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Gamma-hydroxybutyrate increases tryptophan availability and potentiates serotonin turnover in rat brain

Serge Gobaille, Carmen Schleef, Viviane Hechler, Sandrine Viry, Dominique Aunis, Michel Maitre*

Laboratoire de Neurobiologie Moléculaire des Interactions Cellulaires, Faculté de Médecine and INSERM U-338, 11 rue Humann, 67085 Strasbourg Cedex, France

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Abstract

Gamma-hydroxybutyrate (GHB) is both a therapeutic agent and a recreative drug. It has sedative, anxiolytic and euphoric effects. These effects are believed to be due to GHB-induced potentiation of cerebral GABAergic and dopaminergic activities, but the serotonergic system might also be involved. In this study, we examine the effects of pharmacological doses of GHB on the serotonergic activity in rat brain. Administration of 4.0 mmol/kg i.p. GHB to rats induces an accumulation of tryptophan and 5-HIAA (5-hydroxyindole acetic acid) in the frontal cortex, striatum and hippocampus without causing significant change in the tissue serotonin content. In the extracellular space, GHB induced a slight decrease in serotonin release. The tryptophan and 5-HIAA accumulation induced by GHB is mimicked by the GHB receptor agonist para-chlorophenyl-transhydroxycrotonate (NCS-356) and blocked by NCS-382 (6,7,8,9-tetrahydro-5-[H]-benzocycloheptene-5-ol-4-ylidene acetic acid) a selective GHB receptor antagonist. GHB induces the accumulation of either a derivative of or [^{3}H]-tryptophan itself in the extracellular space, possibly by increasing tryptophan transport across the blood–brain barrier. The blood content of certain neutral amino-acids, including tryptophan, is also increased by peripheral GHB administration. Some of the effect of GHB could be reproduced by baclofen and reduced by the $GABA_B$ antagonist CGP 35348. Taken together, these results indicate that the GHB-induced stimulation of tissue serotonin turnover may be due to an increase in tryptophan transport to the brain and in its uptake by serotonergic cells. As the serotonergic system may be involved in the regulation of sleep, mood and anxiety, the stimulation of this system by high doses of GHB may be involved in

* Corresponding author. Institut de Chimie Biologique and INSERM U-338, 11 rue Humann, 67085. Strasbourg, France. Tel.: +33-3-90-24-30-96; fax: +33-03-90-24-31-02.

E-mail address: maitre@neurochem.u-strasbg.fr (M. Maitre).

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certain neuropharmacological events induced by GHB administration.

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Introduction

Gamma-hydroxybutyric acid (GHB) is used clinically to induce anesthesia and for the treatment of narcolepsy [1,2]. However, in recent years it has gained considerable interest among drug abusers and body builders due to its sedative, anxiolytic, euphoric and fat-burning metabolic effects [3]. The mechanism of these pharmacological effects is now beginning to be understood. Massive absorption of GHB (several grams) rapidly induces a sustained high concentration of this substance in the brain which interacts with the endogenous GHB system [4]. Under physiological conditions, this system transforms endogenous GABA into GHB which is released by exocytosis. The resultant GHB pool interacts with specific GHB receptors, modulating predominantly the activity of GABA and dopamine systems in specific brain regions [4–6]. This principally results in a reduction of GABA and dopamine release in these brain regions.

Baclofen, acting trough $GABA_B$ receptors, has a similar effect. As GHB is a weak affinity ligand for $GABA_B$ receptors [7], it is generally assumed that its effects on the GABAergic or dopaminergic systems are mediated via interactions with $GABA_B$ receptors, possibly a presynaptically located subclass. However, if some of these effects are blocked by $GABA_B$ antagonists [8–10], the majority of the GHB effects are strongly reduced by pre- or co-administration of NCS-382, a specific GHB receptor antagonist [11]. This compound has no affinity for $GABA_B$ receptors (unpublished results).

When high cerebral concentrations of GHB are present in the brain following peripheral administration, dopamine synthesis is stimulated mainly in the nigro-striatal and mesocorticolimbic pathways via the activation of tyrosine hydroxylase. This phenomenon induces accumulation of dopamine in these tissues and subsequently increases its release [12–16]. Similarly, GABA release is decreased by low concentrations of GHB, but is increased when its brain concentration is elevated after absorption of large doses of this drug [6]. Thus, the sedative, anxiolytic and euphoric effects of GHB administration reported by drug abusers may be due to an increase in dopamine and/or GABA release. This phenomenon is hard to explain by stimulation of $GABA_B$ receptors and in addition, baclofen has not been reported to possess addictive effects.

