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GET73 modulates rat hippocampal glutamate transmission: evidence for a functional interaction with $mGluR_5$

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Abstract:
In the present study, the effects of the γ-hydroxybutyrate (GHB) analog GET73 on hippocampal glutamate transmission have been evaluated by an approach combining *in vivo* microdialysis with the *in vitro* evaluation of tissue slices. The microdialysis results indicated that local perfusion (60 min) with 10 nM – 1 mM GET73 increased extracellular glutamate levels in the CA1 region of the hippocampus of freely moving rats in a concentration dependent manner. In tissue slices from the rat hippocampus, GET73 (1 μM – 10 μM) did not affect L-[^{3}H]glutamate uptake, whereas treatment with 1 μM GET73 significantly increased K^+-evoked, but not spontaneous, glutamate efflux. The GHB analog did not affect the increase in glutamate efflux induced by 100 μM and 300 μM NMDA. In contrast, 500 nM GET73, a concentration at which it is ineffective alone, partially but significantly counteracted the increase in K^+-evoked glutamate efflux induced by 100 μM CHPG, an mGluR5 agonist. When 500 nM GET73 was coperfused with 100 μM MPEP, it amplified the decrease in K^+-evoked glutamate efflux induced by the mGluR5 antagonist. Interestingly, the increase in K^+-evoked glutamate efflux induced by 1 μM GET73 was counteracted by coperfusion with a low (10 μM) concentration of MPEP, which by itself is ineffective. Finally, 500 nM GET73 did not affect the reduction of K^+-evoked glutamate efflux induced by the mGluR2/3 agonist LY379268.
These findings demonstrate that the GHB analog GET73 significantly affects glutamate transmission in the hippocampus, and its profile of action differs from that of its parent compound.

Key words:
CA1 hippocampus slices, glutamate efflux, mGluR5 antagonist, γ-hydroxybutyrate

Abbreviations: CNS – central nervous system, GHB – γ-hydroxybutyrate, mGluR – metabotropic glutamate receptor

Introduction

γ-Hydroxybutyrate (GHB) is a four-carbon chain monocarboxylic acid that is naturally present in the mammalian central nervous system (CNS). After systemic administration, GHB penetrates to the brain and, depending on the dose, can exert a wide range of neuropharmacological effects, such as euphoria, anxiolysis, hypnosis, anesthesia, memory impairment, absence, seizures, ataxia and amnesia [11, 14, 36, 40]. GHB is clinically used for the treatment of narcolepsy [37, 43, 52] and, in some European countries, for the treatment of alcohol dependence [1, 15]. Indeed, GHB attenuates alcohol consumption, craving and the

symptoms of alcohol withdrawal, both in animal models of alcoholism and human alcoholics [1, 9, 14, 26, 34]. GHB is also abused as recreational drug because it possesses euphorigenic and anabolic properties, induces behavioral disinhibition and enhances sex drive [12, 18].

Several pieces of evidence favor considering GHB to be a neurotransmitter and/or a modulator of neuronal signaling [33, 36]. Studies looking at the mechanisms by which GHB affects neural functioning have indicated that exogenous GHB binds to specific GHB receptors as well as to GABA receptors [14, 36] depending on its concentration in the brain. These interactions are thought to mediate its multiple behavioral effects. The highest density of GHB receptors is present in the hippocampus [3, 29], but significant amounts have also been found in the neocortex, the thalamus and in dopaminergic areas, such as the striatum and substantia nigra. The activation of GHB receptors affects dopamine, serotonin, acetylcholine, GABA and glutamate transmission [14, 20, 22, 26, 27, 38]. GHB regulates glutamate release in different brain areas, such as the nucleus accumbens and the hippocampus, similar to the effects of ethanol. The control of glutamatergic transmission by GHB is multifaceted and seems to depend on the administered dose. Electrophysiological studies show that GHB, when it activates its own receptor, reduces the efficacy of excitatory glutamatergic transmission *via* a significant suppression of NMDA-mediated evoked excitatory postsynaptic currents [35]. In contrast, an *in vivo* microdialysis and *in vitro* synaptosomal study clearly demonstrated that exogenously applied GHB controls hippocampal glutamate levels in a concentration dependent manner: nanomolar GHB concentrations increase glutamate levels, intermediate micromolar concentrations have no effect, and millimolar concentrations reduce glutamate levels [22]. The increase in hippocampal glutamate levels induced by nanomolar GHB concentrations is mediated by the activation of GHB receptors, while at millimolar concentrations, GHB reduces hippocampal glutamate levels by interacting with $GABA_B$ receptors [14, 15, 22]. Behavioral studies have also shown that some of the GHB-mediated effects involve the glutamatergic system and NMDA receptor function [2, 31]. In particular, a specific role for NMDA receptors in the $GABA_B$ receptor-mediated effects of GHB has been suggested [31].

