Selective γ-hydroxybutyric acid receptor ligands increase extracellular glutamate in the hippocampus, but fail to activate G protein and to produce the sedative/hypnotic effect of γ-hydroxybutyric acid

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Abstract
Two γ-hydroxybutyric acid (GHB) analogues, trans-γ-hydroxycrotonic acid (t-HCA) and γ-(p-methoxybenzyl)-γ-hydroxybutyric acid (NCS-435) displaced [3H]GHB from GHB receptors with the same affinity as GHB but, unlike GHB, failed to displace [3H]baclofen from GABA B receptors. The effect of the GHB analogues, GHB and baclofen, on G protein activity and hippocampal extracellular glutamate levels was compared. While GHB and baclofen stimulated 5′-O-(3-[35S]thiotriphosphate) [35S]GTPγS binding both in cortex homogenate and cortical slices, t-HCA and NCS-435 were ineffective up to 1 mM concentration. GHB and baclofen effect was suppressed by the GABA B antagonist CGP 35348 but not by the GHB receptor antagonist NCS-382. Perfused into rat hippocampus, 500 nM and 1 mM GHB increased and decreased extracellular glutamate levels, respectively. GHB stimulation was suppressed by NCS-382, while GHB inhibition by CGP 35348. t-HCA and NCS-435 (0.1–1000 μM) locally perfused into hippocampus increased extracellular glutamate; this effect was inhibited by NCS-382 (10 μM) but not by CGP 35348 (500 μM). The results indicate that GHB-induced G protein activation and reduction of glutamate levels are GABA B-mediated effects, while the increase of glutamate levels is a GHB-mediated effect. Neither t-HCA nor NCS-435 reproduced GHB sedative/hypnotic effect in mice, confirming that this effect is GABA B-mediated. The GHB analogues constitute important tools for understanding the physiological role of endogenous GHB and its receptor.

Keywords: GABA B; GHB analogues; glutamate; GTPγS binding; hippocampus; NCS-382.


γ-Hydroxybutyric acid (GHB) is a putative neurotransmitter or neuromodulator in the mammalian brain (see Maitre 1997; Bernasconi et al. 1999). Exogenously administered it produces a number of physiological, pharmacological and biochemical effects in brain, including anxiolytic effect, sleep, anaesthesia and absence seizures (Bernasconi et al. 1999; Schmidt et al. 1991; Hu et al. 2000). Moreover, GHB modifies the levels or action of different neurotransmitters, most notably dopamine, opioids, glutamate and acetylcholine (Banerjee and Snead 1995; Maitre 1997; Erhardt et al. 1998; Madden and Johnson 1998). Clinically, GHB is used as an anaesthetic agent and in the treatment of sleep disorders and alcohol dependence (see Agabio and Gessa 2002). GHB is abused by humans for its euphoriant

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Abbreviations used: GHB, γ-hydroxybutyric acid; t-HCA, trans-γ-hydroxycrotonic acid; HPLC, high-performance liquid chromatography; NCS-435, γ-(p-methoxybenzyl)-γ-hydroxybutyric acid.
effect and, as expected, is self-administered by rats and mice (see Colombo and Gessa 2000). The mechanisms by which GHB affects neural functioning and acts as a drug of abuse are still unclear. GHB has affinity for two distinct binding sites in the brain, the GHB receptor and, at higher concentrations, the GABA\textsubscript{B} receptor sites (Benavides et al. 1982; Mathivet et al. 1997; Castelli et al. 2000). A recent study by Snead (2000) indicates that GHB receptor, like GABA\textsubscript{B} receptor, is coupled to a G protein, but it differs from GABA\textsubscript{B} receptor, having significantly different anatomical distribution and ontogeny (Snead 1994, 1996). The putative GHB receptor antagonist NCS-382 and different GABA\textsubscript{B} agonists and antagonists have been used to discriminate the relative role of GHB and GABA\textsubscript{B} receptors in the different actions of GHB and to understand the functional meaning of endogenous GHB. NCS-382 has been reported to antagonize some effects of GHB such as GHB-induced seizures and GHB self-administration in mice (Schmidt et al. 1991; Martellotta et al. 1998; Hu et al. 2000).