In this context, it is interesting to explore possible modifications of the activity of the serotonergic system after high doses of GHB. As this system is involved in the regulation of sleep, anxiety and mood, modulation of its activity may participate in the neuropharmacological effects induced by GHB administration. GHB-induced stimulatory effects on serotonergic turnover in the entire rat brain has been reported [17,18]. The present report complements and extends these studies and proposes a mechanism for this potentiation.

Materials and methods

Animals

Male Wistar rats (Charles River, France), weighing 250–300 g at the time of the study, were used. Rats were housed individually and subjected to a 12:12 hours light/dark cycle for two weeks before the experiments. Food and water were available ad libitum. The experimental protocol was in compliance with the European Communities council directive (86/609/EEC).

Extracellular levels of tryptophan and serotonin.

Rats were anaesthetized with ketamine (100 mg/kg i.p.). Two dialysis probes were placed according to Paxinos and Watson [19] in the frontal cortex (coordinates from bregma AP = 3.0, ML = 1.8, DV = −5.5 mm, with an angle of 15°) and in the striatum (AP = 0.5, ML = 3.0, DV = −5.8 mm). The probes (4 mm long, 0.52 mm diameter, 20,000 mol.wt. cut-off, Carnegie CMA 12) were permanently fixed to the skull with stainless steel screws and methacrylic cement. The rats were used 24 hours after surgery. During the experiments, animals were placed in hemispherical bowls of 40 cm diameter and the microdialysis probes were connected to a Carnegie CMA 100 microinjection pump. The probes were perfused with 147 mM NaCl, 4.0 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, pH 6.0, containing 1.0 μM Citalopram which inhibits serotonin uptake. Dialysates (1 μl/min, 30 min collection) were collected in tubes containing 7.5 μl 0.1 M $HClO_4$. 3,4 Dihydroxyhydrocinnamic acid was used as an internal standard and both tryptophan and serotonin (5-HT) were analysed by HPLC chromatography (C_{18} μBondapak column) with electrochemical detection.

At the end of each dialysis experiment, the rats were killed by an overdose of pentobarbital. The brains were removed and stored in buffered formalin. 50 μm thick serial sections were and stained with cresyl violet. Correct placement of the microdialysis probe was verified by microscopic examination of the sections.

Some experiments were carried out in order to follow the kinetics of the accumulation of L-[5-^{3}H]-tryptophan (or of a substance derived from L-[5-^{3}H]-tryptophan metabolism) in the extracellular space after i.v. administration (20 μCi of L-[5-^{3}H]-tryptophan per animal, 31 Ci/mmol) with or without GHB 4.0 mmol/kg injected i.p. The presence of radioactive tryptophan and/or of tryptophan-derived metabolite(s) were quantified in dialysates of the striatum and frontal cortex by scintillation counting.

Measurement of tryptophan, serotonin and 5-HIAA brain tissue levels

Serotonin, 5-HIAA (5 hydroxyindole acetic acid) and tryptophan were simultaneously determined using a reverse phase HPLC procedure coupled to electrochemical detection (Waters electrochemical detector 460) at 900 mV. GHB (4.0 mmol/kg), NCS-356 (2.0 mmol/kg),GHB + NCS-382 (2.0 mmol/kg) or GHB + CGP 35348 (200 mg/kg) were injected i.p. three hours before analysis. NCS-382 and CGP 35348 were injected ten minutes before GHB. Animals were killed by decapitation and the brain was rapidly dissected on an ice-cooled glass

plate. The brain regions were frozen in liquid nitrogen until analysis. The brain samples were weighed and, after thawing, homogenized in 0.1 N $HClO_4$ containing 6 mM Na-metabisulfite and 1 mM EDTA. The homogenates were centrifuged at 10,000 g for 20 min at 4 °C. Aliquots of the supernatant were transferred to the HPLC system with a Wisp automatic injector (Waters). Chromatography was carried out using a Bondapak phenyl column (10 μm, 300× 3.1 mm) with a mobile phase (3% methanol in 0.1 M Na-phosphate pH 2.5, 0.1 mM EDTA and 2 mM 1-octane sulfonic acid, Na salt) pumped at a flow rate of 1.4 ml/min. 3,4-dihydroxyhydrocinnamic acid (Aldrich) was used as internal standard.