Recently, the GHB analog *N*-(4-trifluoromethylbenzyl)-4-methoxybutanamide (GET73) was synthesized in an effort to obtain a compound with a better antialcohol profile than GHB. Unpublished data indicate that GET73 decreases spontaneous alcohol intake in Sardinian alcohol-preferring rats with a clear improvement over GHB in terms of both potency and duration of activity, with the reduction being significant for up to 6 h after administration [42; Colombo et al., unpublished data]. Furthermore, acute and 2–3 week treatments with GET73, at doses not associated with a detrimental effect on gross behavior and motor activity, significantly reduced the consumption and reinforcing effect of sucrose as well as other highly palatable foods [42, 51], suggesting that the compound may affect gratification mechanisms and pathways that mediate both drug and food reward. However, the mechanism underlying these effects of GET73 remains unclear. In fact, although chemically related to GHB, GET73 is not a prodrug of GHB and, in contrast to the parent compound, has no affinity for GHB or $GABA_B$ receptors [42]. Although at present there is no data on the neurochemical profile of action of this new compound, a series of behavioral studies aimed at characterizing the neuropharmacological profile of GET73 [Loche et al., unpublished data], led to the hypothesis that this compound may modulate glutamatergic neurotransmission. The pivotal role of glutamate in drug addiction and reward-related processes and the demonstrated ability of GHB to modulate glutamate levels (see above) prompted us to test this hypothesis by investigating the possible effects of GET73 on hippocampal glutamate transmission. To achieve this aim, we first investigated the role of GET73 in regulating glutamate levels in the CA1 region of hippocampus of alert rats using an *in vivo* microdialysis approach. In view of the results obtained, the profile of action of GET73 on glutamate transmission was pharmacologically characterized in rat hippocampal slices.

Materials and Methods

Animals

Male adult Sprague-Dawley rats (300–320 g, Harlan Italy S.r.l., Zona Ind. Azzida 57, S. Pietro al Natisone, Udine, Italy) were used. The animals were housed in cages in groups of five animals in a temperature- and

relative humidity-controlled environment with a regular 12 h light/dark cycle and had free access to food and water. Following delivery, the animals were allowed to adapt to the environment for at least one week before the experiment commenced.

Experiments were carried out in strict accordance with the European Communities Council Directive (86/609/EEC) and the Guidelines released by the Italian Ministry of Health (D.L. 116/92 and D.L. 111/94-B). A formal approval to conduct the experiments described was obtained by the local Ethic Committee (University of Ferrara, Italy). All efforts were made to minimize the number of animals used and their suffering.

Substances

N-(4-Trifluoromethylbenzyl)-4-methoxybutanamide (GET73) was a kind gift of CT laboratories (Sanremo, Italy). It was dissolved in DMSO and then added to the perfusion medium (final DMSO concentration was 0.01%) shortly before experiments. Control groups were treated with DMSO solution. l-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC), 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP), N-methyl-D-aspartate (NMDA), (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG) and (1S,2R,5R,6R)-2-amino-4-oxabicyclo [3.1.0]hexane-2,6-dicarboxylic acid (LY379268) were purchased from Tocris-Cookson (Bristol, UK). MPEP, LY379268 and CHPG were dissolved in DMSO, while PDC was dissolved in the perfusion medium.

In vivo experiments: microdialysis

Surgery

On the day of surgery, the animals, kept under halothane anesthesia (1.5% mixture of halothane and air), were mounted in a David Kopf stereotaxic frame with the upper incisor bar set at –2.5 mm below the interaural line. After exposing the skull and drilling a hole, a microdialysis probe of concentric design (CMA 12; MW cutoff 20,000 daltons; outer diameter 0.5 mm; length of dialyzing membrane 1 mm; Alfatech S.p.A., Via Scarsellini 97, 16149 Genova, Italy) was implanted into the CA1 region of the right or the left hippocampus. The coordinates relative to the bregma were: A: –3.8; L: ±2.0; V: –3.0 [44]. Following the implantation, the probe was permanently secured to the skull with methacrylic cement and 36 h later the release experiment was carried out.

