors and neuromodulator binding sites, in particular those controlling GABA-mediated inhibition, were evaluated. In addition, we assessed the effects of GBL and of the selective $GABA_B$ receptor agonist R(−)-baclofen either alone or in combination with CGP 35348, on cGMP levels and we used this paradigm to study the potential interactions between GHB and $GABA_B$ receptors in vivo.

2. Material and methods

Animals

Experiments for the development of the GC-MS procedure and for the assessment of cGMP were conducted on male Tif: RAIF (SPF) rats (Tierfarm Sisseln, Switzerland)

weighing 240–280 g. Other experiments on cGMP levels were performed on male Tif: MAGf (SPF) mice, 23–27 g body weight, 5–8 weeks of age (Tierfarm Sisseln, Switzerland). The animals were kept in an air-conditioned room at 21°C, with a 12 hour light-dark cycle and were sacrificed between 8:30 and 10:00 a.m. to avoid circadian variations of the different biochemical parameters measured.

Rats with spontaneous absence-like seizures

Male Wistar rats (350–400 g) from the breeding colony at the Centre de Neurochimie, C.N.R.S., Strasbourg were used in this study. They were chosen from the 9th generation of a strain with spontaneous generalized non-convulsive seizures, in which bilateral SWD (frequency = 7–9 c/sec, amplitude = 300–1,000 μV, mean duration = 6.0 ± 3.4 sec with a variance between 0.5 and 40 sec, occurrence = 1/min) are observed in awake but inactive animals. Controls were also from the 9th generation of a strain which never displayed SWD. Epileptic and non-epileptic rats were of the same age. They were sacrificed after an acclimatization period of 15 days in Basel.

Chemicals for GC and GC-MS

1,3-Diphenyl-1,1,3,3-tetramethyldisilazane (DPTMDS, Cat. No 43340), hexamethylsilazane (HMDS, Cat No 52619), acetonitrile, and acetic acid anhydride were purchased from Fluka. The internal standard for GHB and GBL, GBL-2,2,3,3,4,4-d_6 (GBL-d_6) was from Merck, Sharp & Dohme Ltd, Pointe Claire, Quebec, Canada. The stationary phase CP-51 wax was from Chrompack International (Middleburg, The Netherlands). All other chemicals and reagents were of analytical-reagent grade and were used without purification.

Drugs

GHB (sodium salt) and GBL were purchased from Fluka. [2,3-^{3}H]GHB, potassium salt (spec. act. 100 Ci/mmol) was prepared by the CEA (Gif-sur-Yvette, France). Valproate sodium was synthesized in our laboratories by Dr. H. Allgeier. Drugs were dissolved in saline 0.9% such that the volume of injection was 1 ml/kg and were used on the same day, if necessary the pH was adjusted to pH = 5 with NaOH 1N. We only used subanaesthetic (200–400 mg/kg) doses of GBL, which produce EEG and behavioural changes corresponding to stage 1 and 2 of Snead (1988). Doses larger than 400 mg/kg i.p. are associated with a burst suppression pattern described as stage 3 by Snead (1988).

Sample preparation for GC-MS-analysis

Rats were killed by fast focused microwave irradiation of the head (Püschner GmbH, Schwanewede, F.R.G; 1.6 sec, 7.5 kW). The brains were rapidly removed, cooled on dry ice and dissected immediately into different brain areas according to the method of Glowinski and Iversen (1966). The brain structures were divided into two equal parts (left and right). One part of the samples was homogenized for 10 min at room tempera-

ture in a ground-glass homogenizer with 2 ml acetonirile containing 20 ng of the the internal standard GBL-d_6. Since GHB does not undergo lactonisation under these conditions (Vayer et al., 1988; Snead et al., 1989), any GHB present would not be lactonized and thus not extracted into the acetonitrile. Therefore, the values obtained represent only GBL. The contralateral brain structures were extracted for 10 min at room temperature in the ground-glass homogenizer with a solution of 5% acetic anhydride in acetonitrile containing 80 ng of GBL-d_6. This procedure lactonizes all the GHB present in the sample, such that the value obtained represents GHB plus GBL. Hence, by subtracting the value obtained from the pure acetonitrile extract, it is possible to determine the concentration of GHB. The acetonitrile solutions were allowed to stand for 1 hr at room temperature and were centrifuged at 10,000 g for 1 hr at 4°C. Owing to the selectivity of the GC-MS method, prior purification of these solutions of GBL in acetonitrile or acetic anhydride/acetonitrile is not necessary.

GC-MS assay for GHB and GBL

The GC-MS analyses were carried out on a Finnigan 4500 mass spectrometer interfaced with an Incos data-processing system and coupled to a Carlo Erba gas chromatograph model 5160, Mega series equipped with the Ciba-Geigy injector model 1988 and the A 200S autosampler. The injector developed at Ciba-Geigy (Lauber-Injector) can be variously operated for split/splitless injection mode, for cold quasi on column injection mode or for hot quasi on column injection mode. All three injection techniques were automated by the autosampler A 200S from Carlo Erba Instruments, Milan, Italy for Europe and Leap Technology, Chapel Hill, NC, for USA.

For GBL analysis, the temperature-controlled "cold on column" mode was chosen. The temperature was kept at 20°C. GC analyses were performed with a 50 m × 0.3 mm glass capillary column pretreated and coated with CP 51 Wax at a film thickness of 1 μm according to Grob (1986), with a 25 m retention gap. The GC oven programme started at 70°C, increased at a rate of 7.5°C per minute to 220°C and was kept for 10 min at this temperature. The temperature of the GC-MS interface and the ion source were kept constant at 250°C and 100°C, respectively. Hydrogen was used as carrier gas at a pressure of 80 kPa. The mass spectra were obtained in the total ion current (TIC) mode. The following mass spectrometric conditions were used: positive chemical ionization with methane as reactant gas at an ion source pressure of 45 kPa measured with an uncalibrated thermocouple gauge. The filament current was kept at 200 μA, and the electron energy at 70 eV. The mass spectrometer was scanned from m/z 50 to 250 daltons in 1 sec intervals. Multiple Ion Detection was used for sensitive, selective simultaneous mass specific detection of the GBL-d_0 and GBL-d_6 at m/z 87 and 93. These base peaks were used for the quantitative assessment of GHB and GBL in brain structures (Fig. 1B). Under these conditions the retention time for GBL and the internal standard GBL-d_6 was 8:15 min (Fig. 1A). Every sample was injected twice.