Free amino-acid levels in blood plasma and brains of GHB-treated rats

Samples of blood were taken from 6 male Wistar rats 3 hours after administration of 4.0 mmol/kg GHB or of GHB + NCS-382 (2.0 mmol/kg) i.p. In parallel experiments, the frontal cortex of treated and untreated animals were rapidly dissected after sacrifice and homogenized in 10 volumes of 0.1 N HCl (w/v). After centrifugation, samples of plasma were treated for one hour with ice-cold 0.1 N HCl. Plasma and brain homogenates were then centrifuged to remove proteins. The internal standard was added to the supernatants, which were then used for determination of the levels of free amino-acids by the HPLC method of Allison et al [20] with some modifications [6]. Controls were carried out on rats injected with saline.

Statistical analysis

For statistical evaluation of brain tissue concentrations of tryptophan, serotonin and 5-HIAA, the non parametric Mann–Whitney test was used. Animals treated with GHB, NCS-382 or NCS-356 were compared with control animals treated with saline.

Microdialysis experiments were analyzed using a two-way analysis of variance. If a significant change was found, *post hoc* comparisons were performed using the Newman–Keuls test for multiple comparisons. The release values are expressed as the percentage of the average basal release (the average basal release was reached after two hours of dialysis, then the mean of the three 30 min fractions collected prior to stimulation in each experiment was taken as the basal value).

Results

GHB-induced modifications of the accumulation of tryptophan or of tryptophan-derived metabolite(s) in the extracellular space

Modifications of tryptophan levels in the extracellular space were studied in the striatum by microdialysis in rats treated with GHB and compared to control rats injected with saline. High brain levels of GHB after peripheral injection (4.0 mmol/kg) induced an increase in extracellular tryptophan (2.5 fold increase from basal levels) which was maximal 90 min after injection and remained constant for more than 300 min (Fig. 1). In rats treated with a mixture

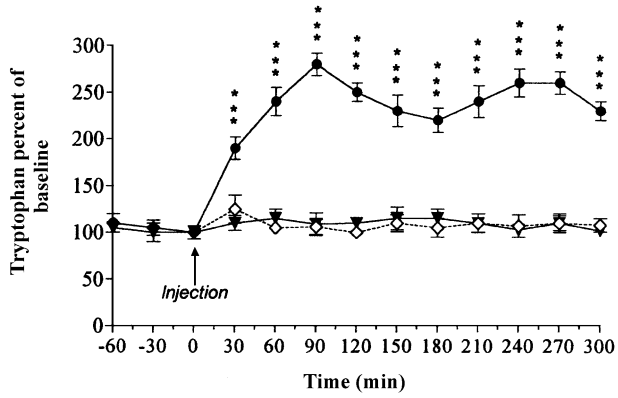

Fig. 1. Effect of administration of GHB (4.0 mmol/kg i.p.) on the extracellular level of tryptophan in striatum. Results are expressed as the percentage of the mean basal release of tryptophan (mean of three 30 min dialysate fractions prior to the first drug injection). Administration of the GHB receptor antagonist NCS-382 (2.0 mmol/kg i.p.) alone is represented by open diamonds (mean basal release±SEM=2.07±0.12 pmol/30 minutes; n=3 rats) while administration of GHB alone (4.0 mmol/kg i.p.) is shown by black circles (mean basal release± SEM=1.95±0.15 pmol/30 min; n=3 rats). The inhibition of the GHB-induced tryptophan increase by NCS-382 (2.0 mmol/kg i.p.) is represented by black triangles (mean basal release±SEM=2.13±0.18 pmol/30 min; n=3 rats). For GHB versus GHB+NCS-382, statistical significance was assessed by ANOVA followed by Newman–Keuls test. ***=$p<0.001$.

of GHB (4.0 mmol/kg) and NCS-382 (2.0 mmol/kg ten minutes before GHB), the extracellular levels of tryptophan were not modified compared to controls (Fig. 1).

Intravenous administration of L-[5-^{3}H]-tryptophan (20 μCi/20 nmol in a total volume of 200 μl) gave the same results. The lifetime of exogenous tryptophan (or of tryptophan-derived metabolite(s)) in the extracellular space of the frontal cortex and the striatum increased when the animals were treated with GHB. Compared to control rats, extracellular radioactivity began to accumulate 60–80 min after GHB administration, with a maximal increase of about 2 fold after 5–6 hours.($p<0.05$; Fig. 2).