Experimental protocol

On the day of the microdialysis experiment, the probe was continuously perfused with Ringer solution (in mM: Na^+ 147; K^+ 4; Ca^{++} 1.4; Cl^- 156; glucose 2.7) at a constant flow rate of 2 µl/min, using a CMA 100 microinfusion pump. This perfusion medium was chosen to compare the effects of GET73 with those previously displayed by its parent compound GHB under the same experimental conditions [22]. However, to compare the *in vivo* results with the *in vitro* ones (see below), control experiments with a Krebs solution as perfusion medium were performed.

The probe was employed for both local treatments and for the collection of perfusate samples.

The collection of perfusate samples commenced 300 min after the onset of perfusion to achieve stable dialysate glutamate levels and perfusates were collected every 15 min. After four stable basal glutamate values were obtained, 10 nM – 1 mM GET73 was added to the perfusate medium for 60 min (four collected samples). This concentration range was chosen to compare the effects of GET73 with those previously displayed, under the same experimental conditions, by its parent compound GHB [22]. This medium was then replaced with the original perfusate and another four samples were collected (60 min).

At the end of each experiment, the brain was removed from the skull, and the position of the probe 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.

In vitro experiments: hippocampus slices

Tissue preparation

On the day of the release experiment, the animals were sacrificed, their brains were promptly isolated and 400 µm thick slices (15–20 mg each) were obtained from both the left and right hippocampi with a McIlwain Tissue Chopper. The tissue was then allowed to equilibrate for 20 min at room temperature in Krebs' solution (composition in mM: NaCl 118; KC1 4.4; $CaC1_2$ 1.2; $MgS0_4$ 1.2; KH_2P0_4 1.2; $NaHCO_3$ 25; glucose 10) and gassed with a mixture of 95% O_2 and 5% CO_2.

L-[^{3}H]glutamate uptake

The effect of GET73 on L-[^{3}H]glutamate uptake in hippocampus slices was analyzed and compared with that of the specific glutamate uptake inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) [8]. For this purpose, the slices were incubated for 15 min at 37°C in Krebs' solution containing L-[^{3}H]glutamate (0.3 μCi) in the absence or presence of 1 nM or 10 nM GET73 or 0.1 mM PDC. Thereafter, the uptake was halted by replacing the incubation medium with ice-cold Krebs' solution. The radioactivity that had accumulated in the cells was extracted by incubation in 0.5 ml of acidic ethanol (95% ethanol/5% 0.1 M HCl) for 30 min at 37°C and quantified by liquid scintillation spectrometry. The specific [^{3}H]glutamate uptake was calculated as the difference between the uptake obtained in the incubation medium as described above and the uptake obtained with a similar incubation medium containing choline chloride instead of NaCl (nonspecific uptake). Na^+-independent uptake was less than 9 ± 3% of the total [17].

Spontaneous glutamate efflux

To determine the spontaneous glutamate efflux, the slices were transferred into oxygenated superfusion chambers (0.6 ml volume each; 2–3 slices/chamber, temperature 37°C) and continuously superfused with an oxygenated Krebs' solution at a flow rate of 0.3 ml/min. After 30 min of superfusion, samples were collected from each chamber every 5 min for 60 min for a total of 12 samples. The first three samples were used to assess basal glutamate release. After these samples were collected, the drugs under investigation (GET73, NMDA, the mGluR2/3 agonist LY379268 and the mGluR5 agonist and antagonist CHPG and MPEP, respectively) were added to the superfusion medium and maintained until the end of the experiment. GET73 was added to the superfusion medium in a wide range of concentrations (100 nM – 10 μM). This concentration range was chosen to compare the effects of GET73 with those previously displayed by its parent compound GHB under the same experimental conditions [22 and personal unpublished data].

When the effect of NMDA on endogenous glutamate levels was assessed, the slices were perfused with a Mg^{++}-free Krebs' solution. Control slices, superfused with Krebs' solution from the beginning till the end of the release experiment, were always assayed in parallel.

K^+-evoked glutamate efflux

To investigate the effect of GET73 on K^+-evoked glutamate efflux, 5 min samples were collected from 30 to 90 min from the onset of superfusion. During this period, the slices were stimulated by pulses (2 min duration) of high potassium (20 mM) Krebs' solution (corrected for osmolarity by replacing KC1 for NaCl) at 45 (St_1) and 70 (St_2) min after the onset of superfusion. The drugs under investigation were added to the superfusion medium 10 min before St_2 and maintained until the end of the experiment. GET73 was added to the superfusion medium in a wide range of concentrations (100 nM – 1 μM). This concentration range was chosen in order to compare the effects of GET73 with those previously displayed under the same experimental conditions by its parent compound GHB [22 and personal unpublished data].

