Journal of Neurochemistry, 2001, **78**, 929–939

γ-Hydroxybutyrate modulation of glutamate levels in the hippocampus: an *in vivo* and *in vitro* study

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Abstract

The effect of γ-hydroxybutyric acid on extracellular glutamate levels in the hippocampus was studied by microdialysis in freely moving rats and in isolated hippocampal synaptosomes. Intra-hippocampal (CA1) perfusion with γ-hydroxybutyric acid (10 nM–1 mM) concentration-dependently influenced glutamate levels: γ-hydroxybutyric acid (100 and 500 nM) increased glutamate levels; 100 and 300 μM concentrations were ineffective; whereas the highest 1 mM concentration reduced local glutamate levels. The stimulant effect of γ-hydroxybutyric acid (100 nM) was suppressed by the locally co-perfused γ-hydroxybutyric acid receptor antagonist NCS-382 (10 μM) but not by the $GABA_B$ receptor antagonist CGP-35348 (500 μM). Furthermore, the γ-hydroxybutyric acid (1 mM)-induced reduction in CA1 glutamate levels was counteracted by NCS-382 (10 μM), and it was also reversed into an increase by CGP-35348. Given alone, neither NCS-382 nor CGP-35348 modified glutamate levels. In hippocampal synaptosomes, γ-hydroxybutyric acid (50 and 100 nM) enhanced both the spontaneous and K^+-evoked glutamate efflux, respectively, both effects being counteracted by NCS-382 (100 nM), but not by CGP-35348 (100 μM). These findings indicate that γ-hydroxybutyric acid exerts a concentration-dependent regulation of hippocampal glutamate transmission via two opposing mechanisms, whereby a direct γ-hydroxybutyric acid receptor mediated facilitation is observed at nanomolar γ-hydroxybutyric acid concentrations, and an indirect $GABA_B$ receptor mediated inhibition predominates at millimolar concentrations.

Keywords: CA1, conscious rat, CGP-35348, microdialysis, NCS-382, synaptosomes.
J. Neurochem. (2001) **78**, 929–939.

γ-Hydroxybutyric acid (GHB) is a natural constituent of mammalian brain (Roth and Giarman 1970), and is synthesized from GABA via GABA-transaminase and a specific succinic semialdehyde dehydrogenase (Gold and Roth 1977; Snead *et al*. 1989).

Evidence is accumulating for the role of GHB as a neurotransmitter or neuromodulator including (i) synaptosomal localization (Snead and Liu 1984); (ii) the presence of release/uptake mechanisms (Vayer *et al*. 1987; Vayer and Maitre 1988); (iii) a heterogeneous brain distribution of specific high- and low-affinity GHB binding sites (Vayer *et al*. 1987; Maitre 1997). As GHB fulfils most of the criteria as a putative neurotransmitter (Maitre *et al*. 2000), its specific binding sites have been termed GHB receptors despite the fact that, to date, no GHB containing neuronal pathway has been demonstrated.

Exogenously administered GHB crosses the blood–brain barrier and causes behavioural, electrophysiological and biochemical effects in a dose-related fashion that appears to recruit the $GABA_B$ receptor at higher doses (Tunnicliff

Received November 17, 2000; revised manuscript received March 28, 2001; accepted April 6, 2001.

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Abbreviations used: CA1, intra-hippocampal; GHB, γ-hydroxybutyric acid; TTX, tetrodotoxin.

1992; Cash 1994; Maitre 1997). Thus, electrophysiological responses to low doses of GHB are blocked by the GHB receptor antagonist NCS-382 (Godbout *et al*. 1995), whereas the effects of high doses are reversed by $GABA_B$ receptor antagonists (Xie and Smart 1992; Williams *et al*. 1995), suggesting that the pharmacological effects of low and high doses of GHB are mediated by GHB and $GABA_B$ receptors, respectively (Maitre 1997; Lingenhoehl *et al*. 1999). Biochemical findings indicate that GHB modifies the activity of different neurotransmitters (Diana *et al*. 1991; Banerjee and Snead 1995; Howard and Feigenbaum 1997; Gobaille *et al*. 1999). In spite of these findings, the precise role of GHB and/or $GABA_B$ receptors in the neurochemical and neurological effects of GHB remains unclear. Furthermore, clinical and behavioural studies reported that the exogenous administration of GHB induced a modification of behaviour and electroencephalogram trace. In fact, the drug possesses hypnotic action and induces amnesia in several species (Maitre 1997; Schwartz *et al*. 2000). Moreover, GHB abuse or intoxication has been associated with a Wernicke–Korsakoff syndrome (Friedman *et al*. 1996), mild euphoria, respiratory depression, aggressive behaviour and coma (Engelsen and Christensen 1999).

Emerging evidence suggests that GHB plays a key role in regulating glutamate transmission in the hippocampus. Thus, autoradiography and binding studies in mammalian and human brain indicate that the highest GHB receptor density is found in the hippocampus (Snead and Liu 1984; Hechler *et al*. 1992). In addition, electrophysiological hippocampal slice studies show that micromolar GHB concentrations depress electrically evoked postsynaptic potentials mediated by NMDA and AMPA/kainate receptors, and that this effect was blocked by NCS-382 (Berton *et al*. 1999). At even higher millimolar concentrations GHB has also been reported to hyperpolarize intra-hippocampal (CA1) glutamate-containing pyramidal neurones, an effect also counteracted by local $GABA_B$ receptor blockade (Xie and Smart 1992). These findings suggest that GHB indirectly reduces hippocampal glutamate transmission via the local $GABA_B$ receptor. At the other end of the dose range, Aizawa *et al*. (1997) reported that the systemic administration of low doses of γ-butyrolactone, a pro-drug of GHB, augments long-term potentiation in the CA1 region and this is also counteracted by NCS-382, suggesting that GHB receptor activation can also increase hippocampal glutamate transmission independently of local $GABA_B$ receptor involvement. Thus, we investigated by microdialysis the effect of the local (CA1) perfusion of a wide range of GHB concentrations on extracellular glutamate levels in the hippocampus, and the influence of the GHB- and $GABA_B$-receptor antagonists, NCS-382 and CGP-356348, respectively, on GHB response.

