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GAMMA HYDROXYBUTYRATE DISTRIBUTION AND TURNOVER RATES IN DISCRETE BRAIN REGIONS OF THE RAT

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Abstract—Gamma-hydroxybutyric acid and trans-gamma-hydroxycrotonic acid levels have been determined in 24 regions of the rat brain after sacrifice by microwave irradiation. Concentration ranges are from 4 pmol/mg protein (frontal cortex) to 46 pmol/mg protein (substantia nigra) for gamma-hydroxybutyric acid and from 0.4 pmol/mg protein (striatum) to 11 pmol/mg protein (hypothalamus) for trans-gamma-hydroxycrotonic acid. It appears that gamma-hydroxybutyric acid levels correlate well with GABA distribution in the same region. However this correlation is not evident with regard to the distribution of the gamma-hydroxybutyric acid synthesizing enzyme, specific succinic semialdehyde reductase. Using the antiepileptic drug, valproate which strongly inhibits gamma-hydroxybutyric acid release and degradation, we estimated the turnover rate of this compound in six regions of the rat brain. Turnover numbers ranged from 6.5 h^{-1} in hippocampus to 0.76 h^{-1} in cerebellum.

Gamma-hydroxybutyrate (GHB) in rat brain is primarily a GABA metabolite (Anderson *et al.*, 1977; Gold and Roth, 1977). During the last 6 years, much evidence has been presented which is in favour of a transmitter role for this substance in brain (Maitre and Mandel, 1984). In particular, characterisation of its synthesis (Cash *et al.*, 1979; Rumigny *et al.*, 1980; Rumigny *et al.*, 1981a; Rumigny *et al.*, 1981b), release (Maitre *et al.*, 1983), transport (Benavides *et al.*, 1982a), turn-over (Gold and Roth, 1977), binding to synaptosomal membranes (Benavides *et al.*, 1982b; Snead and Liu, 1984) and degradation (Vayer *et al.*, 1985b) clearly suggest important functions in neuromediation. This role is also supported by a discrete regional distribution and ontogeny (Snead and Morley, 1981). However, no precise distribution in the brains of animals killed by microwave irradiation has yet been described. Indeed, taking into account the reported kinetics and characteristics of gamma-hydroxybutyrate binding, transport and degradation, it is important to know with precision the distribution pattern and levels of this molecule in brain. These results could constitute, together with studies of local turn-over rate, an index of regional GHBergic functional activity.

In the present study, using microwave irradiation to kill the animals and negative ion mass spectrometry to measure regional endogenous GHB levels, we describe the distribution of GHB in 24 regions of the rat brain. In parallel, a similar study has been made concerning trans-gamma-hydroxycrotonic acid (HCA), a structural analogue of GHB, whose presence in rat brain as an endogenous substance has recently been demonstrated (Vayer *et al.*, 1985a). After treatment of rats with valproate, a time and dose dependent accumulation of GHB is described in the six regions of the rat brain investigated. This rapid accumulation which is due to an inhibition of release and degradation of GHB, has been used as a method to calculate regional turnover numbers for this substance in the rat brain.

EXPERIMENTAL PROCEDURES

1. Determination of GHB regional levels

Male Wistar rats (300–350 g) were sacrificed by focused microwave irradiation for 1.7 s (Püschner Apparatus 10 kW output 75%). Brains were removed and 8 slices were cut at the following coordinates with respect to lambda (zero point): (0, +1); (+1, +3.5); (+3.5, +5.5); (+5.5, +9.5); (+9.5, +11.5); (+11.5, +15.5); (−1, −2); (−2, −8). 24 areas were dissected out on a glass plate over liquid