Glutamate analysis

Endogenous glutamate levels were quantified using a HPLC/fluorimetric detection system, including pre-column derivatization with *o*-phthaldialdehyde reagent and a Chromsep 5 (C18) column [21]. The mobile phase consisted of 0.1 M sodium acetate, methanol (100 ml/l) and tetrahydrofurane (22 ml/l), pH 6.5. The limit of detection for glutamate was 30 fmol/sample.

Tissue slices: endogenous hippocampal glutamate efflux was calculated as nmol/min/g of wet tissue. The effects of treatments on spontaneous glutamate efflux were calculated as percentages of the mean ± SEM of the first three samples. The percentages obtained from treated groups were compared with the corresponding values obtained from untreated (control) slices assayed in parallel. When the effects of the drugs on K^+-evoked glutamate efflux were studied, the St_2/St_1 ratio for treated slices was calculated and compared with the corresponding St_2/St_1 value obtained from control slices assayed in parallel. K^+-evoked glutamate efflux is expressed as the percent increase over the spontaneous (i.e., basal) glutamate efflux, calculated as the mean of the two fractions collected prior to the depolarizing stimulus [23].

Data analysis

Microdialysis: data from individual time points are reported as percentages of the mean ± SEM of the three basal samples collected prior to treatment. The sig-

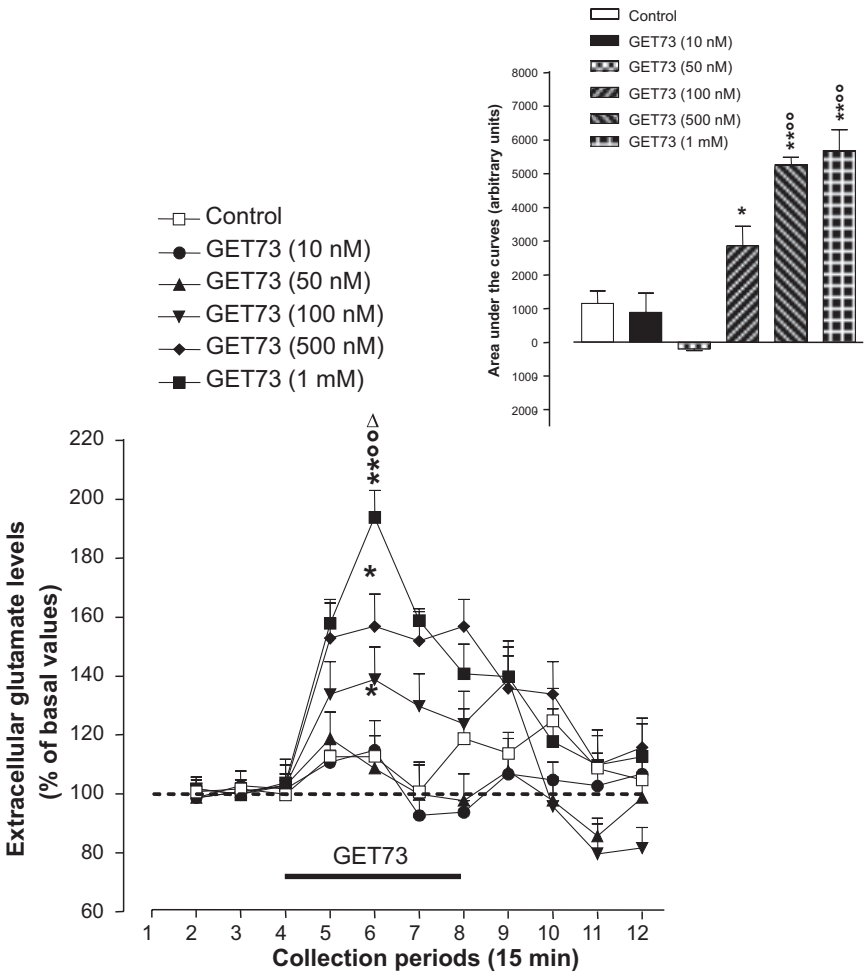

Fig. 1. Effect of intra-CA1 perfusion with GET73 on local dialysate glutamate levels in the alert rat. The solid bar indicates the period of perfusion with GET73 (60 min). Each point represents the mean of percentage changes ± SEM of 5–6 animals. The significance of the peak effect (maximal response) is shown in the figure. The histograms of the areas under the curves, which represent the integrated time-response curve of the effects, are shown in the upper panel. * $p < 0.05$; ** $p < 0.01$ significantly different from control as well as GET73 (10 and 50 nM); ^{oo} $p < 0.01$ significantly different from GET73 (100 nM); $^{\Delta}$ $p < 0.05$ significantly different from GET73 (500 nM), based on ANOVA followed by the Newman-Keuls test for multiple comparisons

nificance with regard to the peak effects (maximal responses) are shown in the figures.