Thus, for total tryptophan or for radioactive tryptophan administration, the increase in GHB-treated rats was about 2.0 to 2.5 fold in the extracellular space. This suggests that the majority of the extracellular radioactivity found in the extracellular space after peripheral administration of radioactive tryptophan was probably authentic tryptophan.

GHB-induced modifications of serotonin and 5-HIAA in the extracellular space

Serotonin and 5-HIAA levels were monitored by simultaneous microdialysis of the frontal cortex and the striatum. There is a significant decrease in 5-HT and 5-HIAA after GHB treatment compared to basal levels (Fig. 3). After 150 to 180 min, a significant decrease in the serotonin release was found in both the striatum and the frontal cortex. In both regions, 5-HIAA and serotonin release were reduced by a maximum of about 30–40%, two to three hours after GHB administration. Then, baseline levels were restored progressively about five hours after treatment.

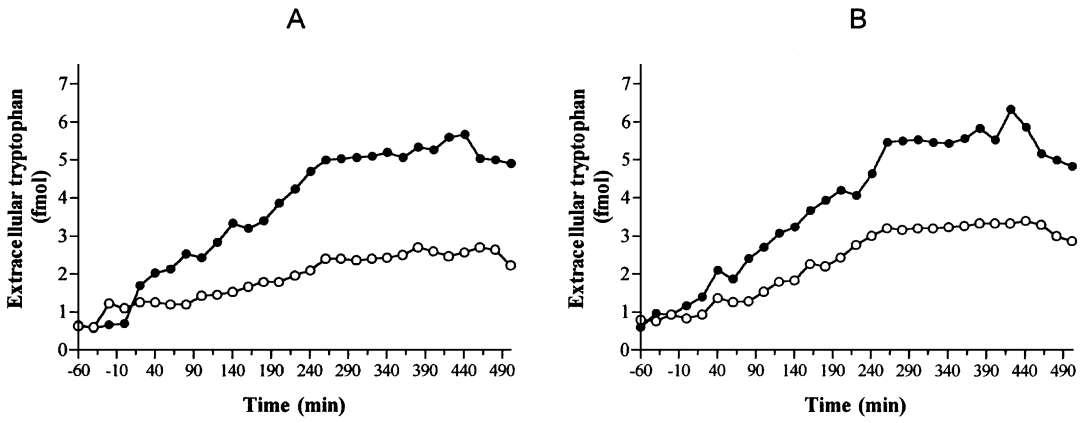

Fig. 2. Time-course of extracellular radioactive tryptophan in the frontal cortex (Fig. 2A) and striatum (Fig. 2B) after injection of L-[3H-5] tryptophan (20 μCi/20 nmol in 200 μl i.v.)±GHB 4.0 mmol/kg i.p. Results are expressed in fmoles of equivalent tryptophan /20 min dialysate. Black circles represent GHB treated animals (4.0 mmol/kg i.p.) and the open circles represent control animals (L-[3H-5] tryptophan+NaCl 0.9%). Results are mean of 3 separate experiments. For each value, SEM are always <20% (not shown).

Variations of brain tissue concentrations of tryptophan, serotonin and 5-HIAA

The administration of 4.0 mmol/kg i.p. GHB to the animals leads to increases in the tryptophan content of the frontal cortex (+35%), striatum (+29%), hippocampus (+26%) and Raphe nuclei (+77%, not shown), 180 minutes after treatment (Fig. 4). This delay was chosen because previous experiments have shown a maximal increase of serotonin turnover during