nitrogen, and stored in liquid nitrogen. The extraction and derivatization of GHB and HCA were carried out as previously described (Ehrhardt *et al.*, 1987) with the following modifications. Homogenization of the various brain regions was performed in a mixture of ethanol–water (80:20 v/v) at 0°C containing the internal standards ([2,3-d_2]GHB and beta-methyl trans-gamma-hydroxycrotonic acid (MHCA)). After centrifugation (20 min 50,000 *g*) supernatants were derivatized with pentafluorobenzyl bromide and N-ter.-butyldimethylsilyl-N-methyltrifluoroacetamide in acetonitrile as previously reported (Ehrhardt *et al.*, 1988). The derivatized products were submitted to mass spectrometry analysis in an LKB 2091 mass spectrometer modified for negative ion detection and equipped with a Pye 104 gas chromatograph. Chromatographic and detection conditions are identical to those previously described (Ehrhardt *et al.*, 1988). Measurements were performed by detection of characteristic ions $m/z = 217$ for GHB, $m/z = 219$ for [2,3-d_2]GHB, $m/z = 215$ for HCA and $m/z = 229$ for MHCA.

Protein was measured by the Lowry method (Lowry *et al.*, 1951) on the centrifugated pellets after solubilization in 2 M NaOH. A schematic representation of the GHB distribution pattern was carried out using an arbitrary colour code. Diagrams of brain regions were computerized (Victor VPC2 equipped with a 8087 coprocessor) using a data tablet and a graphic program (D-Calc from JPK-Conseil).

2. Turnover rate estimation

Valproate (sodium salt) was injected IP into male Wistar rats (300–350 g) at doses 200, 400, 600 mg/kg before sacrifice by focused microwave irradiation under the same conditions as described above 20 min after injection. In parallel experiments, the time dependent accumulation of GHB following a valproate dose of 400 mg/kg was determined from 0 to 90 min at chosen intervals. In every case, GHB levels were determined by the method previously described in 6 regions of the rat brain: dorsal hippocampus, pineal gland, hypothalamus, cerebellum, striatum, temporo-parietal cortex. Turnover rates were estimated by following the kinetics of accumulation of GHB in the linear part of the different curves obtained.

RESULTS

1. GHB levels in discrete rat brain areas

The amount of GHB and HCA in 24 regions of the rat brain are indicated in Table 1. Taking into account the very low level of GHB in blood (Nelson *et al.*, 1981), the errors induced by the variable blood contamination among regions is insignificant when compared to the unavoidable experimental variations. The richest structures for GHB are substantia nigra, hypothalamus, superior colliculi and pineal gland, whereas the cortical regions are relatively poor. The distribution pattern of HCA follows in general that of GHB, except for olfactory tubercles, septum and dorsal hippocampus which contain relatively high amounts of HCA. In contrast striatum, hypothalamus, thalamus and pineal gland contain a lower proportion of HCA compared to GHB. In 18 regions (among the 24 regions studied, i.e. if we eliminate posterior hypothalamus, olfactory tubercles, striatum, pineal gland, superior colliculi and posterior thalamus) a Student t-test reveals a linear correlation between HCA and GHB levels ($r = 0.70$ with $P < 0.01$). Figure 1 shows the general distribution pattern of GHB in rat brain using an arbitrary colour code. This representation illustrates the heterogeneous distribution of GHB in the brain regions investigated.