Moreover, we also investigated the effect of GHB on spontaneous and K^+-evoked glutamate efflux in isolated hippocampal synaptosomes and the influence of NCS-382 and CGP-35348 on GHB response.

Materials and methods

Animals

Adult male Sprague–Dawley rats (280–300 g, Stefano Morini, Reggio Emilia, Italy) were housed at constant temperature (22°C) under a 12 : 12 h light–dark cycle (lights on at 6.00 AM) and provided with food and water *ad libitum*. Following delivery, the rats were allowed to adapt to the environment for at least one week before the experiments commenced. The local ethics committee approved all experiments.

Microdialysis study

On the day of microdialysis probe implantation, the animals were anaesthetized with a halothane/air (1.5%) mixture and mounted in a David Kopf Instruments (Tujunga, CA, USA) stereotaxic frame with the upper incisor bar set at − 2.5 mm below the interaural line. After exposing the dura and drilling one hole, a microdialysis probe of concentric design (molecular weight cut-off, 20 kDa; length of dialysing membrane, 1 mm; outside diameter, 0.24 mm; CMA 11, Carnegie Medicin, Stockholm, Sweden), was implanted into the right or the left CA1 region of the hippocampus. The coordinates relative to the bregma were: A, − 3.4 mm; L, ± 1.8 mm; V, − 2.8 mm (Paxinos and Watson 1986). Following the implantation, the probe was permanently secured to the skull with methacrylic cement and 36 h later microdialysis was carried out.

On the day of the microdialysis experiment, the probe was continuously perfused with Ringer solution (in mM: Na^+ 147; K^+ 4; Ca^{2+} 1.4; Cl^- 156; glucose 2.7) at a constant flow rate of 2 μL/min, by using a CMA 100 microinfusion pump. The probe was employed both for intra-CA1 perfusion with GHB and recovery of perfusate samples for measurement of local glutamate levels. When required, local perfusion with the GHB receptor antagonist NCS-382, and the $GABA_B$ receptor antagonist CGP-35348 (Olpe *et al*. 1990) alone and in the presence of GHB was also performed. 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 four stable basal glutamate values were obtained, GHB (10 nM–1 mM) was included into the perfusate medium for 60 min. This medium was then replaced with the original perfusate and another four samples were collected (60 min). In a parallel set of experiments NCS-382 (10 μM) and CGP-35348 (500 μM) were included in the perfusion medium either alone, or in combination with GHB, 60 min after the onset of sample collection and maintained until the end of the experiment (120 min). The antagonists were included in the perfusion medium 30 min prior to the addition of GHB. When required, tetrodotoxin (TTX; 1 μM) or a low-calcium (0.2 mM) Ringer solution were locally perfused either alone or 40 min prior to the addition of GHB, and lasted until the end of the experiment.

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 was carefully verified in 30-μm-thick coronal

cryostat sections. Only those animals in which the probe was correctly located were included in this study.

Synaptosomal study

On the day of the experiment, the animals were killed by decapitation under light ether anaesthesia, and the hippocampi were rapidly homogenized in ice-cold 0.32 M sucrose buffered at pH 7.4. The homogenate was centrifuged at 1000 *g* (10 min; 4°C) and then the supernatant was again centrifuged at 12 000 *g* (20 min; 4°C) to obtain the crude synaptosomal pellet (P_2). The synaptosomes were then resuspended in oxygenated (95% O_2/5% CO_2) Krebs' solution (mM composition: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10; gassed with 95% O_2 and 5% CO_2) and maintained at 37°C for 20 min. Thereafter, 0.5-mL aliquots of the suspension (protein concentration ≅ 0.45 mg protein/mL) were slowly injected into nylon syringe filters (outer diameter, 13 mm; pore size, 0.45 μm; Micron Separation Inc., Westport, MA, USA). Each filter, placed in a thermostatic bath set at 37°C, was connected to a peristaltic pump, and superfused at a flow rate of 0.3 mL/min with a pre-oxygenated Krebs' solution. Thirty minutes from the onset of the superfusion, samples were collected every 5 min from each filter. After the collection of three basal glutamate values, the effect of GHB on spontaneous glutamate efflux was evaluated by adding GHB (50 and 100 nM) to the superfusion medium for 15 min (three samples). Thereafter, the filters were perfused with the original Krebs' solution and three more samples were collected. The effect of the drug was also evaluated in the presence of the GHB receptor antagonist NCS-382 (100 nM), or the $GABA_B$ receptor antagonist CGP-35348 (100 μM), included in the superfusion medium 5 min before the addition of GHB and maintained until the end of the collection period. The effect of GHB on K^+-evoked glutamate efflux was also evaluated. To this purpose high-concentration K^+ stimulation (10 mM; 1-min pulse) was applied at the onset of the fourth collection period, whereas GHB (50 nM) was added to the perfusion medium 5 min before that. When required, NCS-382 (100 nM) or CGP35348 (100 μM) was added to the perfusion medium 5 min before the addition of GHB.

Glutamate analysis

Glutamate was quantified using an HPLC/fluorimetric detection system, including precolumn derivatization *o*-phtaldialdehyde reagent and a Chromsep 5 (C18) column (Ferraro *et al*. 1997). The mobile phase consisted of 0.1 M sodium acetate, 10% methanol and 2.5% tetrahydrofurane, pH 6.5. The limit of detection for glutamate was 30 fmol/sample.

The inclusion of GHB, 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.