Measurement of GHB rate of synthesis

The time-dependent accumulation of GHB and GBL following a dose of 400 mg/kg valproate was determined from 0 to 240 min at fixed intervals. GHB and GBL levels were determined by the GC-MS method previously described. Turnover rates were estimated by measuring the accumulation of GHB and GBL in the linear part of the curves obtained.

Receptor binding assays

To demonstrate the selectivity of the interactions, GHB and GBL were tested in a battery of 12 assays including $GABA_A$, $GABA_B$, benzodiazepine, α_1, α_2 and β-adrenoceptors, muscarinic cholinergic, $5HT_1$, histamine H1, adenosine A1, opiate μ and substance P receptors. Methods for receptor-binding assays used in the present investigation are documented in table 3. All assays were validated using appropriate reference standards. When testing the affinity of GHB for $GABA_B$ receptors in epileptic as compared to non-epileptic conrol rats, we used the potent and selective tritiated $GABA_B$ receptor agonist 3-aminopropylphosphinic acid, [^{3}H]CGP 27492 (15.0 Ci/mmol, Ciba-Geigy Horsham, UK) as described by Bittiger et al. (1990).

cGMP determination

cGMP assays were performed using a radioimmunoassay kit with [^{3}H]cGMP obtained from Amersham (Amersham, Buckinghamshire, UK). Groups of 8 mice or rats were injected i.p. with test compounds or saline and sacrificed by fast focused microwave irradiation of the head (for mice: 3 sec, 2.8 kW, operating power; 2,450 MHz, 54 cm^{-2}; Medical Engineering Consultants, Lexington, MA) to prevent post mortem changes in levels of cGMP. Each cerebellum was dissected and homogenized by ultrasonication in 1 ml 0.05 M tris buffer with 4 mM EDTA, pH 7.5 (to prevent enzymatic degradation of cGMP), followed by heating 800 μl of the solution for 3 minutes at 120°C in a glycerine bath to coagulate protein. Homogenized samples were then centrifuged for 5 min at 40,000 × g in the cold. cGMP levels in 100 μl aliquots of the supernatants were assayed in duplicate with the radioimmunoassay kit. The procedure involved incubating [^{3}H]cGMP, antiserum and sample at 4°C for 1.5 to 18 hr. The antibody-cGMP complex was pellected by the addition of chilled ammonium sulfate (60% saturated) and centrifugation. Pellets were resuspended in water, the suspension added to a scintillation cocktail, and radioactivity measured. Control experiments were carried out with an acetylated [^{125}I]cGMP RIA kit of Advanced Magnetic (Cambridge, MA).

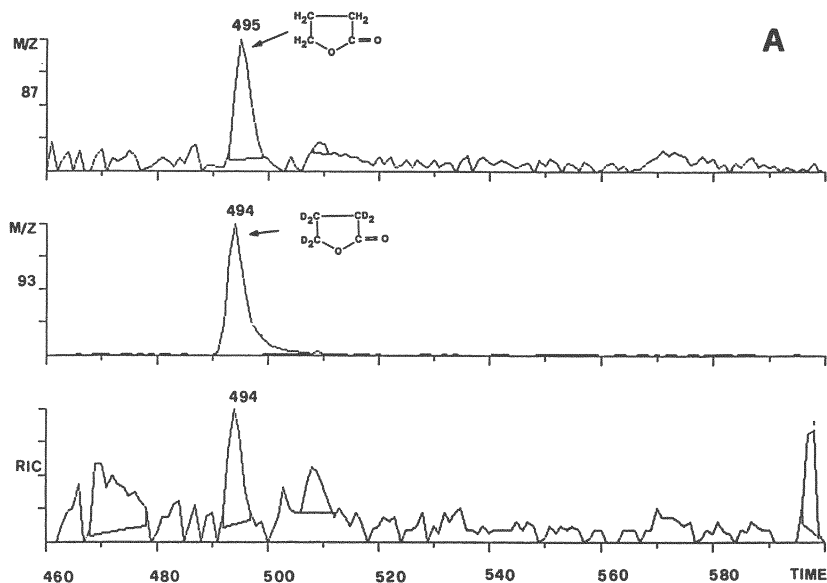
Analysis of data

Results are expressed as means ± standard deviation for 6 to 10 animals per group. Dunnet's multiple comparison two-tailed test (Winer, 1971) was used to assess the significance of differences between several groups and Student's t-test for paired groups. Means ± SEM were considered to be statistically different when $p < 0.05$.

3. Results

Quantification, linearity, recovery and reproducibility

The GC characteristics and mass spectra of GBL are shown in Fig. 1A and 1B. The yield for the extraction of GBL using brain homogenates spiked with pure [2,3^{3}H]GHB was 100% (N = 8). Total recovery of the method, extraction plus derivatization, as estimated by adding different quantities of GHB (sodium salt) to brain extracts, was 100 ± 7.7%. The conversion of