Table 1. Concentrations of GHB and HCA in dissected brain areas

Abbreviations	Structures	GHB levels (pmol/mg protein)	HCA levels (pmol/mg protein)
SN	Substantia nigra	46 ± 6	6.9 ± 0.3
Hyp	Posterior hypothalamus	42 ± 3	11 ± 1
SC	Superior colliculi	40 ± 9	n.d.
Pin	Pineal gland	40 ± 4	n.d.
Hya	Anterior hypothalamus	39 ± 7	3.0 ± 0.4
THa	Anterior thalamus	36 ± 3	4.7 ± 1.2
IC	Inferior colliculi	32 ± 3	4.5 ± 0.5
THp	Posterior thalamus	30 ± 4	n.d.
OB	Olfactory bulbs	27 ± 9	5.1 ± 0.5
A	Amygdala	24 ± 7	4.1 ± 0.1
PCx, CCx	Parietal, Cingulate } Cortex	21 ± 5	4.3 ± 0.7
Rad + m	Raphe	19 ± 3	2.8 ± 0.5
CP.GP	Striatum	18 ± 1	0.4 ± 0.1
Pit	Pituitary gland	15 ± 4	1.9 ± 0.2
S	Septum	15 ± 1	4.7 ± 0.9
TCx, ppCx, pCx	Temporal, Prepiriform, Piriform } Cortex	13 ± 2	2.6 ± 0.3
Hid	Dorsal hippocampus	12 ± 1	6.2 ± 1.2
Hiv	Ventral hippocampus	12 ± 3	1.6 ± 0.5
OCx, Ent.Cx	Occipital, Entorhinal } Cortex	12 ± 2	2.6 ± 0.7
OT	Olfactory tubercles	9 ± 1	4.8 ± 0.4
C	Cerebellum	8 ± 2	1.4 ± 0.2
M	Medulla oblongata	8 ± 1	1.3 ± 0.1
pFCx	Prefrontal cortex	6 ± 1	1.2 ± 0.3
FCx, ppCx	Frontal, Prepiriform } Cortex	4.0 ± 0.5	0.7 ± 0.1

Values are the mean ± SEM of six different rats (n.d. = not detected).

2. Time course of GHB accumulation in rats treated with valproate (400 mg/kg IP)

Rats were treated with valproate (400 mg/kg IP) and killed by microwave irradiation at time 0 to 90 min every 15 minutes. Then, GHB levels were determined in cerebellum, pineal gland, hypothalamus, striatum, temporo-parietal cortex. For hippocampus, the accumulation of GHB is more rapid and GHB levels were determined every 5 min for 45 min. In all regions investigated, valproate induced a rapid and strong increase of GHB level, except for cerebellum where accumulation is much lower. GHB content in all regions increases linearly for 20 min in