However, NCS-382 failed to antagonize the depressant effect of GHB on locomotor activity and its inhibitory effect on operant response rates in mice, and was not able to precipitate withdrawal signs in rats treated repeatedly with GHB (Cook et al. 2002). On the other hand, the majority of the biochemical, electrophysiological and behavioural effects of the high and low doses of GHB were reproduced and potentiated by the GABA\textsubscript{B} Receptor antagonists such as baclofen, and were reversed by the GABA\textsubscript{B} receptor antagonists CGP 35348, CGP 36742 and SCH 50911 (Bernaconi et al. 1992, 2002; Colombo et al. 1998), suggesting that they are mediated by GABA\textsubscript{B} receptors. Thus, the distinct role of GHB and GABA\textsubscript{B} receptors in the pharmacological actions of GHB is still matter of controversy.

The present investigation, aimed at further clarifying this issue, was prompted by two recent observations. The first was the discovery of two GHB analogues, t-HCA and 5-dimethyl-2-morpholineacetic acid, which GHB affects neural functioning and acts as a drug of abuse are still unclear. GHB has affinity for two distinct binding sites in the brain, the GHB receptor and, at higher concentrations, the GABA\textsubscript{B} receptor sites (Benavides et al. 1982; Mathivet et al. 1997; Castelli et al. 2000). A recent study by Snead (2000) indicates that GHB receptor, like GABA\textsubscript{B} receptor, is coupled to a G protein, but it differs from GABA\textsubscript{B} receptor, having significantly different anatomical distribution and ontogeny (Snead 1994, 1996). The putative GHB receptor antagonist NCS-382 and different GABA\textsubscript{B} agonists and antagonists have been used to discriminate the relative role of GHB and GABA\textsubscript{B} receptors in the different actions of GHB and to understand the functional meaning of endogenous GHB. NCS-382 has been reported to antagonize some effects of GHB such as GHB-induced seizures and GHB self-administration in mice (Schmidt et al. 1991; Martellotta et al. 1998; Hu et al. 2000).

Moreover, we investigated whether these two compounds shared with GHB the ability to produce loss of the righting reflex in mice, an effect that has been shown to be mediated by GABA\textsubscript{B} receptors (Carai et al. 2001).

Materials and methods

Materials

GHB sodium salt was purchased from Sigma Chemical Co (St Louis, MO, USA); R-(−)-baclofen, CGP 35348 (3-aminopropyl-diethoxy-methylphosphonic acid) and SCH 50911 [2S(+)-5, 5-dimethyl-2-morpholineacetic acid] from Tocris (Bristol, UK), [\textsuperscript{3}H]GHB sodium salt (60 Ci/mmol), [\textsuperscript{3}H]baclofen (38.7 Ci/mmol) and 5′-O-(3-[\textsuperscript{35}S]thiotriophosphate) ([\textsuperscript{35}S]GTP\textsubscript{S}) (1250 Ci/mmol) were obtained from ARC (St Louis, MO, USA) and New England Nuclear (NEN, Boston, MA, USA), respectively. GDP and GTP\textsubscript{S} were purchased from Sigma/RBI (St Louis, MO, USA). NCS-382 (6,7,8,9-tetrahydro-5-[\textsuperscript{3}H]benzocyclohept-5-ol-4-ylidene acetic acid), t-HCA (trans-\textgreek{gamma}-hydroxycrotonic acid) and NCS-435 (\textgreek{gamma}-(p-methoxybenzil)-\textgreek{gamma}-hydroxybutyric acid) were synthesized by Prof Cignarella (University of Milan, Milan, Italy), as previously described (Bourguignon et al. 1988, 2000; Maitre et al. 1990).

Binding studies

Tissue preparation

Male Sprague–Dawley rats (Charles River, Como, Italy), weighing 200–250 g, were used in all experiments and were maintained on ad libitum food and water. Rats were killed by decapitation, their brains rapidly removed and cerebral cortices dissected on ice.