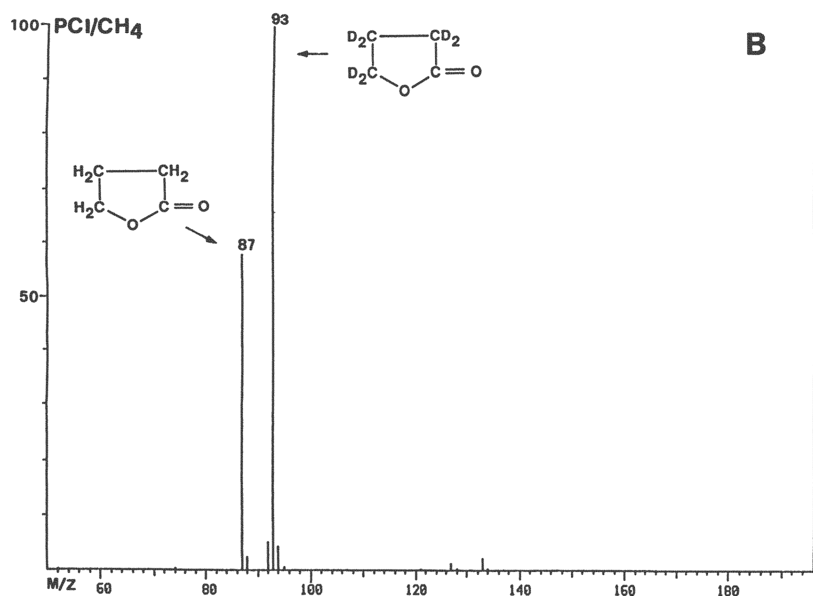


Fig. 1. Gas chromatography and mass spectra of GBL and GBL-d_6. These lactones were analyzed by extracting them from brain tissue with acetonitrile or with the combination acetonitrile and 5% acetic anhydride as described in "Materials and methods". 2 µl was injected into GC/MS system. GC conditions as described in "Materials and methods". **A** Mass spectrum of GBL-d_0 m/z = 87 and GBL-d_6 m/z = 93 used as internal standard for quantification. Ionization conditions are positive ion chemical ionization with methane as reagent gas. Time = retention time in sec. The number at the top of each peak represents the retention time of the corresponding lactone. *RIC* Reconstructed ion current. **B** GBL-d_0 m/z = 87 and GBL-d_6 m/z = 93 as internal standard selected ion mass chromatograms from brain extract. Ordinate = relative intensity in %

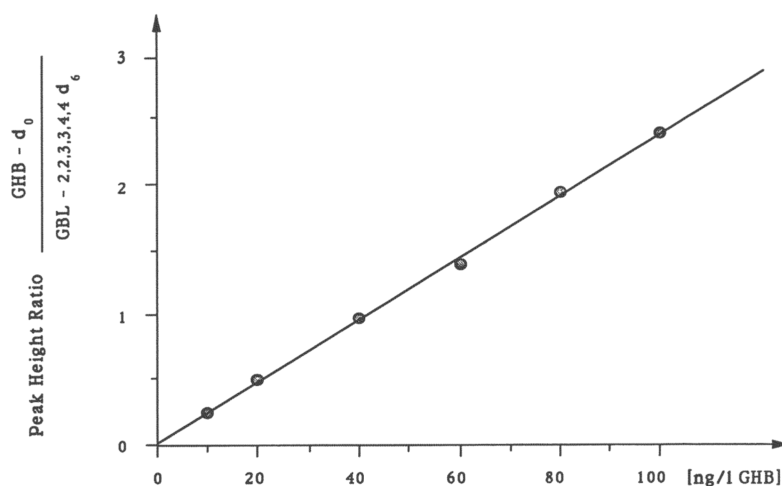


Fig. 2. Calibration curve GHB versus GBL-d_6 for the determination of GHB. Test samples containing various amounts of GHB and constant amounts of internal standard GBL-d_6 were derivatized to GBL-d_0 using conditions described in "Materials and methods" and injected into the gas chromatograph. Every point is the average of two determinations. Data are expressed as peak height ratio GHB-d_0: GBL-d_6

GHB to GBL under the conditions of acidification used was also quantitative. Standard curves were obtained by derivatizing quantities ranging from 5 to 100 ng GHB with 40 GBL-d_6. The calibration curves of GHB/internal standard peak area versus the GHB/internal standard concentration ratio showed a linear response in the range studied. The regression coefficient for the calibration curves was $r \geqslant 0.99$ (Fig. 2).

The sensitivity of the assay is high, as the quantities of GHB-d_6 injected to get the fragmentograms of Fig. 1B are about 125 pg, and the setting of the electron multiplier is very low. In the total reproducibility assay (extraction, derivatization and GC-MS measurement), the quantities of GHB plus GBL measured from a pool of cortices were 2.36 ± 0.09 nmol/g, which corresponded to a coefficient of variation of 3.88% ($N = 40$). This variation coefficient is low because the internal standard is the deuterated derivative and GBL and GBL-d_6 are eluted at the same time (Fig. 1A). The mean cortical concentrations of GHB in all control samples ($N = 57$) was 2.12 ± 0.23 nmol/g, GBL was also found to be present in all brain structures investigated. The cortical concentration was 0.370 ± 0.025 nmol/g ($N = 57$). This is about 15% of the concentration of GHB in this brain area.

Extraction with organic solvents

A study of the most favorable conditions for the isolation and extraction of GBL and GHB from brain tissues was carried out with several organic

Table 1. Regional distribution of GHB and GBL in brain of rats

Brain areas	GBL levels nmol/g wet wt	GHB levels nmol/g wet wt
Cortex	0.37 ± 0.02 (57)	2.12 ± 0.23 (57)
Striatum	0.65 ± 0.06 (12)	4.67 ± 0.25** (12)
Hippocampus	0.39 ± 0.04 (38)	4.49 ± 0.91** (38)
Hypothalamus	N.D.	4.25 ± 0.48** (28)
Thalamus	N.D.	4.28 ± 0.90** (20)
Cerebellum	0.33 ± 0.02 (6)	2.33 ± 0.16 N.S. (6)

Each value represents the mean ± SEM for (N) rats. ND = not determined. Interregional significancies were estimated relative to cortical GHB level by the Student's t-test for paired groups. ** $p < 0.01$

solvents by adding GBL and GHB before the homogenisation of the irradiated tissue and analysis with the GC-MS method. Best recovery (>95%) was obtained with acetonitrile. The acetonitrile extracts yield much cleaner chromatograms than do extracts prepared from other organic solvents (ethanol, methanol, chloroform, dioxane and tetrahydrofurane).

Assay of GHB and GBL in different brain structures

The amounts of GHB and GBL in 6 regions of the rat brain are indicated in Table 1. The structures richest in GHB are striatum, hypothalamus, hippocampus and thalamus, whereas cortex and cerebellum had a relatively