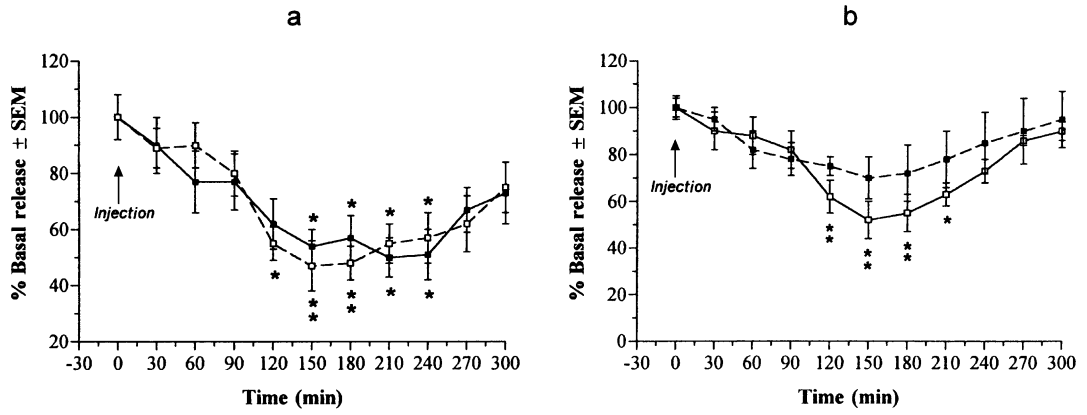

Fig. 3. Time-course of extracellular serotonin (black squares) and 5-HIAA (open squares) in frontal cortex (Fig. 3a) and striatum (Fig. 3b) after administration of 4.0 mmol/kg GHB i.p. a: Results are expressed as percentages of basal levels (mean of 6×30 min dialysates ± SEM = 287 ± 29 fmoles/30 min for serotonin; n = 3 rats and 14.1 ± 0.9 pmoles/30 min for 5-HIAA; n = 3 rats). b: Results are expressed as percentages of basal levels (mean of 6×30 min dialysates ± SEM = 108 ± 4 fmoles/30 min for serotonin; n = 3 rats and 27 ± 1.4 pmoles/30 min for 5-HIAA; n = 3 rats). Statistical significance was assessed by ANOVA followed by Newman–Keuls. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$.

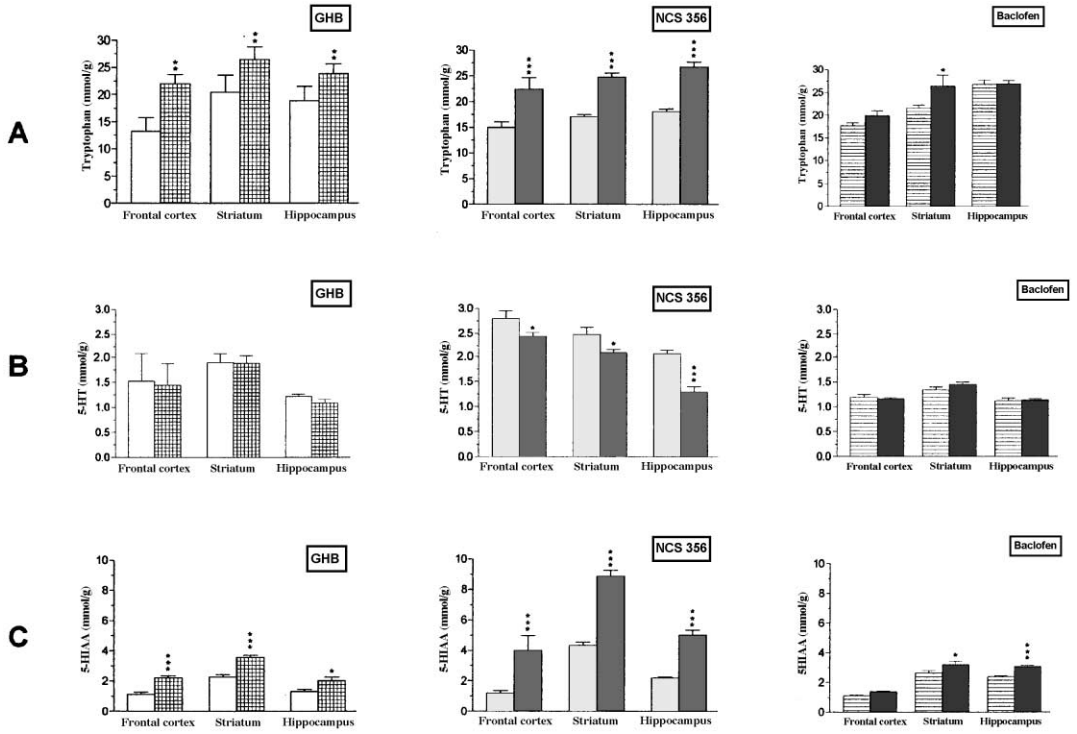

Fig. 4. Effects of GHB (4 mmol/kg i.p.), NCS-356 (2 mmol/kg i.p.) or Baclofen (10 mg/kg i.p.) on tryptophan (line A), 5-HT (line B) and 5-HIAA (line C) in three brain regions (frontal cortex, striatum and hippocampus). White bar: control rats (n = 6), squared bar: treated rats (n = 6). Results are expressed in nmol/g wet weight ± SEM. For treated animals versus controls, statistical significance was assessed by Man–Withney test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