Cortical tissue was homogenized using an homogenizer system (Glas-Col, Terre Haute, IN, USA) in 20 volumes (v/w) of ice-cold 0.32 m sucrose containing 1 mM EDTA. The homogenate was centrifuged at 1000 g for 10 min and the supernatant collected and recentrifuged at 20 000 g for 20 min. The pellet was resuspended in 20 volumes (v/w) of ice-cold water, homogenized using a Polytron homogenizer and centrifuged at 8000 g for 20 min. The supernatant together with the buffy layer on the pellet was then centrifuged at 45 000 g for 20 min. The resulting pellet was resuspended in ice-cold distilled water and once more centrifuged at 45 000 g for 30 min. Homogenization and centrifugation in water was performed to lyse the tissue and washout endogenous GHB and GABA. The final pellet was frozen and stored at −80°C for at least 18 h before use both for [\textsuperscript{3}H]baclofen and [\textsuperscript{3}H]GHB binding assay and [\textsuperscript{35}S]GTP\textsubscript{S} binding assay.

\textsuperscript{3}H]Baclofen and \textsuperscript{3}H]GHB binding assay

For binding assay, membrane pellets were allowed to thaw at 4°C before resuspension in 20 volumes (v/w) of both 50 mM KH\textsubscript{2}PO\textsubscript{4} buffer (pH 6.5) containing 1 mM EDTA and 50 mM Tris–HCl, pH 7.4, CaCl\textsubscript{2} 2.5 mM for [\textsuperscript{3}H]GHB and [\textsuperscript{3}H]baclofen, respectively. The suspension was incubated for 20 min at 20°C before centrifugation at 7000 g for 10 min. The washing step was repeated three more times allowing 15 min incubation with each addition of the same buffer to further remove the residual endogenous ligand GABA or GHB. The final pellet was then resuspended in appropriate binding buffer to a final concentration of 200–300 μg of membrane protein for both [\textsuperscript{3}H]GHB and
In the presence of 1 mM unlabelled GHB, \([3H]\)baclofen binding of 0.6 nM at 450 mM Tris–HCl, pH 7.4, 2.5 mM CaCl₂, or 0 mM KH₂PO₄, respectively, was assessed in duplicate in a volume of 0.6 mL at 4°C for 30 min. Non-specific binding was estimated in the presence of 1 mM unlabelled GHB. \([3H]\)Baclofen binding was assayed in duplicate in a volume of 1 mL at 20–22°C for 20 min, and R-(−)baclofen (100 µM) was used to define non-specific binding. In both binding assays, free ligand was separated from bound ligand by rapid filtration through Whatman GF/B glass filters using a Brandell 96-sample harvester (Gaithesburg, MD, USA). Filters were then rinsed twice with either ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 2.5 mM CaCl₂, or 0 mM KH₂PO₄ buffer (pH 6.5) for \([3H]\)baclofen and \([3H]\)GHB, respectively. Filter-bound radioactivity was counted in a liquid scintillation counter (Packard Tricarb 2900, Packard, Meriden, CT, USA), using 3 mL of scintillation fluid (Packard Ultima Gold MV). We used filtration instead of centrifugation also for membrane preparations from at least three different brains.

Independent experiments were repeated on membrane preparations by incubating tissue slices for 2 h at 25°C with either baclofen, GHB, t-HCA, NCS-435 in fresh buffer containing 0.04 nM of \([35S]\)GTPyS and 30 µM GDP. Agonist-stimulated activity was measured in the presence of different concentrations of baclofen, GHB, t-HCA, NCS-435 in the presence of 0.2 nM of \([35S]\)GTPyS and 30 µM GDP. Agonist-stimulated activity was performed in the presence of either the GABAB antagonist CGP 35348 (200 nM) or the GHB receptor antagonist NCS-382 (100 µM). The stimulation by agonist was defined as a percentage increase above basal levels (i.e. \([\text{dpm(agonist)}–\text{dpm (no agonist)}]/\text{dpm (no agonist)}) \times 100).

Data are reported as mean ± SEM of three to six experiments, performed in triplicate. Non-linear regression analysis of concentration–response data was performed using Prism 2.0 software (GraphPad Prism Program, GraphPad, San Diego, CA, USA) to calculate \(E_{\text{max}}\) and \(EC_{50}\) values.