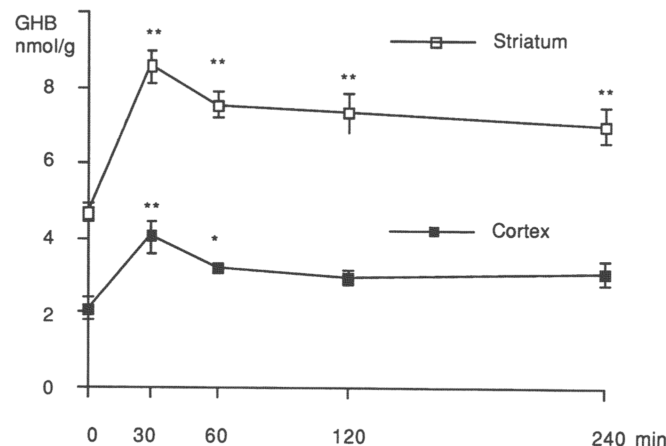


Fig. 3. Time course of GHB accumulation in cortex and striatum of rats treated with valproate (400 mg/kg i.p.). Results are the mean ± S.E.M. for six animals. Statistical significance was calculated by Dunnett's test: *$p < 0.05$, **$p < 0.01$ when compared to the control group at $t = 0$. The initial rate of GHB synthesis in the cortex and striatum were 3.84 nmol/g/h and 7.78 nmol/g/h, respectively

low content of GHB. Statistically significant differences ($p < 0.01$; Student's t test for paired group) were observed between the cortex (= 100%) versus the following areas: striatum, 220%; hippocampus 210%; hypothalamus 200% and thalamus 200%. In general, the distribution pattern for GBL seems to follow that of GHB.

Time course of GHB accumulation in rats treated with valproate

Rats were treated with valproate (400 mg/kg i.p.), killed by microwave irradiation 0, 30, 60, 120 and 240 minutes later; GBL and GHB levels were determined in cortex and striatum. In these two regions, valproate induced a rapid and strong increase of GHB and GBL levels (about 180%) for 30 minutes; then the content of the two GABA metabolites decreased slightly until a plateau was reached (Fig. 3). This rapid accumulation of GHB observed 30 minutes after enzymatic inhibition of its metabolization was used to determine the rate of GHB synthesis by calculating the difference between GHB content 30 minutes after treatment with valproate and the control level. In the different regions investigated, the accumulation of GBL caused by valproate was of the same order of magnitude as for GHB (180%); but these increases never reached the level of significance (results not shown).

GHB and GBL content and rate of synthesis in rats with SWD compared to controls

The concentrations of endogenous GHB and of its prodrug GBL were measured in hippocampus, thalamus and frontal cortex in GAERS and compared to those observed in rats from the selected control group (Table 2, Fig. 4). Levels of GHB in GAERS were never different from those observed in control animals. Cortical and hippocampal GBL concentrations were also similar in both strains (results not shown).

The rate of GHB synthesis was assessed in GAERS as well as in control rats by reference to the valproate-induced accumulation of GHB (Table 2 and Fig. 4). The increases in GHB concentrations in GAERS were not different from control rats (Table 2, Fig. 4). The same is true of the valproate-induced increases in cortical and hippocampal GBL content in rats with SWD and in those without SWD (results not shown).

Selectivity of interactions of GHB with $GABA_B$ receptors

GHB interacted with the $GABA_B$ receptors with an IC_{50} of 1.5×10^{-4} M. This value was obtained in three different experiments using [^{3}H]baclofen as radioligand and membranes prepared from cerebral cortices according to

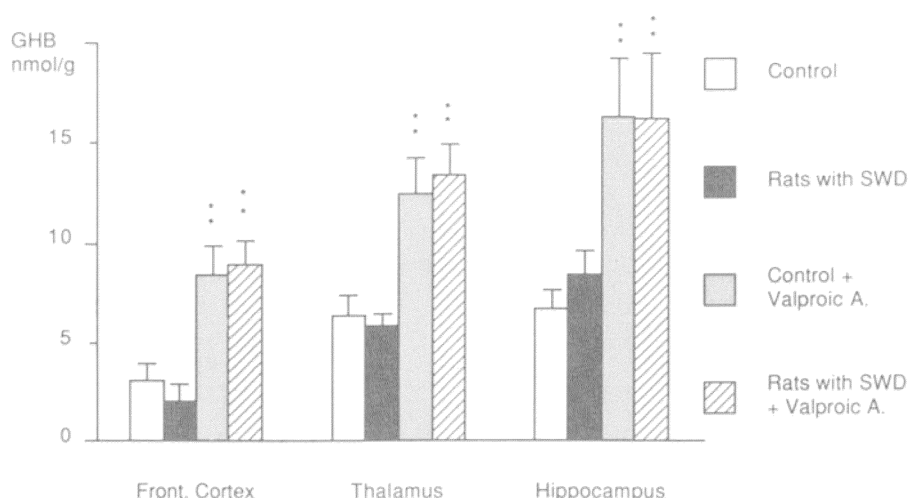


Fig. 4. GHB levels and GHB rate of synthesis in rats with SWD as compared to "non-epileptic" control rats. Animals treated with valproate were sacrificed 30 min later. Results are the mean ± S.E.M. for groups of ten rats. $^{**}p < 0.01$ when compared to the respective control group (Dunnett's test)

Table 2. Kinetic parameters for the synthesis of GHB in rats with SWD as compared to controls

Brain areas	Control GHB content nmol/g	GHB content after valproate nmol/g	Initial rate of GHB synthesis nmol/g/h	Turnover time h
Cortex GAERS	2.02 ± 0.83	8.90 ± 1.14**	13.76	0.15
Cortex control	3.06 ± 0.84	8.31 ± 1.54**	10.50	0.29
Hippoc.GAERS	8.34 ± 1.15	16.17 ± 3.34**	15.68	0.53
Hippoc.control	6.67 ± 0.91	16.30 ± 2.86**	19.24	0.35
Thalam.GAERS	5.69 ± 0.71	13.38 ± 1.46**	15.38	0.37
Thalam.control	6.25 ± 1.05	12.47 ± 1.83**	12.44	0.50

Wistar rats from the colony of Strasbourg (GAERS) were treated with valproate, killed 30 min later and GHB levels were determined in dissected brain regions. Control GHB concentrations were determined in animals receiving saline. All values are means ± S.E.M. for 10 animals per group and refer to wet weight.
Statistical significance of difference was calculated by Dunnet's test: $^{**}p < 0.01$

Bernasconi et al. (1986). Interactions with other receptors (including $GABA_A$ and central benzodiazepine receptors) were absent at a concentration of 100 μM (Table 3). GBL did not interact with the 12 receptors listed in Table 3, including $GABA_B$ receptors, at a concentration of 100 μM. Thus, the interaction of GHB with $GABA_B$ receptors appears to be selective.