this period [21]. At the same time and in the same brain regions, 5-HIAA also increased by 39%, 57%, 53% and 60% respectively. Serotonin levels were not significantly altered. NCS-356 (2.0 mmol/kg, i.p.), which is a GHB receptor agonist, induced similar changes in tryptophan tissue levels (+49%;+45% and +48% in frontal cortex, striatum and hippocampus respectively), however 5-HIAA levels were further increased (+231%; +105% and +128% respectively in the same regions) and significant decreases in serotonin tissue concentrations were observed (−13%; −16% and and −37%; Fig. 4). Under the same conditions, the $GABA_B$ agonist baclofen (10 mg/kg i.p.) induces only a slight effect on tryptophan content in the striatum (+22%) and an increase in 5-HIAA in striatum (+22%) and hippocampus (+29%) 3 hours after treatment.

Effects of NCS-382 and of CGP 35348 on the GHB-induced modification of tissue serotonin turnover

Animals were pre-treated with NCS-382 (2.0 mmol/kg i.p.) or with CGP 35348 (200 mg/kg) 10 minutes before GHB treatment (4.0 mmol/kg) and sacrificed 3 hours later. The blockade of

GHB receptors by NCS-382 strongly reduced both the GHB-induced tryptophan accumulation (and – 18%, – 26% and and – 16% in the striatum, frontal cortex and hippocampus respectively) and the GHB-induced increased in 5-HIAA levels (and – 5%, – 36% and – 3% respectively in the same regions, see Fig. 5). In the presence of CGP 35348, the effect of GHB was also reduced (– 21%, – 21% and and – 11% for the striatum, frontal cortex and hippocampus respectively for tryptophan, and – 27%, – 56% and and – 15% in the same regions for 5-HIAA (Fig. 5).