Data analysis

Data from individual time points were reported as percentages of the mean of either four (*in vivo* experiments) or three (*in vitro* experiments) basal perfusate values collected prior to treatments with GHB, NCS-382 or CGP-35348. The data were calculated as mean ± SEM and the significance with regard to the peak effects (maximal responses) is shown in the figures. In the *in vitro* study, K^+-evoked glutamate overflow was calculated by subtracting the presumed basal efflux (obtained by the interpolation between the

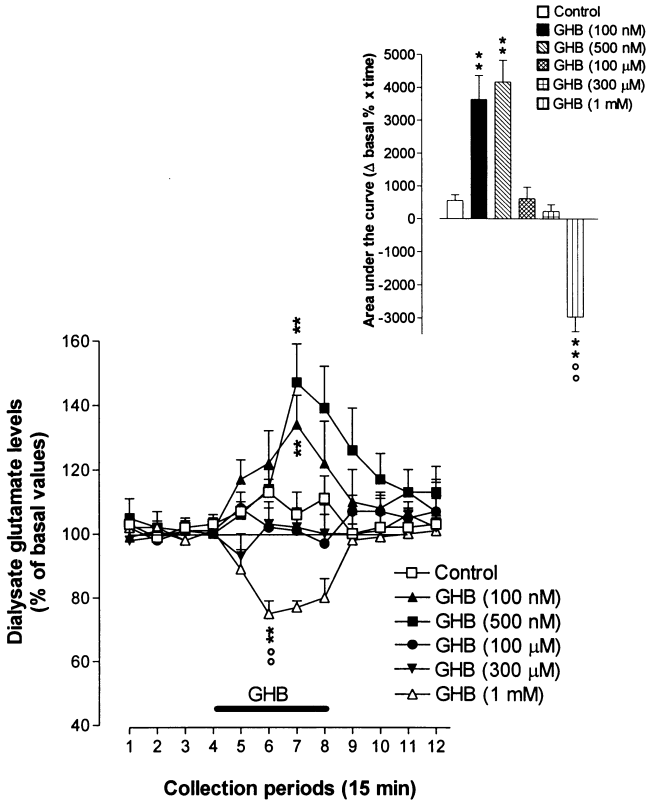

Fig. 1 Effects of intra-CA1 perfusion with γ-hydroxybutyric acid (GHB) on local dialysate glutamate levels in the conscious rat. Each point represents the mean ± SEM of either five or six animals, expressed as percentages of the mean of the four basal values before treatment. The histograms of the areas under the curves, which represent the integrated time-response curve of the overall effects, are shown in the upper 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. $^{**}p < 0.01$ significantly different from control as well as GHB 100 and 300 μM; $^{\circ\circ}p < 0.01$ significantly different from GHB 100 and 500 nM according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons. The significance with regard to the peak effects (maximal responses) is shown in the figures. The effects of the drug remain significant until the end of the drug perfusion.

Table 1 Effects of local perfusion with tetrodotoxin (TTX) and a low-concentration Ca^{2+} (0.2 mM) perfusion medium on basal (A) and γ-hydroxybutyric acid (GHB)-induced changes (B) in dialysate glutamate levels in the hippocampus of the awake rat

	Glutamate	
Treatment	peak effect (% of basal values)	area (Δ basal X time)
A		
Control	110 ± 6	521 ± 396
TTX (1 μM)	93 ± 7	652 ± 654
Ringer's low Ca^{2+} (0.2 mM)	111 ± 9	854 ± 425
B		
GHB (100 nM)	135 ± 6**	3754 ± 462**
TTX (1 μM) + GHB (100 nM)	109 ± 7	951 ± 647
Ringer's low Ca^{2+} (0.2 mM) + GHB (100 nM)	110 ± 4	856 ± 451
GHB (1 mM)	74 ± 4**	− 3012 ± 378**
TTX (1 μM) + GHB (1 mM)	87 ± 3*,°	− 1895 ± 351*,°
Ringer's low Ca^{2+} (0.2 mM) + GHB (1 mM)	87 ± 4*,°	− 1745 ± 344*,°

(A) Basal levels: TTX or low-concentration Ca^{2+} Ringer's solution was added to the perfusion medium following four stable basal samples (15 min/sample; 2 μL/min) and remained in the perfusion medium until the end of the collection period. Control rats were perfused with Ringer's solution throughout the experimental period (180 min). The data are expressed as a percentage of the mean of the four basal values before treatment. (B) GHB: GHB was locally perfused after four stable basal samples were obtained. Thereafter, eight more perfusate samples were collected. When necessary, TTX (1 μM) or low-concentraion Ca^{2+} Ringer's solution was added to the perfusion medium 40 min prior to GHB perfusion, and remained in the perfusion medium until the end of the experiment (160 min). The data are expressed as a percentage of the final baseline sample before GHB perfusion. The maximal peak changes and the areas under the curves, calculated as percentage changes in basal values over time (Δ basal percentage × time) by using the trapezoidal rule, are shown. Each value represents the mean ± SEM of either five or six experiments. *$p < 0.05$, **$p < 0.01$, significantly different from control group; °$p < 0.05$, significantly different from the respective GHB-treated group according to a one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

sample before and after the stimulus) from the total efflux observed in the stimulated samples (fourth).

In addition, the area under the curve, which reflects the duration of the effect over the entire experimental period (120 min), was calculated for each animal. The area values (overall effects) were calculated as percentage changes in basal value over time (Δ basal percentage × time) by using the trapezoidal rule.

In both sets of experiments, the statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for multiple comparisons.

Materials

The GHB (Laboratorio Farmaceutico CT, Sanremo, Italy), NCS-382 (6,7,8,9-tetrahydro-5-[*H*]benzocyclohepte-5-ol-4-ylidene acetic acid) and CGP-35348 (3-aminopropyl-diethoxy-methilphosponic acid; Tocris Cookson Ltd, Bristol, UK) were dissolved in saline and Ringer solution, respectively. At all concentrations tested, the compounds did not affect the pH of the perfusion medium (pH = 7.0). The solutions were freshly prepared just before each experiment and were used only once. All other chemicals used were of analytical grade and purchased from Sigma Chemical Co. (St Louis, MO, USA) or Merck & Co., Inc. (Whitehouse Station, NJ, USA).