\([35S]\)GTPyS binding autoradiography

\([35S]\)GTPyS binding autoradiography was performed as previously described by Sim et al. (1995). Briefly, rats were killed by decapitation and the brains removed and immediately immersed in isopentane at −35°C. Twenty-micrometer coronal sections were cut on a cryostat at −20°C and thaw-mounted onto Superfrost Plus slides (BDH, Lutterworth, UK). Tissue slices were pre-incubated in assay buffer (50 mM Tris–HCl, 3 mM MgCl₂, 0.2 mM EGTA and 100 mM NaCl, pH 7.4) at 25°C for 10 min and pre-incubated further with 2 mM GDP for 15 min in assay buffer at 25°C. Agonist-stimulated activity was determined by incubating tissue slices for 2 h at 25°C with either baclofen, GHB, t-HCA, NCS-435 in fresh buffer containing 0.04 nM of \([35S]\)GTPyS and 2 mM of GDP. Agonist studies were also performed in the presence of either the GABAB antagonist CGP 35348 (100 µM) or the GHB receptor antagonist NCS-382 (100 µM). Basal activity was assayed in the absence of agonist and in the presence of 2 mM GDP. Non-specific binding was determined in adjacent brain sections in the presence of 10 µM of unlabelled GTPyS. Following incubation, tissue slices were rinsed twice in ice-cold Tris–HCl buffer (50 mM, pH 7.4) and once in deionized water and air-dried. Dried tissue sections and \(14^C\)Micro-scales standards (Amersham, Arlington Heights, IL, USA) were placed in a Fujifilm BAS cassette with a BAS-5000 imaging plate. The resulting images were analysed with the Fujifilm-BAS 5000 imaging system (AIDA, Raytest, Wilmington, NC, USA), and optical densities were transformed into levels of bound radioactivity (fentomoles per milligram of tissue equivalent) with gray values generated by co-exposed \(14^C\)standards. Agonist-stimulated activity in brain sections was calculated by subtracting the optical density in basal sections (incubated with GDP alone) from that of agonist-stimulated sections; results were expressed as percentage of basal activity.

Data are mean values ± SEM of duplicate sections of brains from five to six animals. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by either the posthoc Newman–Keuls or Dunn’s test.

**Micrdialysis study**

**Animals**

Adult male Sprague-Dawley rats (280–300 g, Charles River, Como, Italy) were housed at constant temperature (22°C) under a
12:12 h light–dark cycle (lights on at 06.00 h) with food and water ad libitum. Following delivery, the rats were allowed to adapt to the environment for at least 1 week before the experiments commenced. Experiments were carried out in strict accordance with guidelines issued by the Italian Ministry of Health (DL116/92) and (DL111/94-B), the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health (Bethesda, MD, USA).

Surgery
Under halothane anaesthesia (1.5% mixture of halothane/air), animals were mounted in a David Kopf stereotaxic apparatus and a microdialysis probe of concentric design (MW cut-off 20 000 Da; CMA 11, Carnegie Medicin, Stockholm, Sweden), was implanted into the right or left CA1 region of the hippocampus. The co-ordinates relative to the bregma were: A −3.4; L ±1.8; V −2.8 (Paxinos and Watson 1986). After implantation, the probe was secured to the skull with methacryl cement. Microdialysis measures were performed after at least 36 h of recovery.

Experimental procedures
On the day of the experiment, the probe was perfused with an artificial cerebrospinal fluid (NaCl 148 mM; KCl 2.7 mM; CaCl2 1.2 mM; MgCl2 0.85 mM; glucose 2.7 mM) at a constant flow rate (2 μL/min) via a microinfusion pump. In order to achieve stable dialysate glutamate levels, collection of perfusate samples commenced 300 min after the onset of perfusion and perfusates were collected every 15 min thereafter. After the obtaining of stable basal glutamate values, different concentrations of GHB (500 nm and 1 μM), t-HCA or NCS-435 (0.1–1000 μM) were included into the perfusate medium for 60 min. This medium was then replaced with the original one and a further four samples collected (60 min). In parallel sets of experiments the GHB receptor antagonist NCS-382 (10 μM) or the GABA<sub>B</sub> receptor antagonist CGP 35348 (500 μM) was included into the perfusion medium either alone or in combination with t-HCA or NCS-435. The antagonists were included in the perfusion medium 15 min prior to GHB receptor ligands.