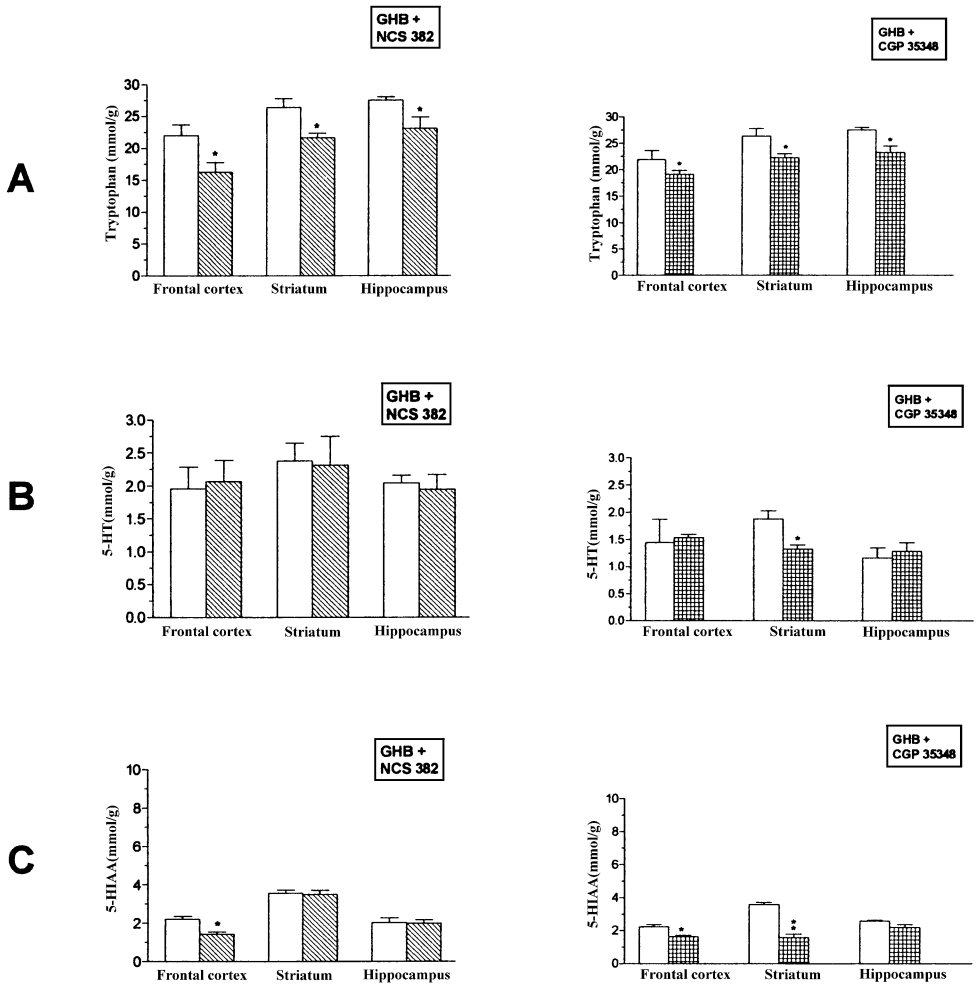

Fig. 5. Effects of GHB (4.0 mmol/kg i.p.) in the presence of either NCS-382 (2 mmol/kg) or CGP 35348 (200 mg/kg) on tryptophan (line A), 5-HT (line B) and 5-HHIA (line C) in three brain regions (frontal cortex, striatum, and hippocampus). White bars: GHB treated rats (n = 6); hached bars: GHB+NCS-382 and squared bars: GHB + CGP 35348. Results are expressed in nmol/g wet weight ± SEM. For GHB treated animals versus GHB + NCS-382 or GHB+CGP 35348, statistical significance was assessed by Man–Withney test. * $p < 0,05$ ** $p < 0,01$ *** $p < 0,005$.

Table 1
Effect of GHB (4.0 mmol/kg i.p.) or GHB + NCS-382 (2.0 mmol/kg) on neutral amino-acid concentrations in plasma and in frontal cortex. Results are expressed in μmol/litre for plasma and in μmol in the supernatant of 100 mg frontal cortex tissue homogenate (w/v). For teated animals versus saline controls, statistical significance was assessed by Man–Withney test. (n = 6 rats per group)

	Plasma (μmoles/l)			Frontal Cortex (μmoles/l)		
Amino Acids	NaCl	GHB	GHB + NCS-382	NaCl	GHB	GHB + NCS-382
Isoleucine	274 ± 25	423 ± 19**	298 ± 31	2.0 ± 0.1	3.2 ± 0.4*	2.5 ± 0.3
Leucine	423 ± 19	630 ± 37**	416 ± 29	5.5 ± 0.5	7.0 ± 0.9*	6.1 ± 0.8
Phenylalanine	208 ± 10	264 ± 15**	195 ± 21	6.0 ± 0.5	8.3 ± 0.6*	5.5 ± 0.6
Tryptophan	186 ± 13	226 ± 32**	192 ± 18	15.1 ± 0.6	20.3 ± 1.2*	17.1 ± 1.8
Valine	134 ± 6	179 ± 18**	142 ± 11	10.0 ± 0.1	12.0 ± 0.7*	9.7 ± 0.6

* = $p < 0.05$.
** = $p < 0.01$.

GHB-induced changes in neutral amino-acid concentrations of the rat blood plasma and frontal cortex

Three hours after administration of GHB (4.0 mmol/kg i.p.) most of the neutral amino-acid concentrations of blood plasma increase by 20–60%. Isoleucine, leucine, phenylalanine, tryptophan and valine increase similarly whereas alanine, glycine, histidine, threonine, methionine and tyrosine remain constant (Table 1). A similar increase of the same amino-acids was observed in the frontal cortex of treated rats. When GHB was co-administered with NCS-382 (2.0 mmol/kg), no significant changes in the levels of neutral aminoacids were found.

Discussion

Considerable evidence suggests the existence of an endogenous GHB signalling system in brain [22]. This system can be easily stimulated by peripheral administration of GHB. Anaesthetic doses of GHB increase serotonin turnover in the adult rat and some studies report an accumulation of serotonin in certain brain regions [17,18]. The present results confirm the GHB-induced increase in serotonin turnover, as GHB or NCS-356 treatment increased brain tissue levels of 5-HIAA. Serotonin contents of the three regions investigated do not change significantly, except after NCS-356 treatment, which induces a slight decrease in the frontal cortex and the striatum, accompanied by an even more pronounced decrease in the hippocampus. The absence of serotonin increase may be due to rapid intracellular metabolism of serotonin after potentiation of its synthesis. 5-HIAA is an index of the rate of serotonin metabolism in tissue because a decrease in the extracellular concentration of serotonin was seen after GHB treatment. The work of Spano and Przegalinski [17] indicate that this accumulation of 5-HIAA is not due to inhibition of transport from the brain, since probenecid and GHB treated rats show a further significant increase of 5-HIAA in the telediencephalon and the brainstem. Thus the accumulation of 5-HIAA after GHB or NCS-356 treatment

probably results from intraneuronal deamination of newly synthesized serotonin which remains in the cytoplasmic pool.