Results

Microdialysis

Basal dialysate glutamate levels

Basal CA1 glutamate levels were 0.223 ± 0.027 μM ($n = 31$), and in the control group the levels remained stable throughout the 180-min time period of the experiment with a fluctuation of no more than 3–5%.

GHB alone

Intra-CA1 GHB (10 nM–1 mM; 60 min) differently influenced local glutamate levels in a concentration-dependent manner. As shown in Fig. 1, nanomolar concentrations (100–500 nM) increased glutamate levels (+38 ± 8% and + 58 ± 6% of basal values, respectively), with the maximum effect observed 45 min after the onset of GHB perfusion. In contrast, micromolar GHB concentrations (100 and 300 μM) had no effect, whereas the highest 1 mM concentration reduced glutamate levels (− 23 ± 4%) with the maximum effect observed 30 min after the onset of perfusion, and glutamate levels rapidly returned to basal values after the switch back to normal Ringer perfusion

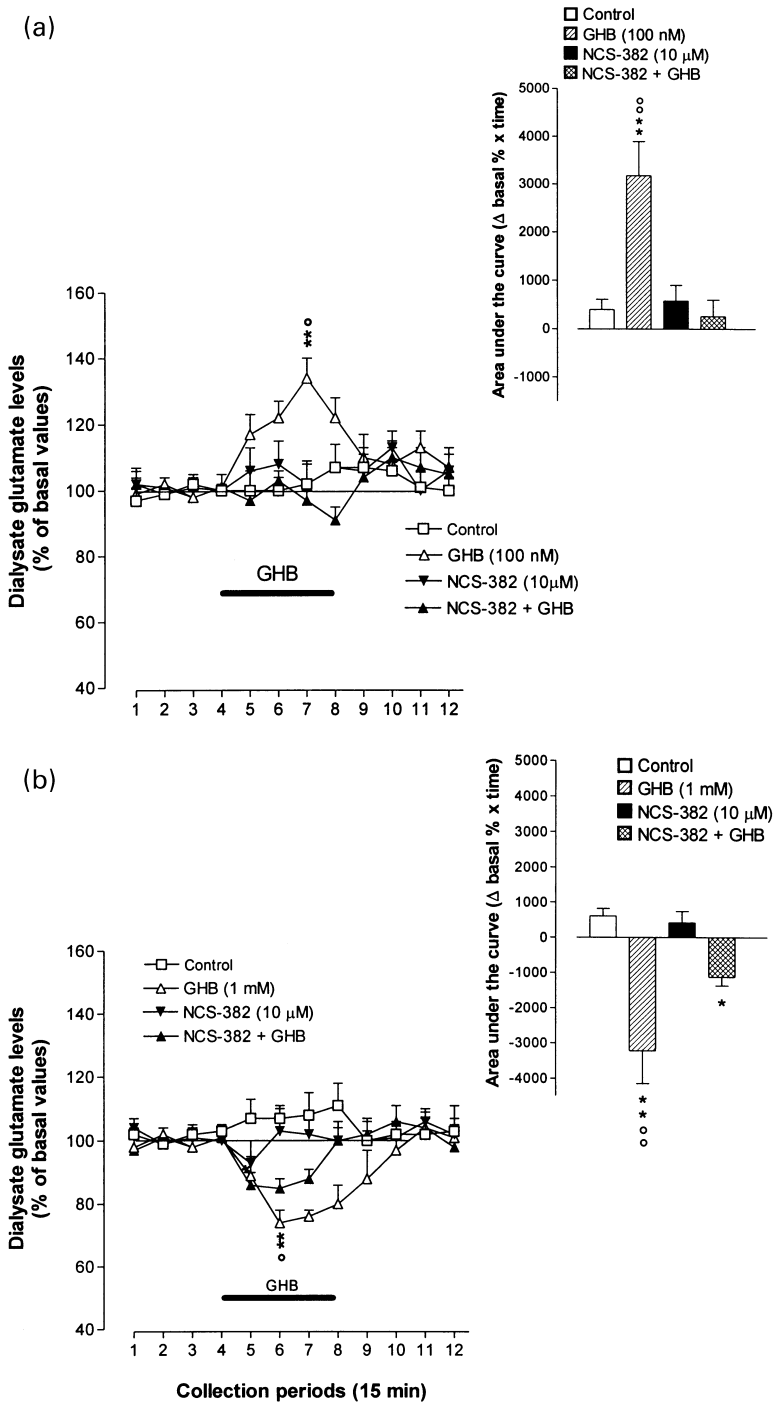

Fig. 2 (a) Counteraction by NCS-382 (10 μM) of the stimulant effect of intra-CA1 γ-hydroxybutyric acid (GHB; 100 nM) perfusion on local dialysate glutamate levels in the conscious rat. (b) Partial counteraction by NCS-382 (10 μM) of intra–CA1 GHB (1 mM)-induced reduction on local dialysate glutamate levels in the conscious rat. Each point represents the mean ± SEM of either seven or eight animals, expressed as percentages of the mean of the four basal values before treatment. The antagonist was included in the perfusion medium 30 min prior to GHB and maintained till the end of the experiment. The histograms of the areas under the curves that represent the integrated time-response curve of the overall effects are shown in the upper panels of (a) and (b). 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, NCS-382 alone group, respectively; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, significantly different from the respective GHB + NCS-382 group according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

medium. The lower 10 nM GHB concentration was without effect (data not shown).

TTX and low-calcium

Intra-CA1 TTX (1 μM, 120 min) or low-calcium (0.2 mM, 120 min) Ringer's solution did not affect local dialysate glutamate levels (Table 1). However, the GHB (100 nM)-induced increase in glutamate levels was counteracted when TTX or low-calcium medium were perfused into the CA1 40 min prior to the addition of GHB. Furthermore, both TTX and low-calcium solution partially counteracted the GHB (1 mM)-induced decrease in glutamate levels.