For the measurement of the interaction of GHB with $GABA_B$ receptors in GAERS as compared to control rats, the potent and highly selective

Table 3. Inhibition of binding by γ-butyrolactone (GBL) and by γ-hydroxybutyric acid (GHB) in 12 receptor binding assays

Putative receptor	Radioligand	Inhibition of binding (% at 10^{-4} M) GHB	GBL	Method
α_1-Adrenergic	[^{3}H]prazosin	0	0	Greengrass and Bremner (1979)
α_2-Adrenergic	[^{3}H]clonidine	0	0	Tanaka and Starke (1980)
β-Adrenergic	[^{3}H]DHA	0	0	Bylund et al. (1976)
5-HT_1	[^{3}H]5-HT	0	0	Nelson et al. (1978)
$Histamine_1$	[^{3}H]doxepine	5*	0	Tran et al. (1981)
Muscarinic	[^{3}H]QNB	0	0	Yamamura et al. (1974)
Mu-opiate	[^{3}H]naloxone	0	0	Bradbury et al. (1976)
$GABA_A$	[^{3}H]muscimol	0	0	Beaumont et al. (1978)
$GABA_B$	[^{3}H]baclofen	44**	0	Bernasconi et al. (1986)
Benzodiazepine	[^{3}H]flunitrazepam	0	0	Speth et al. (1978)
Adenosine A1	[^{3}H]CHA	0	0	Patel et al. (1982)
Substance P	[^{3}H]substance P	0	0	Bittiger et al. (1982)

The receptor binding assays were performed essentially as described in the references. Abbreviations: *DHA* dihydro-alprenolol; *5-HT* serotonin; *QNB* quinuclidinyl benzylate; *CHA* cyclohexyl-adenosine. * =55% at 10^{-3} M; ** IC_{50} = 1.5×10^{-4} M obtained from 3 inhibition curves

Table 4. Interactions of γ-hydroxybutyric acid with $GABA_B$ receptors in rats with SWD and in rats from the selected control group
IC_{50} in μM

Brain structures	Control rats	Rats with SWD
Cortex	152.5	132.2
Cerebellum	138.0	168.4
Thalamus	166.7	157.2

Membranes were prepared from male Wistar rats from the breeding colony at the Centre de Neurochimie, C.N.R.S., Strasbourg according to Bittiger et al. (1990). The radioreceptor assay was performed with [^{3}H]CGP 27492 as radioligand according to Bittiger et al. (1990)

$GABA_B$ radioligand, [^{3}H]CGP 27492, was used (Bittiger et al., 1988, 1990). The IC_{50} ranged from 1.38×10^{-4} M in the cerebellum to 1.66×10^{-4} M in the thalamus and were similar in GAERS and in control rats (Table 4) and not different from the IC_{50} values obtained with [^{3}H]baclofen as radioligand and membranes prepared from cerebral cortices (Table 4).

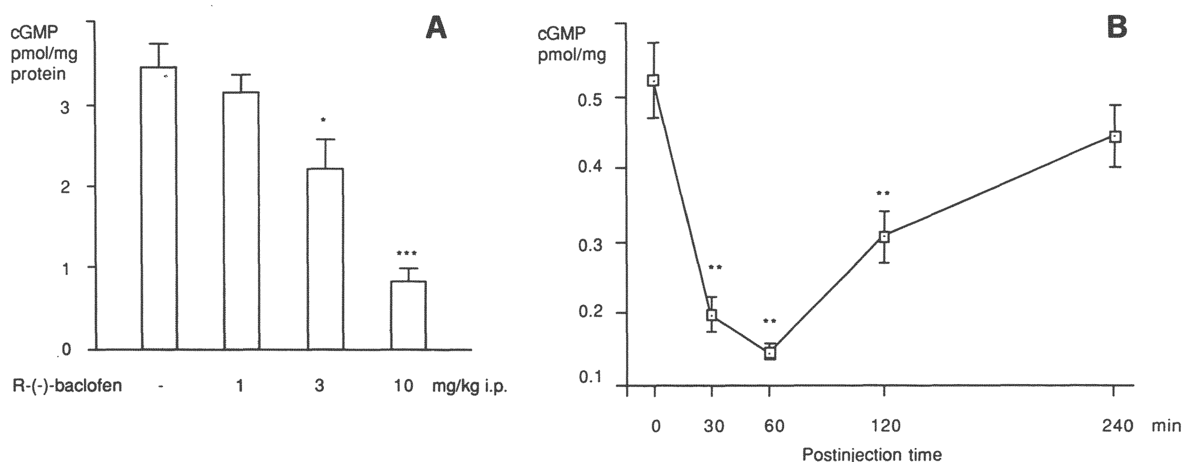


Fig. 5. Cerebellar cGMP concentrations in mice exposed to R(−)-baclofen. **A** Dose-dependent decrease in cGMP content. Animals (n = 8) were administered R(−)-baclofen and killed 60 min later, controls received 0.9% saline. cGMP levels were determined by radioimmunoassay and expressed as mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.001$ (Dunnett's test). **B** Time course of cGMP levels in the cerebellum following administration of R(−)-baclofen (6 mg/kg, i.p.). Each value represents the mean ± S.E.M. of 8 mice. Controls received 0.9% saline and were killed 30 min later

Effects of GHB and the $GABA_B$ receptor agonist R(−)-baclofen on cerebellar cGMP content

The $GABA_B$ receptor agonist R(−)-baclofen dose-dependently decreased cerebellar cGMP levels (Fig. 5A). The threshold dose 60 min after injection was between 1 and 3 mg/kg i.p. (56% of control at 3 mg/kg) and the content of cGMP after 6 mg/kg R(−)-baclofen was 28% of control value and decreased to 20% at 10 mg/kg. Figure 5B shows the time-course for the decrease of cerebellar cGMP observed after injection of 6 mg/kg of the agonist. The onset of the decrease of cGMP content caused by R(−)-baclofen was very rapid; 30 min after administration of R(−)-balcofen cGMP levels were 38% of controls and decreased further to 28% 60 min after drug treatment. Then, levels of the second messenger increased again and reached 59% of control values 2 hours after administration of R(−)-baclofen. After 4 hours cerebellar cGMP concentrations were normalized and ataxia had disappeared in mice. This suggests that the behavioural effects induced by R(−)-baclofen correlate with the decrease in cGMP.