GHB and the GHB agonist NCS-356 facilitate tryptophan accumulation in brain tissue. This was demonstrated not only by the extracellular increase of tryptophan in brain, but also by the rapid accumulation of radioactivity in the extracellular fluid after peripheral administration of [^{3}H]-tryptophan. Since L [5-^{3}H] tryptophan looses its radioactivity when transformed into 5-HTP, the radioactivity which appears rapidly in the extracellular space is most probably authentic tryptophan. However, the presence of a possible metabolite of tryptophan derived from the kynurenine or the tryptamine pathways cannot be rulled out. In vivo, the accumulation of tryptophan in tissue and in the extracellular space may be due to an increase of tryptophan transport through the blood brain barrier and the neuronal membrane. It is currently thought that most essential neutral amino-acids enter the brain via the L-type amino-acid transporter, primarily located on endothelial cell membranes [23]. This system is Na^+-independent and transports leucine, isoleucine, methionine, valine, phenylalanine, histidine, and threonine as well as tyrosine and tryptophan. The transporter has been cloned from rat kidney and is expressed in the brain, where there are unrelated transporters which carry leucine and tryptophan with the same efficiency (Km about 20 to 30 μM) [24]. It is generally accepted that L-tryptophan loading stimulates serotonin synthesis and increases 5-HIAA concentrations [25].

An interrelationship between both serotonergic and dopaminergic systems has been suggested which would implicate the latter in some of the serotonergic effects of GHB. Projections from the substantia nigra and the ventral tegmentum area to the dorsal raphe nucleus have been described and, in the striatum, dopamine release has been shown to be increased by serotonin and serotonin agonists [26,27]. Nigrostriatal and mesocorticolimbic stimulation of dopaminergic activity has been described for sedative doses of GHB [13] and dopamine receptor-mediated modulation of serotonin metabolism could participate in the adaptation of serotonin turnover.

Thus the increase in tryptophan brain tissue concentrations could stimulate the biosynthesis of serotonin or could result from the GHB-induced increase in serotonin turnover. GHB could increase, via direct or indirect mechanisms, tryptophan uptake in addition to the uptake of other neutral amino-acids using the same transporter [28]. The endothelial barrier of capillaries is more permeable to tryptophan and thus may explain why blood and brain accumulate this amino-acid. In addition, the presence of GHB in blood could facilitate the dissociation of tryptophan from its albumin binding sites that are sensitive to free fatty acids and certain anesthetics. These possible changes in the equilibrium conditions between free and bound tryptophan could facilitate the uptake of this substance into the brain [29,30].

GHB or NCS-356 could also interfer with the vesicular accumulation of serotonin at specific synaptic sites. This interference could explain the rapid tissue conversion of serotonin into 5-HIAA and the decrease of the serotonin concentration in the extracellular space. Under these conditions, the antagonist NCS-382 appears to abolish GHB or NCS-356-induced modifications in the vesicular accumulation of serotonin since the elevated tissue 5-HIAA was considerably decreased in its presence.

The present study shows that the GHB effects on tryptophan levels both in brain and in blood are blocked by NCS-382, a specific GHB receptor antagonist [11]. This effect is repro-

duced by NCS-356 that exhibits agonistic properties at the GHB receptor [31] and modulates dopaminergic activity in a way similar to that of GHB. NCS-356 exhibits a stronger affinity for the GHB binding site than GHB itself [31] and it exerts a greater impact on 5-HIAA accumulation and serotonin turnover than GHB, even at a lower dose. These results imply a role of pharmacological doses of GHB in the modulation of serotonergic activity. The specific role of GHB and its analogues (NCS-356 and NCS-382) demonstrate the implication of GHB receptors in controlling serotonin turnover, because these two last compounds have no affinity for GABA receptors, including $GABA_B$ receptors. Nevertheless, this last class of receptors seems to play a similar role to that of GHB receptors in regulating tryptophan bioavailability and 5-HIAA synthesis. In that respect, the effect of baclofen and of CGP 35348 are demonstrative. However, GHB or baclofen act either on separate receptors or on a class of receptors sensitive to both compounds. The classical set of $GABA_B$ receptors seems to possess too low affinity for GHB [32,33] and no affinity at all for NCS-382 and NCS-356 to be implicated in the neuropharmacological mechanism of action of GHB regulating serotonin turnover. In addition, despite significant influence of baclofen on several brain neurotransmitters [34], it is not addictive and thus contrasts significantly with the pharmacological profile of GHB [35].

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