NCS-382 alone and together with GHB

In order to determine the extent to which the intra-CA1 GHB induced facilitatory and inhibitory effects on local

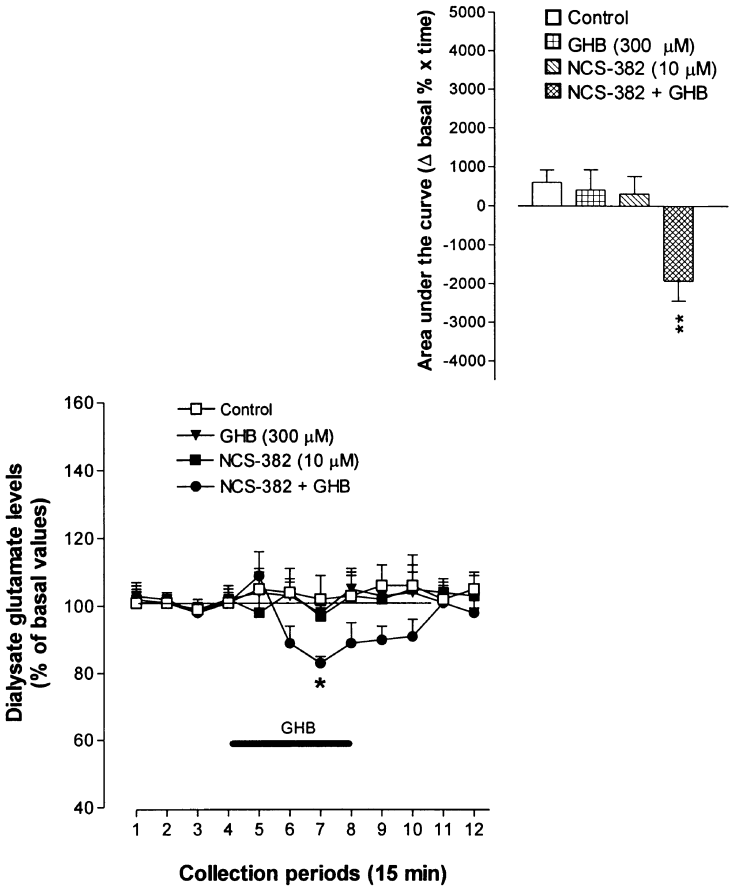

Fig. 3 Inhibitory effect of intra-CA1 GHB (300 μM) on local dialysate glutamate levels when co-perfused with NCS-382 (10 μM). Each point represents the mean ± SEM of either four or five animals, expressed as percentages of the mean of the four basal values before treatment. The antagonist was included in the perfusion medium 30 min prior to the addition of GHB and maintained until the end of the experiment. The histograms of the areas under the curves that represent the integrated time-response curve of the overall effects are shown in the upper panel of the figure. 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 other groups according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

glutamate levels was mediated by local GHB receptors, the GHB receptor antagonist NCS-382 (10 μM) was locally coperfused starting 30 min prior to GHB (100 nM and 1 mM). The addition of NCS-382 alone failed to influence local glutamate levels; however, it totally suppressed the GHB (100 nM)-induced increase in glutamate levels (Fig. 2a), and at least partially counteracted the GHB (1 mM)-induced reduction in local glutamate levels (Fig. 2b). Finally, NCS-382 converted the previously ineffective GHB (300 μM) effect to a decrease in glutamate levels (− 17 ± 2%) (Fig. 3)

CGP-35348 alone and together with GHB

In order to determine the extent to which the intra-CA1 GHB (1 mM)-induced reduction in local glutamate levels was influenced by local $GABA_B$ receptors, the $GABA_B$ receptor antagonist CGP-35348 (500 μM) was locally co-perfused with GHB starting 30 min prior to the addition of GHB. The addition of CGP-35348 alone failed to modify local glutamate levels, whereas it reversed the GHB-induced reduction into an increase (+29 ± 6%; Fig. 4a), and it converted the previously ineffective GHB (300 μM) effect to an increase in glutamate levels (+40 ± 5%) (Fig. 4b). In contrast, CGP-35348 failed to modify the ability of nanomolar GHB concentrations to increase local glutamate levels (data not shown).

Synaptosomal preparation

The effect of exogenously applied GHB on spontaneous and K^+-evoked glutamate efflux in an isolated hippocampal synaptosomal preparation was studied in order to determine the extent to which the ability of GHB to increase hippocampal glutamate levels was mediated by the activation of presynaptic GHB receptors on local glutamatergic terminals.

Basal glutamate efflux

Basal glutamate efflux, as calculated from the mean of the three samples collected before drug treatment (0–15 min), was 52.2 ± 1.1 pmol/mg protein/min and declined slightly over the duration of the experiment (45 min; Fig. 5).

The effect of GHB alone, and together with NCS-382 and CGP-35348, on basal glutamate efflux

The addition of GHB (100 nM, 15 min) to the perfusion medium induced an increase (+33 ± 3%) in glutamate efflux, which was maximal 5 min after onset of exposure to GHB and returned to basal values upon switching back to normal Krebs' perfusion medium. At the lower 50 nM