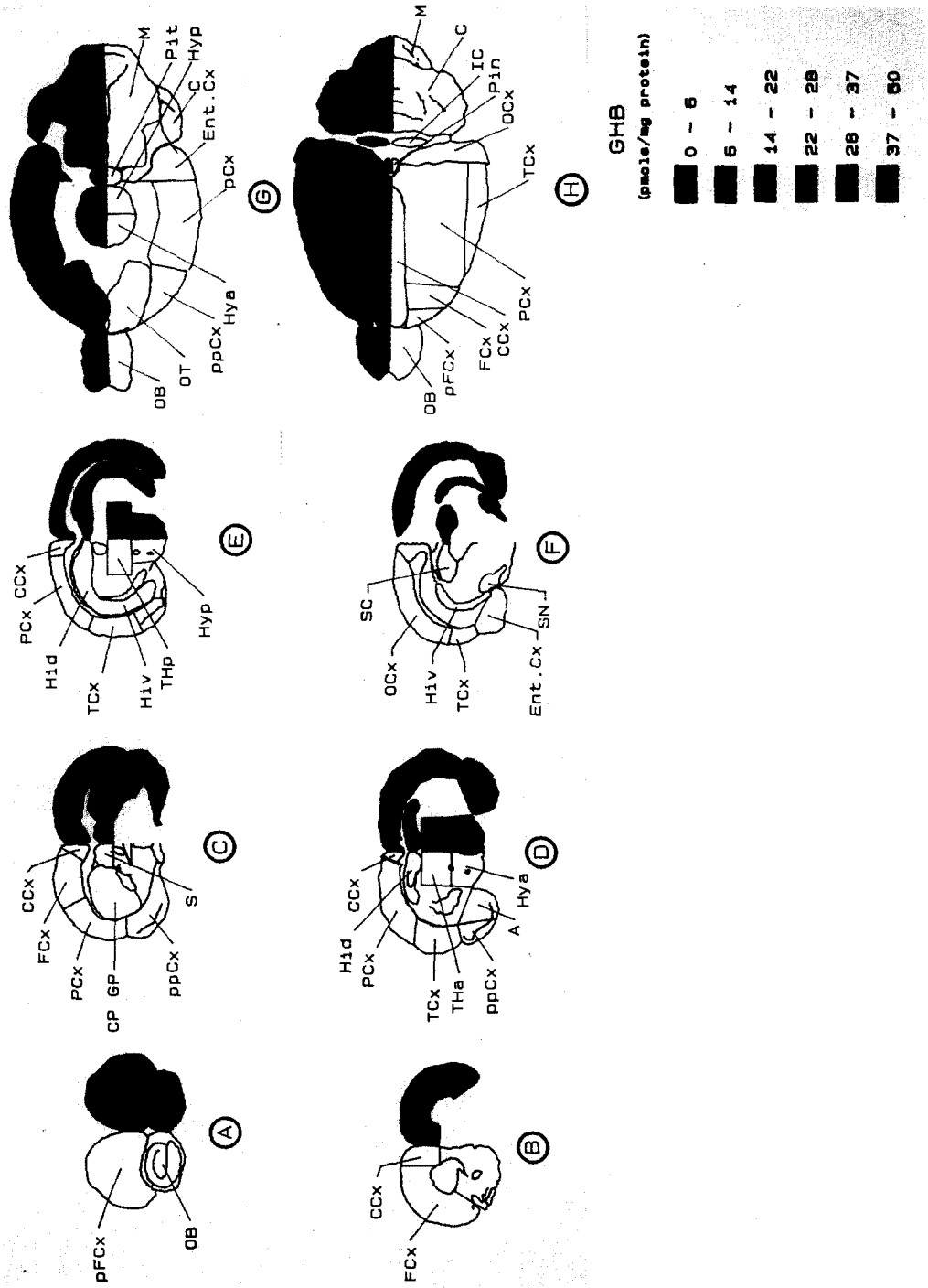

Fig. 1. Distribution of GHB in rat brain represented by an arbitrary colour code. Stereotaxic coordinates of sections (with respect to lambda-zero point-): A (+11.5, +15.5); B (+9.5, +11.5); C (+5.5, +9.5); D (+3.5, +5.5); E (+1, +3.5); F (0, +1); G (ventral view); H (dorsal view).

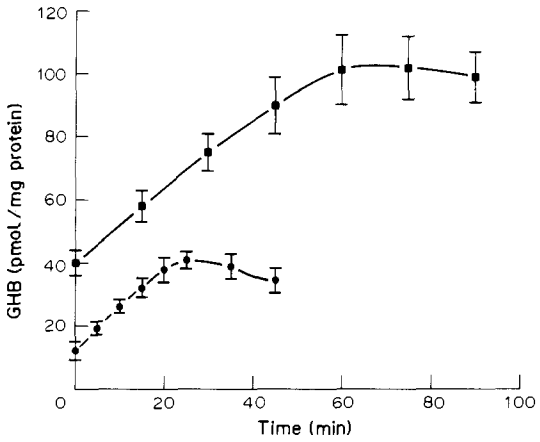

Fig. 2. Time course of GHB accumulation in dorsal hippocampus (●) and in hypothalamus (■) of rats treated with valproate (400 mg/kg IP). Results are the mean ± SEM from three animals.

hippocampus and at least for 60 min in the other regions tested. Then a plateau was reached, more rapidly in hippocampus (Fig. 2).

3. *Dose–response curve of valproate*

GHB levels in hypothalamus were determined 20 minutes after treatment with valproate (0—200—400—600 mg/kg IP). As shown in Fig. 3, GHB levels increased linearly in function of valproate doses. As the LD_{50} for valproate in rats is about 800 mg/kg, higher doses than 600 mg/kg were not tested. Twenty minutes after 400 mg/kg valproate, the increase of GHB in hypothalamus is around 75%.