GBL decreased cGMP levels in a dose-dependent manner (Fig. 6A). While 100 mg/kg GBL i.p. did not alter cerebellar cGMP levels significantly 45 min after administration of the drug (74%), the reductions by 200 mg/kg GBL i.p. (42%) and 400 mg/kg GBL i.p. (24%) were statistically significant ($p < 0.01$). The time course of the effect of GBL on levels of cerebellar cGMP is shown in Fig. 6B. After intraperitoneal administration of

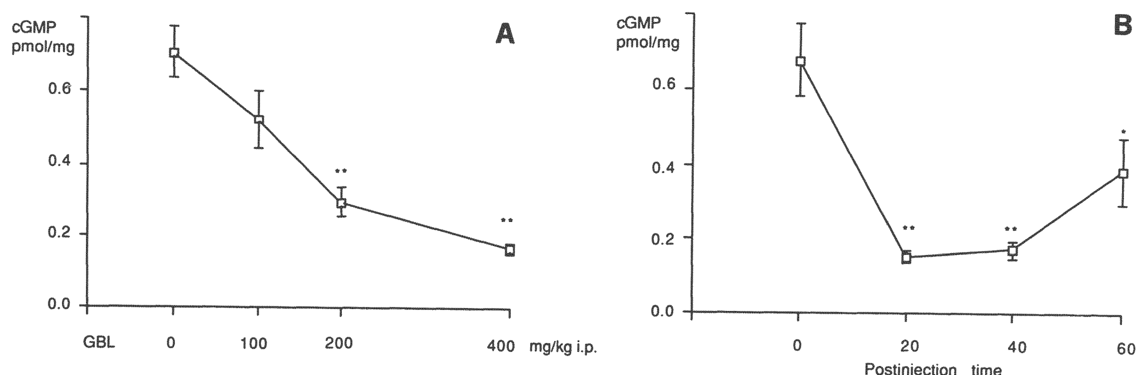


Fig. 6. Effect of GBL on cGMP content in the cerebellum of mice. **A** Decrease in cGMP in function of the doses of GBL. The drug was administered 45 min before microwave irradiation. Each value represents the mean ± S.E.M. of 8 animals. Statistical significance was calculated by Dunnett's test. **B** Time course of cGMP concentrations in the cerebellum of mice following injection of 200 mg/kg GBL. Each point represents the mean ± S.E.M. of 8 animals. $^{*}p < 0.05$, $^{**}p < 0.01$

200 mg/kg of GBL, there was a rapid and marked decrease in the content of cGMP, the maximal effect being achieved after 20 min (22%). This effect lasted up to 40 min (25%); then levels of cGMP increased slightly and reached 57% of control value after 60 min. Similar results were also observed in cerebellum and thalamus of rats after treatment with 3.5 mmol/kg GBL (results not shown).

Antagonism of the decrease of cGMP caused by R(−)-baclofen and GBL by the $GABA_B$ receptor antagonist CGP 35348

The $GABA_B$ receptor antagonist CGP 35348 at doses of 100 and 200 mg/kg i.p. did not alter cerebellar cGMP levels of mice; at 400 mg/kg a slight (about 20%) and occasionally significant increase of cGMP was observed (results not shown). However, the 60% decrease of cGMP content induced by 4 mg/kg i.p. R(−)-baclofen was completely antagonized by 200 mg/kg i.p. CGP 35348 (Fig. 7).

The marked decrease (35% of control value) of cerebellar cGMP concentration observed in mice 40 min after injection of 200 mg/kg i.p. GBL was significant, but only partially antagonized (58% of control value, = 36% antagonism, N = 3) by 200 mg/kg i.p. CGP 35348 given 5 min before GBL (Fig. 8). Similar results were observed in mice when GBL (200 mg/kg) was injected 40 min after 200 mg/kg CGP 35348 and the animals were killed 20 min later by microwave irradiation (results not shown). In addition, the decrease of cGMP caused by 200 mg/kg GBL was also only partially, but significantly, antagonized by pretreatment with the high dose of 400 mg/kg CGP 35348 (CGP 35348 was given 30 min before GBL and 50 min before

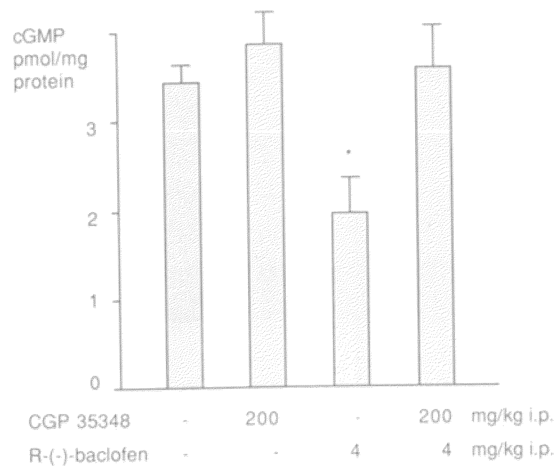


Fig. 7. Antagonism by CGP 35348 of the decrease in levels of cGMP in the cerebellum induced by R(−)-baclofen. CGP 35348 was injected 20 min before R(−)-baclofen and mice were sacrificed 60 min after treatment with the $GABA_B$ receptor antagonist. Each group represents the mean ± S.E.M. of 8 animals. $^{*}p < 0.01$

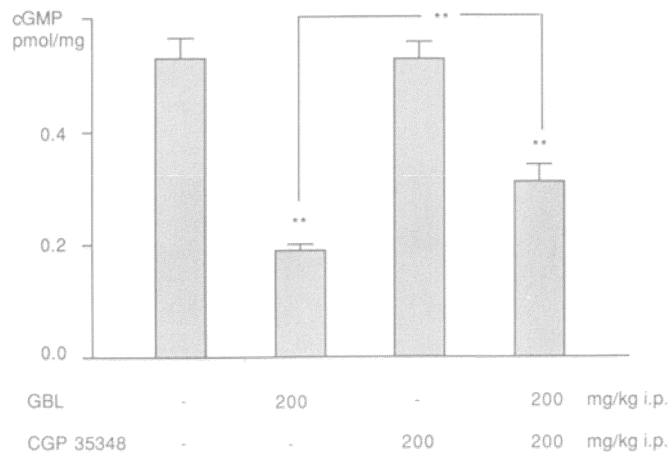


Fig. 8. Partial antagonistic effect of CGP 35348 on the decrease of cerebellar cGMP produced by GBL. CGP 35348 was injected 5 min before GBL and mice were killed 40 min later. The results are the mean ± S.E.M. of duplicate determination. $^{**}p < 0.01$ (Dunnett's test)

sacrifice, control values = 100%, CGP 35348 = 123% N.S., GBL = 23%*, CGP 35348 + GBL = 35%$^{*,\#}$; $^{*}p < 0.01$ versus control, $^{\#}p < 0.05$ versus GBL group). A partial antagonism of the decrease of cerebellar cGMP was observed in rats when 3.5 mmol/kg GBL were injected 30 min after 200 mg/kg i.p. CGP 35348 and animals sacrificed 60 min after the administration of the $GABA_B$ receptor antagonist (results not shown).