The statistical analysis was carried out by analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons or Dunnett's *post-hoc* test.

Results

In vivo experiments: microdialysis

Basal dialysate hippocampal glutamate levels

Basal glutamate levels measured in 15 min fractions from the alert rat hippocampus CA1 region were 0.287 ± 0.023 μM (n = 48) and remained essentially stable over the duration of the experiment (180 min).

Effects of local perfusion with GET73 on dialysate glutamate levels from the hippocampus CA1 region of alert rats

GET73, in a wide range of concentrations (10 nM – 1 mM), was locally perfused by reverse dialysis into the CA1 region of the hippocampus. As shown in Figure 1, intra-CA1 perfusion with GET73 (60 min) significantly increased the local extracellular glutamate levels in a concentration-dependent manner. The maximal effect occurred 30 min after the onset of GET73 perfusion. This profile of action was confirmed in control experiments in which a Krebs solution was used as perfusion medium (data not shown).

Because the profile of action of GET73 was markedly different from that displayed by GHB, it was pharmacologically characterized in a simple and less integrated preparation than microdialysis, such as tissue slices.

In vitro experiments: hippocampus slices

Effect of GET73 on L-[^{3}H]glutamate uptake in rat hippocampus slices

In view of the above results and in order to further evaluate the biochemical mechanisms underlying the modulation of extracellular glutamate levels by GET73, the effect of the compound on L-[^{3}H]glutamate uptake in hippocampal slices was analyzed and compared with the effect of the specific glutamate uptake inhibitor PDC. As expected, the inclusion of 0.1 mM PDC in the medium markedly reduced L-[^{3}H]glutamate uptake. In contrast, 1 μM and 10 μM GET73 failed to affect the L-[^{3}H]glutamate uptake (Fig. 2).

Effects of GET73 on spontaneous glutamate efflux from rat hippocampus slices

Spontaneous hippocampal glutamate efflux, calculated from the mean of the first three samples collected from control slices, was 1.11 ± 0.22 nmol/min/g of fresh tissue (n = 38) and declined slightly over the duration of the experiment (Fig. 3).

The addition of 100 nM – 10 μM GET73 to the perfusion medium did not significantly affect the spontaneous glutamate efflux from rat hippocampus slices (Fig. 3).

Effect of NMDA, alone and in combination with GET73, on spontaneous glutamate efflux from rat hippocampus slices

In order to evaluate whether GET73 functionally interacts with NMDA receptors, the effect of the compound on NMDA-induced glutamate efflux was evaluated. As expected [39], the addition of various concentrations of NMDA (25, 100 and 300 μM) to the perfusion medium induced a concentration-dependent increase in spontaneous glutamate efflux (101 ± 4, 122 ± 8 and 148 ± 11% of basal values, respectively). A second stimulation with NDMA 25 min after the first (St_1) reproduced this effect, with the St_2/St_1 ratio close to unity (NMDA 25 μM = 1.02 ± 0.06; NMDA 100 μM = 1.05 ± 0.06; NMDA 300 μM = 1.03 ± 0.07). When 500 nM or 1 μM GET73, doses that do not affect spontaneous glutamate efflux, was added to the perfusion medium 5 min before St_2, the glutamate efflux from rat hippocampus slices caused by 100 μM NMDA was not affected (St_2/St_1 ratio = 1.07 ± 0.05 and 1.05 ± 0.04%, respectively).