A 15-μL aliquot from each perfusate sample (30 μL) was used for measurement of glutamate. At the end of each microdialysis experiment, the animals received an overdose of anaesthetic, the brain was removed and the position of the probe in the CA1 region of the hippocampus verified in 30-μm-thick coronal cryostat sections. Only those animals in which the probe was correctly located were included in this study.

Glutamate analysis
Glutamate was quantified using a high-performance liquid chromatography (HPLC)/fluorimetric detection system, including precolumn derivatization o-phthalaldehyde reagent and a Chrom sep 5 (C18) column (Ferraro et al. 2001). The mobile phase consisted of 0.1 M sodium acetate, 10% methanol and 2.5% tetrahydrofuran, pH 6.5. The limit of detection for glutamate was 30 fmol/sample.

The inclusion of GHB, t-HCA, NCS-435, NCS-382 and CGP-35348 did not alter the pH of the perfusion medium or interfere with the qualitative and quantitative HPLC assay for glutamate.

Statistical analysis
With regard to neurochemical data, individual time points were reported as percentages of the mean of the four basal samples prior to treatments. The data were calculated as mean ± SEM and significance with regard to the peak responses (maximal responses) is shown in the figures. In addition, the area under the curve, which reflects the duration of the effect over the entire experimental period (180 min), was calculated for each animal. The area values (overall effects) were calculated as percentage changes in basal value over time (D basal percentage × time) by using the trapezoidal rule.

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons.

Loss of the righting reflex in mice
Male DBA/2Jco mice weighing 25–30 g, were used. T-HCA (1000 mg/kg), NCS-435 (2000 mg/kg) and GHB (1000 mg/kg) were acutely administered intraperitoneally (i.p.) to groups of n = 6–12 mice.

In all experiments, after drug injection each mouse was placed on its back once every 60 s until it was unable to right itself within 60 s. The time between drug injection and the start of the 60-s interval when the mouse was unable to right itself was measured as onset of the righting reflex loss. Each mouse was then left undisturbed on its back until it spontaneously regained its righting reflex (sleep time; determined as having at least three paws under its body). Complete recovery of the righting reflex was defined as the mouse being able to turn itself upright twice more within 60 s.

In a parallel set of experiments, either the GABA<sub>B</sub> receptor antagonist SCH 50911 (100 mg/kg; i.p.; Bolser et al. 1995) or the GHB receptor antagonist NCS-382 (250 mg/kg; i.p.) were administered 15 min before the i.p. injection of 1000 mg/kg GHB.

Results
Binding studies
From the displacement curves of [3H]GHB (60 nm, 60 Ci/mmol), GHB, t-HCA and NCS-435 evidenced two populations of binding sites, one of high and the other of low affinity (Fig. 1).

The displacing potency of the GHB analogues against [3H]GHB and [3H]baclofen was compared with that of GHB and baclofen. As shown in Table 1, t-HCA and NCS-435 showed similar potency to GHB in displacing [3H]GHB from the high-affinity binding sites, while at low-affinity binding sites the K<sub>i</sub> values of both GHB analogues were higher than GHB. In contrast, confirming previous studies (Benavides et al. 1982; Snead 1996), baclofen up to the concentration of 1 mM failed to displace [3H]GHB.

As expected from previous studies (Mathivet et al. 1997), GHB inhibited [3H]baclofen binding with an K<sub>i</sub> of 51 ± 2.1 μM, whereas neither t-HCA nor NCS-435 up to the concentration of 1 mM modified [3H]baclofen binding (Table 1).
Effect of baclofen, GHB, NCS-435 and t-HCA on[^35]S-GTP\_cS binding

Basal levels of[^35]S-GTP\_cS binding in frontal cortex of adult rats were 60 ± 3 fmol/mg (n = 10) protein. Baclofen stimulated the specific binding of[^35]S-GTP\_cS in a concentration-dependent manner; as shown in Fig. 2(a), 100, 300 and 600 μM baclofen increased[^35]S-GTP\_cS binding to 141 ± 6.0%, 166 ± 9.0% and 179 ± 15% the basal values, respectively. On the other hand, GHB (Fig. 2b) at concentrations of 500 and 1000 μM stimulated[^35]S-GTP\_cS binding to 137 ± 3.4% and 134 ± 5.2% the basal values, respectively, whereas a 100-μM concentration was ineffective (data not shown). Neither NCS-435 nor t-HCA, up to a concentration of 1 mM, stimulated[^35]S-GTP\_cS binding (Fig. 2b).