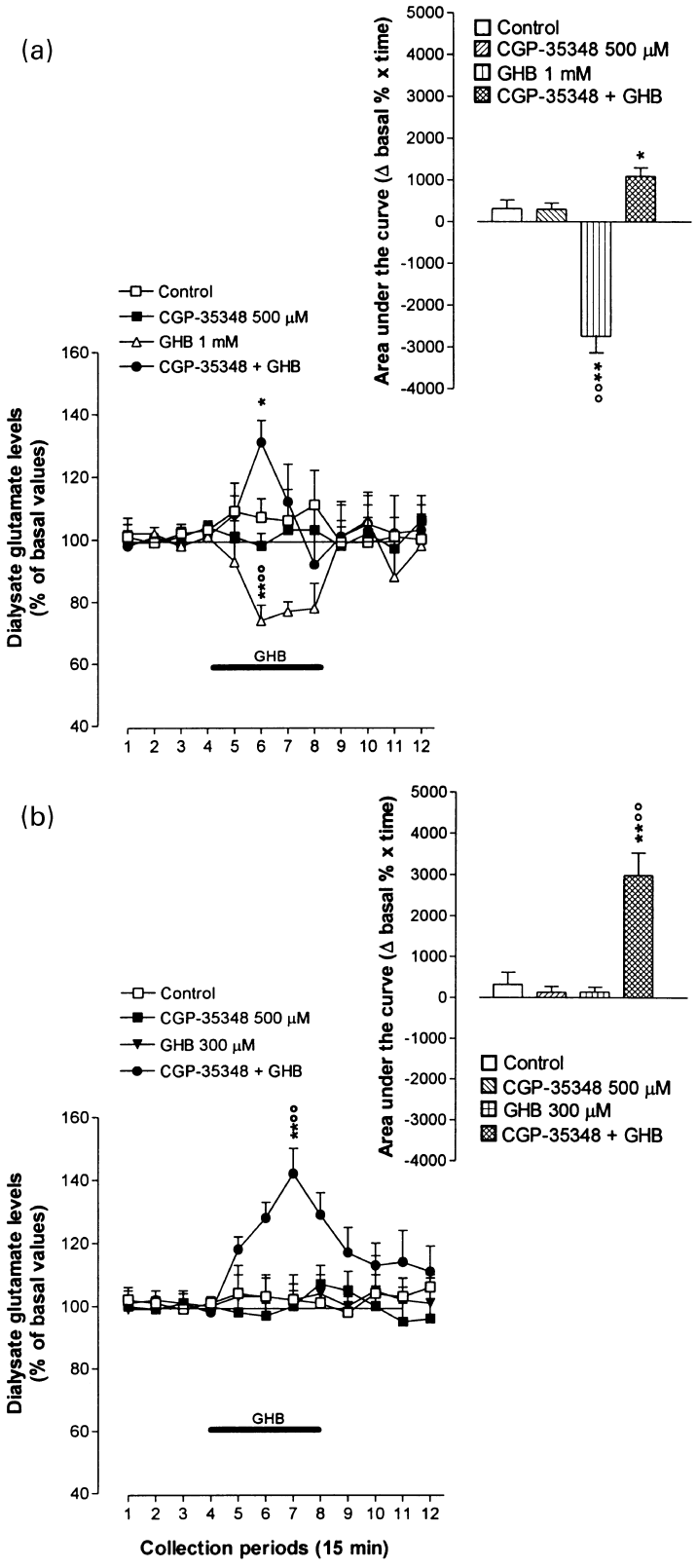

Fig. 4 (a) Counteraction and reversal by the $GABA_B$ receptor antagonist CGP-35348 (500 μM) of the intra-CA1 γ-hydroxybutyric acid (GHB; 1 mM)-induced reduction in local dialysate glutamate levels in the conscious rat. (b) Stimulatory effect of intra-CA1 GHB (300 μM) on local dialysate glutamate levels when co-perfused with CGP-35348 (500 μM). Each point represents the mean ± SEM of either six or seven animals, expressed as percentages of the mean of the four basal values before treatment. The antagonist was included in the perfusion medium 30 min prior to the addition of GHB and maintained until the end of the experiment. The histograms of the areas under the curves that represent the integrated time-response curve of the overall effects are shown in the upper panels of (a) and (b). 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 and CGP-35348-alone group, respectively; $^{\circ\circ}p < 0.01$ significantly different from the respective CGP-35348 + GHB group according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