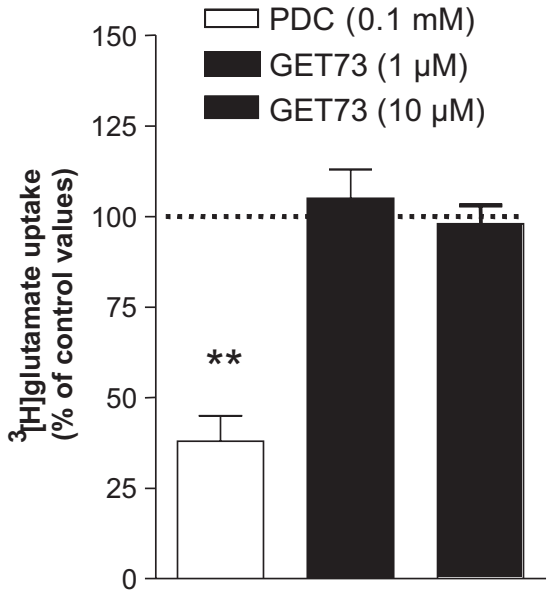

Fig. 2. Effect of the specific glutamate uptake inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) and GET73 on L-[^{3}H]glutamate uptake in rat hippocampal slices. Slices were preincubated at 37°C with PDC or GET73 in 2.5 ml of Krebs' solution for 5 min. The uptake process was initiated by the addition of L-[^{3}H]glutamate and was interrupted after 20 min by dilution with 10 ml of cold (0°C) Krebs' solution. The radioactivity of the slices (0.1 M NaOH extracts) was determined by liquid scintillation spectrometry. Blanks were prepared by incubating the preparation used with L-[^{3}H]glutamate at 0°C. Each column represents the mean of percentage changes ± SEM of 4–5 experiments run in triplicate. ** $p < 0.01$ significantly different from control, 1 μM and 10 μM GET73, based on an ANOVA followed by the Newman-Keuls test for multiple comparisons

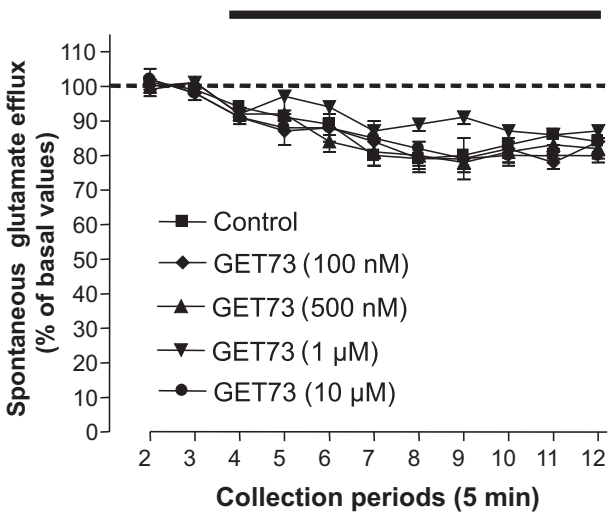

Fig. 3. Effect of GET73 on basal glutamate efflux from rat hippocampal slices. The solid bar represents the period of perfusion with GET73 (40 min). Each point represents the mean ± SEM of 5–6 animals

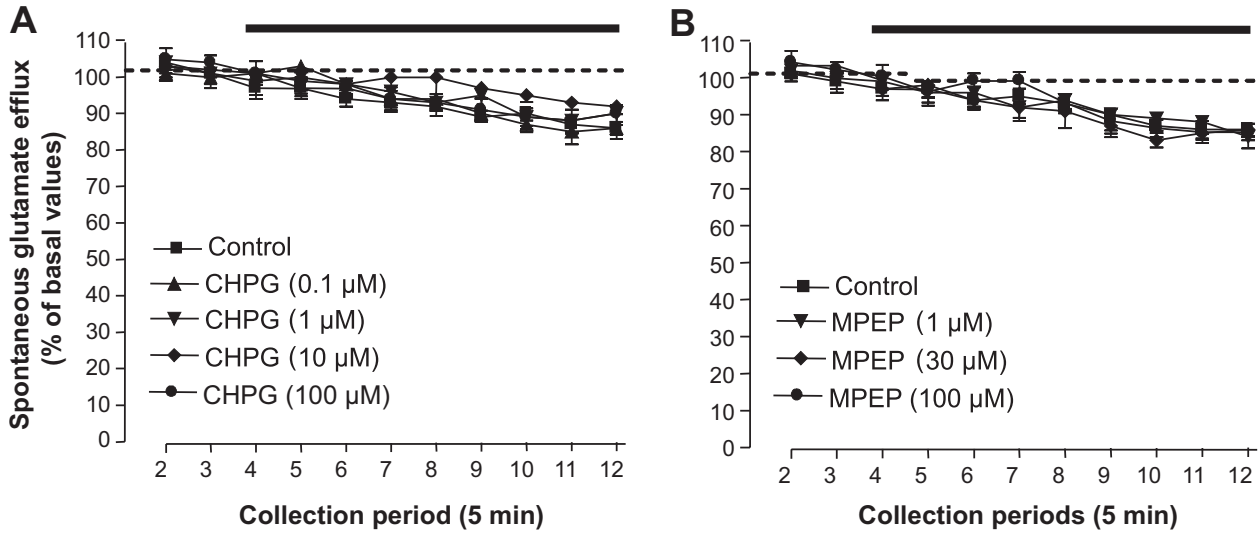

Fig. 4. Effect of CHPG (Panel **A**) or MPEP (Panel **B**) on spontaneous glutamate efflux from rat hippocampal slices. The solid bars represent the period of perfusion with the mGluR5 receptor agonist or antagonist. Each point represents the mean of percentage changes ± SEM of 5–8 animals