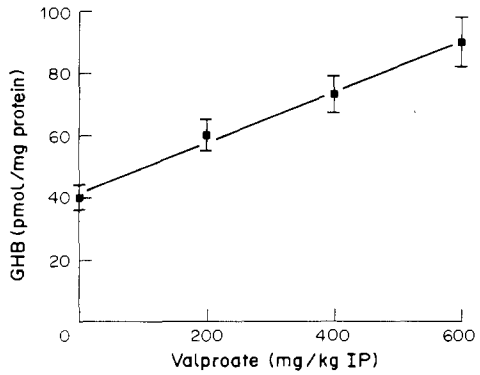

Fig. 3. Effect of valproate on GHB levels in hypothalamus. Rats were treated with different doses of valproate. GHB levels were determined 20 min after treatment with valproate. Each point represents the mean value ± SEM from three animals.

For valproate dosages of 400 mg/kg, the drug concentration in brain after 15 min is around 1400 μM (about 20 times the K_i for GHB dehydrogenase inhibition). Thus GHB degradation is completely inhibited. In fact, we have demonstrated the total inhibition of SSR_1 *in vitro* with 1 mM valproate (Rumigny *et al.*, 1980). Additional effect on GHB accumulation for 600 mg/kg valproate appears to be due to an enhanced inhibition of release from presynaptic stores (Fig. 3).

4. *Estimation of GHB turnover time*

After valproate 400 mg/kg IP, the time dependent accumulation of GHB in the different regions tested was used to determine the rate of GHB synthesis by the difference between GHB level after treatment and the control level. Usually, the linear GHB increase after 20 min treatment was used to calculate the rate of GHB accumulation (Table 2). In pineal gland, hypothalamus, temporo-parietal cortex and striatum, turnover times are of the same order of magnitude. In hippocampus, turnover is much more rapid but much more lower in cerebellum.

In the different regions tested, the accumulation of GHB starts rapidly (except for cerebellum) and is linear in every case for at least 20 min. At an early time, curves exhibit only one slope. Linear regression analysis of initial accumulation of GHB gives the following turnover times (Table 2): Hippocampus 9 min, pineal gland 35 min, hypothalamus 34 min, temporo-parietal cortex 47 min, striatum 30 min and cerebellum 78 min. Turnover time in hippocampus is about 8 times shorter than in cerebellum and about 3 to 4 times lower compared to other regions.

Table 2.

Brain region	Initial rate of GHB synthesis (pmol/min/mg protein)	Turnover time (min)	Turnover number (h^{-1})
Dorsal hippocampus	1.34 ± 0.30	9 ± 3	6.5
Striatum	0.60 ± 0.08	30 ± 5	2.0
Pineal gland	1.14 ± 0.25	35 ± 17	1.7
Hypothalamus	1.16 ± 0.20	34 ± 5	1.7
Temporo-parietal cortex	0.32 ± 0.03	47 ± 10	1.2
Cerebellum	0.10 ± 0.02	78 ± 14	0.7

The animals received 400 mg/kg valproate IP at starting time. Then three animals were killed every 15 min for 90 min and GHB was determined in dissected brain regions. For dorsal hippocampus, three animals were killed every 5 min for 20 min. All values are means ± SEM. Control GHB concentrations were determined in animals receiving saline.

Turnover time: time necessary to renew the whole compartment.

Turnover number: number of times the compartment was renewed per hour.