4. Discussion

GHB and GBL levels and rates of synthesis

In order to test the possibility that GBL and GHB may be present at different concentrations in GAERS and in control rats, a sensitive method for the determination of these two compounds had to be developed. The difficulty of measuring small amounts of GBL and GHB lies in the capacity of the two substances to interconvert as a function of pH, the free acid (GHB) being stable only at pH > 7 and the lactone (GBL) and pH < 7. Consequently, the synthesis of any ester derivatives of GHB under acidic conditions leads inevitably to their conversion into the thermodynamically more stable cyclic derivative GBL. Control experiments under various conditions of the derivatization, followed by GC-MS analysis demonstrated that the yields of ester derivatives of GHB were less than 1% (J. Lauber, unpublished results). We utilized this characteristic of the GHB/GBL system and measured only GBL, that was present normally, and that which was formed from GHB.

The use of the GC/MS assay procedure for GHB and GBL described herein has several advantages. It is simple and gives high recovery yields since it does not require derivatization of the GHB molecule (Eli and Cattabeni, 1983; Ehrhardt et al., 1988) nor a series of washings and re-extraction (Snead et al., 1982). This procedure is very similar to the one recently described by Snead et al. (1989). Like this latter method, it allows clear differentiation between GHB and GBL by altering the conditions of extraction of the brain. The regional levels of GHB (between 2.12 nmol/g in the cortex and 4.67 nmol/g in the striatum) found in the present study are close to the values reported by Eli and Cattabeni (1983) and by Vayer et al. (1988) for age-matched rats killed by microwave irradiation.

There are several lines of evidence that, unlike experimental models of generalized convulsive and partial seizures where the hippocampus plays a dominant role, clinical and experimental absence seizures are characterized by perturbations of thalamocortical mechanisms with no significant involvement of the hippocampus (Avoli and Gloor, 1982; Gloor and Fariello, 1988; Steriade and Llinas, 1988; Vergnes et al., 1987, 1990). If an alteration of GHB and GBL levels, or of their rate of synthesis, is implicated in the pathogenesis of petit-mal epilepsy it should in all probability occur in the frontal cortex and in the thalamus, and no effect should be observed in the hippocampus.

Tissue concentration of GHB in the cortex at the onset of SWD induced with threshold doses of either 3.5 mmol/kg i.p. GHB or GBL was reported by Snead (1991) to be 240 ± 31 nmol/g. This threshold cortical concentration is one hundredfold higher than the naturally occurring content found in this and earlier studies. Thalamic GHB levels were not reported in Snead's study. Assuming that GHB is disributed more or less evenly throughout the frontal cortex and thalamic nuclei, and that high local concentrations do not

exist it would seem unlikely that the endogenous concentrations observed in the present study could exert a significant inhibitory effect capable of inducing SWD. This study has also demonstrated that GHB and GBL levels measured in the three structures were not different in GAERS from the levels measured in control rats (Fig. 4). These findings suggest that endogenous GHB or GBL concentrations do not play an important role in the pathogenesis of absence epilepsy. This hypothesis is also supported by the fact that ethosuximide and valproate have opposite effects on endogenous GHB levels of rat brains. The concentration of GHB decreases after chronic treatment with ethosuximide (Snead et al., 1980), but dose-dependently increases after administration of valproate (Vayer et al., 1988; this study).

Measurement of the rate of GHB synthesis would be a more reliable index of GHBergic functional activity than assessment of regional concentrations. The first observation of an accumulation of GHB following acute treatment with valproate was reported by Snead et al. (1980), and Vayer et al. (1988) used this model to estimate GHB turnover rates in discrete brain regions of the rat. Our results confirm their findings and describe regional variations in the kinetics of these accumulations. The initial rates of GHB and GBL synthesis observed in GAERS and rats from the selected control group are of the same order of magnitude as those reported by Vayer et al. (1988). The data of this study are also in good accordance with the turnover time of GHB in total brain (26 min) determined by Gold and Roth (1977) using [^{3}H]GABA as the GHB precursor.

This increase of GHB levels is due to the fact that valproate does not alter the biosynthesis of GHB, but inhibits its degradation, causing a rapid accumulation of the substance (Vayer et al., 1988). GHB accumulation does not induce a feedback inhibition of GHB synthesis, since no significant effect of GHB has been observed on its synthetic enzyme (Rumigny et al., 1980). Thus, the use of valproate for the determination of GHB turnover seems to be justified. However, these measurements do not necessarily reflect the turnover rate of GHB in the synapses, but rather the overal rate of GHB synthesis in brain.

The rate of GHB synthesis in rats in GAERS was not significantly different from the one observed in rats from the selected control group. These data indicate that the pharmacological basis for the mechanism underlying the occurrence of cortical SWD in the genetic model of absence seizures developed in Strasbourg lies elsewhere, and Snead et al. (1990) have reported an increase in the density of low affinity GHB binding sites in GAERS. However, this observation does not exclude other mechanisms.

Interactions between GHB and $GABA_B$ receptors

As CGP 35348 antagonized the SWD caused by GHB (Marescaux et al., 1992) we tested the affinity of GHB and GBL for $GABA_A$, $GABA_B$ and

benzodiazepine receptors. We observed a selective, but weak affinity of GHB for $GABA_B$ receptors. Similar IC_{50} values of GHB for displacing $[^3H]$CGP 27492 from $GABA_B$ binding sites were observed in three different brain structures in GAERS and in control rats (Table 4). Analysis of the binding data is consistent with a single binding site and does not suggest allosteric interactions between GHB and $GABA_B$ receptors.