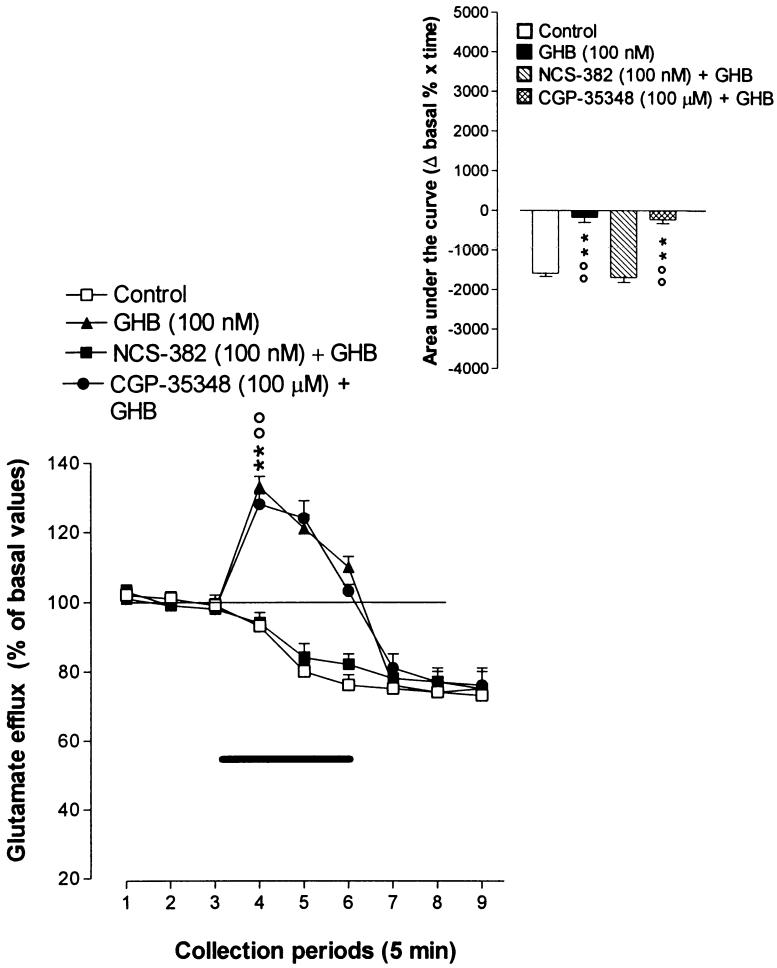

Fig. 5 Effects of γ-hydroxybutyric acid (GHB; 100 nM) on spontaneous glutamate efflux from rat hippocampal synaptosomes. Inhibition of GHB induced increase by NCS-382 (100 nM) but not by CGP-35348 (100 μM). The black bar indicates the period of perfusion with GHB (15 min). The antagonists were included in the perfusion medium 5 min prior to the addition of GHB and maintained until the end of the experiment (35 min). Each point represents the mean ± SEM of either six or seven experiments, expressed as percentages of the mean of the two basal values before treatment. The histograms of the areas under the curves that represent the integrated time-response curve of the overall effects are shown in the upper panel of the figure. The areas under the curves were calculated as percentage changes in basal value over time (Δ basal percentage × time) by using the trapezoidal rule. $^{**}p < 0.01$ significantly different from the control; $^{\circ\circ}p < 0.01$ significantly different from the NCS-382 + GHB group according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

concentration GHB did not affect basal glutamate efflux (data not shown). The GHB (100 nM)-induced increase in spontaneous glutamate efflux was counteracted by co-perfusion with NCS-382 (100 nM). In contrast, co-perfusion with CGP-35348 (100 μM) together with GHB (100 nM) did not alter the GHB-induced increase in glutamate efflux (Fig. 5). At the concentrations used, neither NCS-382 nor CGP-35348 alone altered the basal glutamate efflux (data not shown).

The effect of GHB alone, and in combination with NCS-382 and CGP-35348, on K^+-evoked glutamate efflux

As expected, a 1-min pulse of high K^+ concentration (10 mM) Krebs' solution significantly increased glutamate efflux (+46 ± 4%). Interestingly, the addition of 50 nM GHB to the perfusion medium 5 min before the 10 mM K^+ pulse enhanced the K^+-evoked glutamate efflux (+28 ± 4%; Fig. 6), and this effect was suppressed by co-perfusion with a 100-nM concentration of NCS-382, but not by CGP-35348 (100 μM; Fig. 6). At the concentrations used, neither NCS-382 nor CGP-35348 alone significantly altered K^+-evoked glutamate efflux (data not shown).

Discussion

The present combined *in vivo* microdialysis and *in vitro* synaptosomal study clearly demonstrates that exogenously applied GHB concentration-dependently controls hippocampal glutamate levels: where nanomolar GHB concentrations increase glutamate levels; intermediate micromolar concentrations have no effect and millimolar concentrations reduce glutamate levels.

The finding that the GHB (100 nM)-induced increase in dialysate CA1 glutamate levels is counteracted by the GHB receptor antagonist NCS-382, but not by the $GABA_B$ receptor antagonist CGP-35348, suggests that in this concentration range GHB increases local hippocampal glutamate levels via a direct activation of local GHB receptors, without the involvement of the local $GABA_B$ receptor. This view is supported by findings from the *in vitro* hippocampal synaptosomal studies indicating that nanomolar GHB concentrations increase both spontaneous and K^+-evoked hippocampal glutamate efflux, and that these effects are also fully counteracted by NCS-382 but not by CGP-35348. In this context it is worth noting that

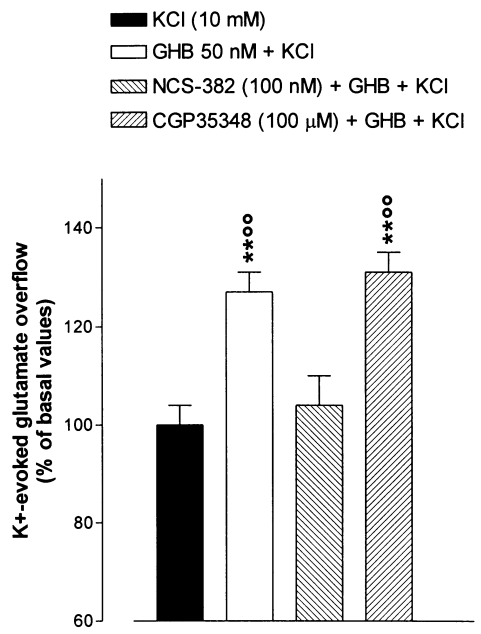

Fig. 6 Effects of GHB (50 nM) on K^+-evoked glutamate efflux from rat hippocampal synaptosomes. Inhibition of GHB-induced increase by NCS-382 (100 nM), but not by CGP-35348 (100 μM). High K^+ stimulation (10 mM; 1-min pulse) was applied after three basal samples had been collected, whereas GHB (50 nM) was added to the perfusion medium 5 min before. When required, NCS-382 or CGP-35348 were added to the perfusion medium 5 min before GHB. The effects of the treatments are expressed as percentage of K^+-evoked overflow under control conditions (filled column). Each point represents the mean ± SEM of either six or eight experiments, expressed as percentages of the mean of the three basal values before treatment. $^{**}p < 0.01$, significantly different from the KCl (10 mM) group; $^{\circ\circ}p < 0.01$ significantly different from NCS-382 + GHB + KCl group according to one-way ANOVA followed by the Newman–Keuls test for multiple comparisons.

previous findings demonstrated that in similar synaptosomal preparations (Zhou *et al*. 1995; Muzzolini *et al*. 1997; Sbrenna *et al*. 1999) high potassium concentrations evoked an overflow of endogenous glutamate levels in a concentration-dependent manner. Furthermore, this K^+-induced glutamate overflow has been suggested to be largely calcium dependent and therefore, at least in part, an expression of a vesicular release from the nerve terminals.