DISCUSSION

Conflicting reports exist about the possible post-mortem changes in brain GHB levels of animals sacrificed by decapitation. In particular, Eli and Cattabeni (1983) reported a lower level of GHB in the brain of animals dissected after irradiation in a microwave oven. For this reason, in order to avoid GHB increase during the time required for tissue dissection, the rats used in the present study were killed by microwave irradiation. In addition, sample preparation was performed by homogenization in a mixture of ethanol–water (80:20 v/v), thus we could exclude any possible lactonization of GHB during the extraction procedure. GHB is present in all the 24 brain areas studied and is distributed unevenly. The ratio of the highest (substantia nigra and hypothalamus) to lowest concentrations (frontal cortex) is about 10 to 1. This distribution pattern of GHB in rat brain can be compared with the similar study made for GABA, the main precursor of GHB in brain, on approximately the same regions of rat brain killed by microwave irradiation (Balcom *et al.*, 1975). The two distributions appear to be similar, the richest regions being in both cases substantia nigra, hypothalamus, colliculi and the poorest are cerebellum, medulla and cortex. The other regions contain intermediate concentrations. Thus the ratio GABA/GHB is always about 1000, increasing respectively to 1600 and 2000 for cerebellum and medulla. The correlation between the regional distribution of GHB and the amount of its synthesizing enzyme, succinic semialdehyde reductase (SSR_2) seems to be more erratic (Rumigny *et al.*, 1982). SSR_2 specific activity and GHB levels are well correlated for hypothalamus, colliculi (high levels), amygdala, hippocampus, striatum (medium levels) and frontal cortex (low levels). However, no correlation seems to exist in cerebellum, pons medulla, septum and olfactory bulbs. If we compare the regional density of binding sites for GHB (Benavides *et al.*, 1982b) and concentrations of this substance in the same region, no apparent correlation exists and this seems to indicate that GHB could have a different functional role in the different brain regions studied.

The regional levels of GHB given in the present study are close to the values reported by Eli and Cattabeni (1983) which concern however only 5 regions of the brains of rats killed by microwave irradiation. The values given by Snead and Morley (1981) are somewhat higher. This discrepancy could be the result of the difference in the time required for dissection of the tissue after decapitation. Values for HCA regional concentrations are about 50 times lower than the value of 220 pmol/mg protein for total brain reported in a previous publication (Vayer *et al.*, 1985a). Preliminary experiments have shown that this discrepancy cannot be attributed to the difference in the mode of sacrifice (decapitation versus microwave irradiation), in the extraction procedure (0.1 M formic acid versus 80% ethanol solution) or to the difference in the age of the rats used in the two studies. Although the method used in the present work for derivatization and quantification is somewhat different from those previously used (Vayer *et al.*, 1985a), it seems most probable that the first reported value for HCA was overestimated. It is possible that an interfering peak is the reason for the too high value reported for HCA. The actual amounts of HCA are about 1/10 of those for GHB in the same region. Thus, it seems that the relative importance of HCA in interfering with GHB functional activity in brain can be considered less credible.

The first report on an accumulation of GHB following acute treatment by valproate was reported by Snead *et al.* (1980). Our results confirm these findings and describe regional variations in the kinetics of these accumulations. The GHB increase can be explained by the two following phenomena. First, we have reported that valproate inhibits GHB release evoked by 40 mM K^+ from hippocampal and striatal slices preloaded with [^{3}H]GHB. The IC_{50} are respectively 500 μM for hippocampus and 250 μM for striatum (Vayer *et al.*, 1987). These values must be compared to the plasma level of valproate (837 μM) 1 h after 150 mg/kg IP (Patsolos and Lascelles, 1981). In addition, the distribution of valproate in brain 15 min after 200 mg/kg IP is homogenous at about 700 μM (Hariton *et al.*, 1984). This effect of valproate on GHB release probably induces a rapid synaptic increase of this substance. Secondly, Kaufman *et al.* (1983) have described a strong inhibition of valproate on GHB dehydrogenase with a K_i of 60 μM. We have demonstrated that this GHB dehydrogenase is in fact the high K_m aldehyde reductase (ALR_1) which is identical to non specific SSR_1 and glucuronate reductase (Vayer *et al.*, 1985c). We measured a K_i of 80 μM for valproate on this enzyme activity (competitive inhibition). At the dosage of valproate used, GHB dehydrogenase inhibition is mainly responsible for the GHB accumulation (valproate concentration equal about $20 \times K_i$ for GHB dehydrogenase inhibition). Under these conditions, accumulation of GHB is mainly due to dehydrogenase inhibition at 400 mg/kg of valproate. The dose effect observed in GHB accumulation for valproate dosages higher

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