Effects of CHPG or MPEP, alone and in combination with GET73, on spontaneous glutamate efflux from rat hippocampus slices

The addition of 0.1–100 μM of the mGluR5 receptor agonist CHPG or 1–100 μM of the antagonist MPEP to the perfusion medium did not significantly affect the spontaneous hippocampal glutamate efflux (Fig. 4). When 500 nM or 1 μM GET73, concentrations that do not affect spontaneous glutamate efflux, was added to the perfusion medium 10 min before 10 or 100 μM CHPG or 30 or 100 μM MPEP, no significant change in the spontaneous glutamate efflux was observed (data not shown).

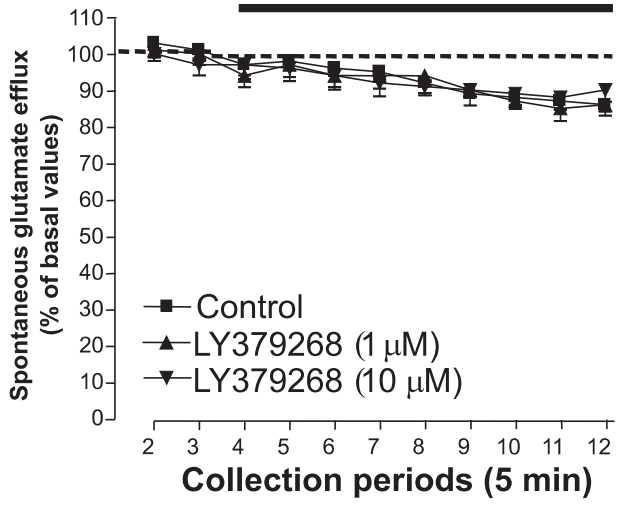

Fig. 5. Effect of LY379268 on spontaneous glutamate efflux from rat hippocampal slices. The solid bar represents the period of perfusion with the mGluR2/3 agonist. Each point represents the mean of percentage changes ± SEM of 5–7 animals

Effects of LY379268, alone and in combination with GET73, on spontaneous glutamate efflux from rat hippocampus slices

The addition of 1 and 10 μM of the mGluR2/3 receptor agonist LY379268 to the perfusion medium did not significantly affect spontaneous hippocampal glutamate efflux (Fig. 5). When 500 nM and 1 μM GET73, doses that do not affect the spontaneous glutamate efflux, was added to the perfusion medium 5 min before the mGluR2/3 receptor agonist, no significant change in the spontaneous glutamate efflux was observed (data not shown).

Effects of GET73 on K^+-evoked glutamate efflux from rat hippocampus slices

In control slices, the first 2 min period of stimulation with 20 mM KCl (St_1) induced a significant increase in glutamate efflux (140 ± 7% of basal values), which was quite similar to that observed during a second period of stimulation (St_2), with the St_2/St_1 ratio being close to unity (1.04 ± 0.05).

When 1 μM GET73 was added to the perfusion medium 10 min before St_2, a significant increase in K^+-evoked glutamate efflux was observed. At lower concentrations (100 and 500 nM), GET73 did not significantly affect K^+-evoked glutamate efflux (Fig. 6).

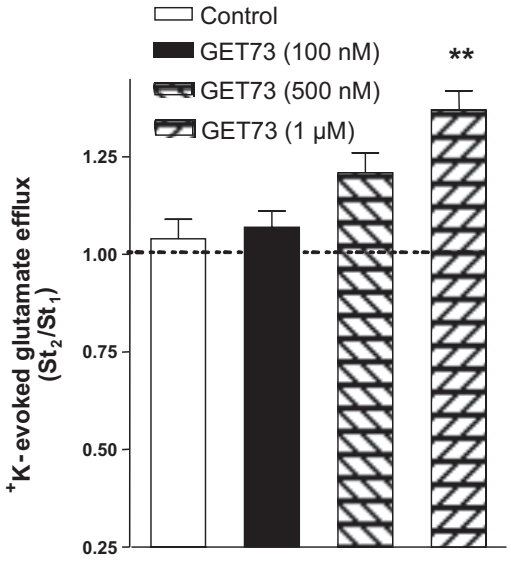

Fig. 6. Effect of GET73 on K^+-evoked glutamate efflux from rat hippocampal slices. The compound was added to the perfusion medium 10 min before St_2 and maintained until the end of the experiment. Each column represents the mean of percentage changes ± SEM of 5–7 experiments. ** $p < 0.01$ significantly different from control group based on ANOVA followed by Dunnett's *post-hoc* test