[^35]S-GTP\_cS autoradiography

Effect of baclofen, GHB, NCS-435 and t-HCA on[^35]S-GTP\_cS binding

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To verify whether GABA<sub>B</sub> and/or GHB receptors mediate the effect of GHB on [<sup>35</sup>S]GTPγS binding, frontal cortical sections were incubated with either baclofen (100 µM) or GHB (500 µM) in the presence of the GABA<sub>B</sub> receptor antagonist CGP 35348 (100 µM) or the GHB receptor antagonist NCS-382 (100 µM). As shown in Fig. 2(c), the GHB receptor antagonist NCS-382 failed to inhibit GHB-stimulated [<sup>35</sup>S]GTPγS binding, while the GABA<sub>B</sub> receptor antagonist CGP 35348 completely abolished both the GHB- and baclofen-induced effect. The results indicate that the G protein activated by GHB is not connected to GHB receptor but to GABA<sub>B</sub> receptor. Neither NCS-382 nor CGP 35348 affected [<sup>35</sup>S]GTPγS binding (data not shown).

**<sup>35</sup>S]GTPγS binding assay**

Baclofen (0.1–1000 µM) stimulated [<sup>35</sup>S]GTPγS binding to rat cortex membranes with an EC<sub>50</sub> value of 3.67 ± 0.39 µM; maximal stimulation being 166 ± 5.2% the basal value (Fig. 3a). In contrast, GHB (1–1000 µM) stimulated [<sup>35</sup>S]GTPγS binding only at the highest concentration tested; maximal increase being 130% the basal value (Fig. 3b). Neither NCS-382 nor t-HCA (1–1000 µM) modified [<sup>35</sup>S]GTPγS binding (Fig. 3b). GHB receptor antagonist NCS-382 (100 µM) failed to modify GHB-induced [<sup>35</sup>S]GTPγS stimulation, while the GABA<sub>B</sub> receptor antagonist CGP 35348 (200 nM) suppressed not only baclofen but also GHB-induced GTPγS activation (Fig. 3c).
Microdialysis experiments

Effects of local perfusion with GHB, NCS-435 and t-HCA on hippocampal (CA1) extracellular glutamate levels

Extracellular glutamate in the CA1 were 0.208 ± 0.018 μM (n = 39) and remained stable throughout the 180-min time period of the experiment.

Intrahippocampal (CA1) perfusion of GHB (500 nM and 1 mM for 60 min) differently influenced extracellular glutamate. Namely, while the nanomolar GHB concentration increased glutamate levels up to 149 ± 11% the basal values, the millimolar concentration reduced glutamate levels down to 75 ± 5% the basal levels; maximum effects were observed about 30–45 min after the onset of perfusion (Fig. 4).

In contrast to the biphasic effect of GHB, the intrahippocampal perfusion of NCS-435 at the concentrations of 500 nM, 300 μM and 1 mM raised extracellular glutamate levels to 132 ± 8%, 159 ± 9% and 355 ± 34% the basal values, respectively (Fig. 5a). The local perfusion of t-HCA also increased extracellular glutamate levels in a concentration-dependent manner, similarly to NCS-435, except that at the highest concentration tested (1 mM) the effect of t-HCA was more marked and persistent (Fig. 5b).