An IC_{50} value of the order of 140 µm for GHB and brain levels of 2.12 nmol/g GHB suggest that GHB stimulation of $GABA_B$ receptors is not the mechanism underlying the pathogenesis of SWD in GAERS. A functional involvement of GHB in the pathogenesis of GAERS would appear improbably unless GHB were highly concentrated locally (about 200 nmol/g) in brain structures such as the lateral thalamus from where SWD emanate, and in view of the average thalamic concentration of 4 nmol/g found in this and other studies that seems unlikely.

On the other hand, according to Snead (1991), the cortical GHB concentrations at the onset of SWD after injection of the threshold dose of 3.5 mmol/kg i.p. GBL are 240 ± 31 nmol/g, i.e., sufficient to stimulate $GABA_B$ receptors. Thus, non-anaesthetic doses of GBL could stimulate $GABA_B$ receptors, and the generation of SWD in the GHB model of absence epilepsy might be due to the $GABA_B$ receptor agonist-like properties of GHB.

These results suggest that $GABA_B$ receptors could assume an important role in absence epilepsy. A dose-related increase in SWD after stimulation of $GABA_B$ receptors with R(−)-baclofen was first described by Vergnes et al. (1984). In human studies, absence attacks have been found to occur more frequently during treatment with baclofen (Gloor and Fariello, 1988).

As $GABA_A$ receptor agonists such as muscimol and THIP exacerbate experimental absence seizures, it was originally thought that $GABA_A$-mediated mechanisms must be involved in the pathogenesis of absences. $GABA_A$ receptor antagonists do not block experimental absence (Micheletti et al., 1985). Because of these negative results and because of the aggravating effects of R(−)-baclofen in experimental absences, we tested the novel, centrally active $GABA_B$ receptor antagonist, CGP 35348, in several animal model of absence seizures. CGP 35348 markedly and dose-dependently decreases SWD in GAERS and antagonizes the aggravating effects of agents which enhance GABA-mediated inhibition (vigabatrin, muscimol, THIP, SKF 89976). In addition, SWD induced by GHB were also antagonized by this $GABA_B$ receptor antagonist (Marescaux et al., 1992; Snead, personal communication). These results demonstrate that $GABA_B$ receptors are primarily involved in the genesis of SWD (Marescaux et al., 1992) and confirm the hypothesis of Crunelli and Leresche (1991), that activation of $GABA_B$ receptors mediates a late and long-lasting inhibitory postsynaptic potential (IPSP) which is critical for the generation of SWD.

The evaluation of the functional relevance of the in vitro binding results requires in vivo paradigms in which the effects of GHB on $GABA_B$ receptors can be demonstrated. The alterations of cerebellar cGMP levels induced by agonists and antagonists of several receptors, and in particular

of $GABA_B$ receptors, have been used as a biochemical index of the signal transduction (Wood, 1991). Recent autoradiographic studies have demonstrated a clear topographic $GABA_B$ receptor distribution with parasagittal zones of high and low density of binding (Albin and Gilman, 1989; Bowery et al., 1987). These cerebellar $GABA_B$ receptors, when activated, decrease cGMP levels (Gumulka et al., 1979). We have confirmed the findings of Gumulka et al. demonstrating that the potent and selective $GABA_B$ receptor agonist R(−)-baclofen decreases cerebellar cGMP in a dose- and time-dependent fashion (Fig. 5A and 5B). GBL produces the same effect (Fig. 6A and 6B).

In this biochemical paradigm, R(−)-baclofen and GHB are roughly equal in efficacy, although the decrease observed after injection of GHB occurs faster than that induced by R(−)-baclofen. The ratio of the doses of R(−)-baclofen (6 mg/kg i.p.) and GBL (400 mg/kg i.p.) needed to bring about the same decrease in cGMP does not correspond to ratio of IC_{50} binding values (30 nM for baclofen as against 140 μM for GHB). This discrepancy may be due to better penetration of GBL into the brain and/or to the fact that GHB interacts not only with $GABA_B$ receptors, but also with several neurotransmitter systems, including the noradrenergic, dopaminergic, serotonergic and cholinergic systems (Snead, 1977). All these neurotransmitter systems have been implicated in the control of cerebellar cGMP levels (Wood, 1991). Hence, GHB could exert an effect through partly different neurochemical systems to produce SWD and to decrease cerebellar cGMP content.

This led us to check whether the decrease in cGMP levels induced by GBL and R(−)-baclofen could be solely attributable to $GABA_B$ receptor activation. CGP 35348 should differentiate between effects of these two compounds that are related and those not related to the $GABA_B$ receptor. The results shown in Fig. 7 demonstrate that 200 mg/kg i.p. CGP 35348 fully antagonizes the decrease of cerebellar cGMP produced by 4 mg/kg i.p. R(−)-baclofen. The hitherto available evidence indicated that the dose of CGP 35348 needed to suppress the SWD induced by administration of 4 mg/kg i.p. R(−)-baclofen was also 200 mg/kg i.p. (Marescaux et al., 1992). This relation between the doses of the $GABA_B$ receptor agonist and antagonist is the same in the two experiments. By contrast, CGP 35348, which by itself has no effect on cGMP levels at doses up to 200 mg/kg i.p., only partially antagonizes the decrease caused by 200 mg/kg i.p. GBL at 200 mg/kg (Fig. 8) and at the very high dose of 400 mg/kg i.p. (results not shown). Thus, CGP 35348 blocked all the effects caused by the $GABA_B$ receptor agonist R(−)-baclofen, but totally antagonized only the increase in the duration of SWD induced by GHB, indicating that these effects may be $GABA_B$ receptor mediated. The decrease in cerebellar cGMP concentrations elicited by GHB was only partially antagonized by CGP 35348 and is therefore only partially mediated through $GABA_B$ receptors.

In sum, the results of this study do not support the hypothesis that endogenous GHB plays an important role in the pathogenesis and control of generalized absence seizures in GAERS. The cortical levels of GHB

reached after administration of GBL are likely to stimulate $GABA_B$ receptors and thus to induce SWD. These findings, in conjunction with those of Marescaux et al. (1992) underline the primary importance of $GABA_B$ receptors in experimental absence epilepsy and in the genesis and control of spontaneous SWD in GAERS (Crunelli and Leresche, 1991). $GABA_B$ receptor antagonists may represent a new class of anti-absence drugs.

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