According to previous *in vivo* microdialysis studies (Moghaddam 1993; Falkenberg *et al*. 1996; Rocher *et al*. 1999; Timmerman *et al*. 1999), basal dialysate CA1 glutamate levels are not affected by local perfusion of TTX (1 μM) or a low calcium concentration perfusion medium, thus suggesting that basal dialysate CA1 glutamate levels do not mainly originate from an exocytotically releasable pool (Timmerman *et al*. 1999). This is despite the fact that the present microdialysis preparation allowed for an extended 6-h wash-out period prior to sample collection. However, the present microdialysis findings showing that the GHB-induced facilitatory effects on glutamate levels are TTX sensitive and calcium dependent suggest that GHB receptor activation influences hippocampal neuronal glutamate transmission. Together with the *in vitro* observations, these results indicating a facilitatory effect of GHB lead us to hypothesize that the presynaptic GHB receptors can modulate an action potential dependent phenomenon in the hippocampal glutamate transmission.

The present *in vivo* and *in vitro* findings also provide strong evidence that the GHB (100 nM)-induced increase in hippocampal glutamate levels is directly mediated by the activation of GHB receptors located on CA1 afferent glutamate nerve terminals. In agreement with this hypothesis, the presence of GHB binding sites on nerve terminals in rat forebrain has already been demonstrated (Maitre *et al*. 1983; Maitre 1997), and a presynaptic GHB receptor-mediated action on the regulation of glutamate release in the ventro-basal nucleus thalamic and hippocampus of the rat, has been recently postulated (Banerjee and Snead 1995; Berton *et al*. 1999).

The mechanism by which the high (1 mM) GHB concentration decreases *in vivo* CA1 glutamate levels appears to employ an indirect mechanism involving the activation of local $GABA_B$ receptors as the reduction is only partially antagonized by NCS-382, and is reversed to increased levels of glutamate in the presence of CGP-35348. This is also in agreement with evidence from biochemical and electrophysiological studies showing that GHB is a weak agonist at both native and recombinant $GABA_B$ R_1/R_2 receptors (Bernasconi *et al*. 1992; Lingenhoehl *et al*. 1999). However, it has been reported that the GABA-like effects of GHB are a result of its conversion by tissue or the tissue extract into GABA (Hechler *et al*. 1997; Maitre 1997). Thus, further studies are necessary to determine if the high concentrations of exogenously administered GHB elevate hippocampal GABA levels. Finally, it cannot be ruled out that additive mechanisms such as the reduction of glutamate transmission, changes in glutamate metabolism, uptake and/or in the activity of its neuronal or glial carrier could participate in the observed inhibition of glutamate basal levels.

The lack of effect for the intermediate (100 and 300 μM) GHB concentrations may reflect a balanced activation of local GHB and $GABA_B$ receptors, resulting in a cancellation of their respective excitatory and inhibitory effects on CA1 glutamate levels. The finding that a CGP-35348-induced local $GABA_B$ receptor blockade converts the previously ineffective intermediate GHB concentrations into an increase in CA1 glutamate levels strongly supports this hypothesis. On the other hand, the observation that a NCS-382-induced local GHB receptor blockade unmasks the previously ineffective GHB 300 μM concentration into a decrease of glutamate levels, further confirm this view.

A possible explanation for the finding that NCS-382 only partially antagonizes the GHB (1 mM)-induced reduction in hippocampal glutamate levels is that two GHB binding sites may exist in the present preparations; one possessing high nanomolar, and the other low micromillimolar, affinity for GHB. In fact several studies have already demonstrated the existence of high- and low-affinity GHB bindings sites with nanomalar and millimolar dissociation constant (K_D) values, respectively (Benavides *et al*. 1982; Hechler *et al*. 1990, 1992; Maitre 1997). Thus, the activation of the high-affinity binding site increases local glutamate levels, whereas the activation of the low-affinity site facilitates a $GABA_B$ receptor mediated reduction in glutamate levels. However, NCS-382 itself also binds to the $GABA_B$ receptor with a K_i value of approximately 100 μM, and may also act as a weak $GABA_B$ receptor antagonist (unpublished findings).

Does the ability of endogenous GHB to influence CA1 glutamate levels underlie its clinical effects?

In the present study, we show that a complex intra-CA1 GHB receptor modulation of local glutamate levels is observed when exogenously administered GHB concentrations are sufficient to activate both GHB and $GABA_B$ receptors. However, the question remains as to whether the concentrations of endogenous GHB under physiological conditions are sufficient to activate the GHB receptor, and not the $GABA_B$ receptor. The synaptic concentration of endogenous GHB may reach millimolar concentrations, and a concomitant activation of GHB and $GABA_B$ receptors will occur in those brain regions where the two receptors coexist resulting in a reduction in local glutamate levels. However, autoradiographic studies have shown that in general the two receptors are not co-localized in the brain (Maitre 1997), suggesting that a differential activation predominates.

The hippocampus has the highest GHB concentrations and receptor densities in rats and man (Maitre 1997), and because hippocampal glutamate has been reported to play a crucial role in memory processes (Javitt and Zukin 1990) it is possible that by modulating CA1 glutamate levels, endogenous GHB may play a key role in cognitive processes. In fact, anecdotal clinical reports indicate that amnesia is associated with GHB intoxication and that GHB abuse is associated with Wernicke–Korsakoff syndrome (Friedman *et al*. 1996). Taken together with the present findings from the microdialysis and synaptosomal studies, these data lead us to hypothesize that the GHB-induced memory impairment may result from the ability of the drug to influence hippocampal glutamate transmission.

Conclusion

For the first time we have provided strong *in vivo* and *in vitro* evidence that exogenously administered GHB regulates CA1 glutamate levels. This finding suggests a physiological role for endogenous GHB in excitatory amino acid transmission in the CA1 and its possible relevance to the clinical manifestations of CA1-related pathology. Further studies are necessary in order to determine whether a similar modulation of CA1 glutamate levels also occurs in other brain regions following acute and repeated administration.

Acknowledgements

This work was supported by Laboratorio Farmaceutico CT (Sanremo, Italy), The Stanley Foundation (USA), The Charitable Infirmary Charitable Trust (Ireland) and Italian 60% grants.

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