Reversal of NCS-435 and t-HCA effect by the GHB antagonist NCS-382

To determine if the effect of the GHB analogues on extracellular glutamate levels was mediated by GHB receptor, the GHB receptor antagonist NCS-382 (10 μM) was co-perfused with either t-HCA or NCS-435 into the CA1 hippocampal region. The GHB antagonist NCS-382 was most effective in inhibiting the effect of the GHB analogues. In fact, the co-perfusion of 10 μM NCS-382 resulted in the almost total suppression of the effect of both compounds at the highest concentration tested (1 mM) the effect of t-HCA was more marked and persistent (Fig. 5b).

Failure of GABA<sub>B</sub> antagonist CGP 35348 to inhibit NCS-435 and t-HCA effect

To determine whether GABA<sub>B</sub> receptor was involved in the effect of NCS-435- and t-HCA on glutamate, the GABA<sub>B</sub> receptor antagonist CGP 35348 was co-perfused with both GHB analogues.

The intrahippocampal co-perfusion of CGP 35348 (500 μM) failed to antagonize the stimulant effect of NCS-435 or t-HCA (300 μM) on extracellular glutamate levels.
Fig. 6  Inhibition by NCS-382 (10 µM) of the stimulant effect of locally perfused NCS-435 (300 µM and 1 mM; a) and t-HCA (300 µM and 1 mM; b) on dialysate glutamate levels in CA1. Each point represents the mean ± SEM of six to seven animals, expressed as percentages of the mean of the four basal values before treatment. The antagonist was included in the perfusion medium 15 min prior to NCS-435 or t-HCA and maintained till the end of the experiment. The histograms of the areas under the curves are shown on the right side of each panel. The areas under the curves were calculated as percentage changes in basal value over time (Δ basal percentage × time) by using the trapezoidal rule. (a) *p < 0.05, **p < 0.01 significantly different from the control as well as NCS-382 alone group; ***p < 0.01 significantly different from the respective NCS-382 + NCS-435 group. (b) *p < 0.05, **p < 0.01 significantly different from the control as well as NCS-382 alone group; ***p < 0.01 significantly different from the respective NCS-382 + t-HCA group according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

Fig. 7  Failure by the GABA<sub>B</sub> receptor antagonist CGP-35348 (500 µM) to affect the intra-CA1 NCS-435 (300 µM; a) and t-HCA (300 µM; b) induced increase in local dialysate glutamate levels in the awake rat. Each point represents the mean ± SEM of four to six animals, expressed as percentages of the mean of the four basal values before treatment. The antagonist was included in the perfusion medium 15 min prior to NCS-435 or t-HCA and maintained till the end of the experiment. The histograms of the areas under the curves which represent the integrated time-response curve of the overall effects are shown on the right sides of each panel. The areas under the curves were calculated as percentage changes in basal value over time (Δ basal percentage × time) by using the trapezoidal rule. *p < 0.05, **p < 0.01 significantly different from the control as well as CGP-35348 alone groups according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

Table 2 Lack of sedative/hypnotic effect of t-HCA and NCS-435

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mice with LRR*/</th>
<th>Onset*</th>
<th>Sleep time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td>mice tested</td>
<td>(min)</td>
</tr>
<tr>
<td>GHB (1000)</td>
<td>0/9</td>
<td>36.5 ± 2.2</td>
<td>115.3 ± 10.1</td>
</tr>
<tr>
<td>t-HCA (1000)</td>
<td>0/5</td>
<td>Not determined</td>
<td>0</td>
</tr>
<tr>
<td>NCS-435 (2000)</td>
<td>0/6</td>
<td>Not determined</td>
<td>0</td>
</tr>
</tbody>
</table>

In the Onset column, values represent the time interval between the treatment and the onset of the loss of righting reflex. Each value is the mean ± SEM of the animals presenting LRR. *LRR, loss of righting reflex. *Onset, time interval between treatment and onset of LRR.

levels (Figs 7a and b). The intrahippocampal perfusion of CGP 35348 per se did not affect extracellular glutamate levels.

Loss of righting reflex in mice

Table 2 shows that neither t-HCA nor NCS-435 induced loss of the righting reflex in mice up to the dose of 1000 and 2000 mg/kg i.p., respectively. Conversely, as expected from a previous study (Carai et al. 2001), all mice treated with 1000 mg/kg of GHB lost the righting reflex, this effect being reversed by the GABA_B receptor antagonists SCH 50 911 (Bolser et al. 1995), but not by the GHB receptor NCS-382 (data not shown).

Discussion

The present results confirm previous observations (Bena-vides et al. 1982; Hechler et al. 1993; Bourguignon et al. 2000) that the GHB analogues t-HCA and NCS-435 bind to GHB receptor, displacing [3H]GHB from both the high- and the low-affinity GHB binding sites with a comparable potency to GHB.

However, in contrast to GHB, t-HCA and NCS-435 are completely ineffective in displacing [3H]baclofen from GABA_B receptors, indicating that they are selective ligands for GHB binding sites.

Moreover, our results have shown that the two GHB analogues share with GHB the ability to increase extracellular glutamate in the hippocampus. As for GHB (EC_50 = 125 nM, Ferraro et al. 2001), the stimulant effect of the GHB analogues was inhibited by NCS-382 but not by CGP 35348, suggesting that it is a GHB receptor-mediated response.

However, while GHB at high concentrations reduced extracellular glutamate levels via GABA_B receptors (EC_50 = 428 μM, Ferraro et al. 2001), the two GHB analogues, consistent with their lack of affinity for GABA_B receptors, only exhibited a stimulant effect on extracellular glutamate, even at the millimolar concentrations.

The results indicate that the two GHB analogues have agonistic properties at GHB receptor sites controlling extracellular glutamate in the hippocampus and that both high- and low-affinity GHB binding sites are involved in their stimulatory effect. Because t-HCA is normally present in the mammalian CNS (Vayer et al. 1985) with a distribution similar to that of GHB (Vayer et al. 1988), it might be suggested that it represents a naturally occurring ligand for the GHB receptors controlling glutamate release.

Results of the experiments performed with GTPγS binding either in cortex membrane homogenates or by autoradiography confirmed Snead’s (2000) observation that GHB shares with baclofen the ability to stimulate G protein activity in the CNS. However, GHB effect was found to be rather modest; a maximal stimulation of about 40% and 30% of GTPγS binding being produced by GHB concentration of 1 mM in the autoradiography and homogenate assay, respectively.

Moreover, in contrast to Snead’s (2000) observations, GHB effect was suppressed, similarly to that of baclofen, by CGP 35348 but not by the GHB-receptor antagonist NCS-382. These results indicate that GHB weakly activates a G protein-coupled to GABA_B and not GHB receptor.

Therefore, it was not surprising that, consistent with their lack of affinity for GABA_B receptors, the GHB analogues were completely ineffective in activating the G protein.

We have no convincing explanation for the discrepancy between our findings and Snead’s (2000) observations. The discordant results might be attributed to the different lines of rats used, as great individual differences in the sensitivity to GHB sedative–hypnotic effect in Sprague–Dawley rats have been observed (Colombo et al. 2001).

The availability of GHB analogues capable of selectively activating GHB receptors might offer a new tool to further clarify different problems such as the possible role of the GHB receptors and endogenous GHB in the control of glutamate release in the hippocampus, as well as in the sedative–hypnotic effect of GHB. To this purpose, the present study has demonstrated that t-HCA and NCS-435, contrary to GHB, failed to produce loss of the righting reflex in mice, constituting further evidence that GABA_B, but not GHB receptor, mediates this GHB response (Carai et al. 2001).

Moreover, previous studies (Hechler et al. 1992; Castelli et al. 2000) indicate that the highest GHB receptor densities in rat and human brain are present in brain areas, such as the hippocampal formation (CA1 fields, dentate gyrus) and the adjacent temporal and enthorinal cortex, that are involved in the control of cognitive functions (Victor and Agamanolis 1990; Bunsey and Eichenbaum 1993). Because hippocampal glutamate is implicated in memory and learning processes (Javitt and Zukin 1990; Akhondzadeh 1999) and GHB is known to have amnestic effect (Schwartz et al. 2000), possibly by an action on GABA_B receptors, it will be of great interest to determine whether t-HCA and NCS-435 would be able to improve cognitive processes.
Finally, the results suggest that the effect on hippocampal glutamate levels may be useful in discriminating agonistic or antagonistic properties of GHB receptor ligands.

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