Effects of CHPG, alone and in combination with GET73, on K^+-evoked glutamate efflux from rat hippocampus slices

The addition of 100 μM of the selective mGluR5 receptor agonist CHPG to the perfusion medium 10 min before St_2 induced a significant increase in K^+-evoked glutamate efflux, though the lower concentrations tested (0.1–10 μM) were ineffective (Fig. 7A). The increase in K^+-evoked glutamate efflux induced by 100 μM CHPG was partially, but significantly, counteracted by 500 nM GET73 (Fig. 7B). In contrast, 1 μM GET73 induced a nonsignificant amplification of the increase in K^+-evoked glutamate efflux provoked by 100 μM CHPG (Fig. 7C).

Effects of MPEP, alone and in combination with GET73, on K^+-evoked glutamate efflux from rat hippocampus slices

When 100 μM of the selective mGluR5 receptor antagonist MPEP was added to the superfusion medium 10 min before St_2, it induced a significant decrease in K^+-evoked glutamate efflux (Fig. 8A). In contrast, at lower concentrations (1–10 μM) MPEP did not affect K^+-evoked glutamate efflux. Interestingly, the increase of K^+-evoked glutamate efflux induced by 1 μM GET73 was counteracted by coperfusion with 1 μM (102 ± 6% of basal values) or 10 μM (Fig. 8B) MPEP.

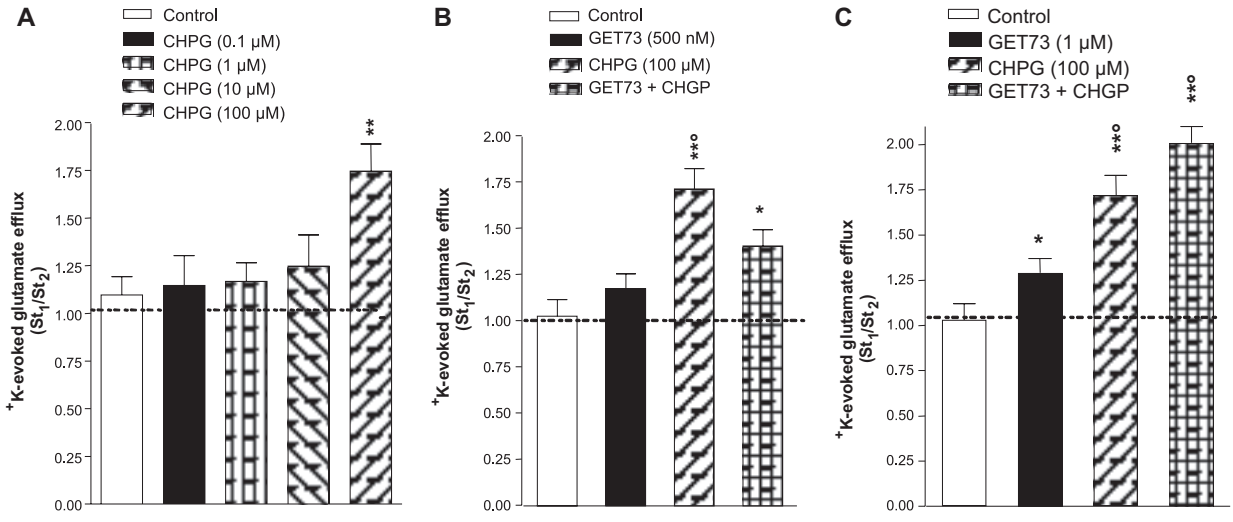

Fig. 7. Effect of CHPG, alone (Panel **A**) or in combination with 500 nM (Panel **B**) or 1 μM (Panel **C**) GET73 on K^+-evoked glutamate efflux from rat hippocampal slices. The selective mGluR5 receptor agonist was added to the perfusion medium 10 min before St_2 and maintained until the end of the experiment, while GET73 was added 5 min before CHPG. Each point represents the mean of percentage changes ± SEM of 4–7 animals. Panel **A**: ** $p < 0.01$ significantly different from the other groups; Panel **B**: * $p < 0.05$; ** $p < 0.01$ significantly different from control as well as from GET73 alone; ^{o} $p < 0.05$ significantly different from GET73 + CHPG; Panel **C**: * $p < 0.05$; ** $p < 0.01$ significantly different from control; ^{o} $p < 0.05$ significantly different from GET73 alone, based on ANOVA followed by the Newman-Keuls test for multiple comparisons

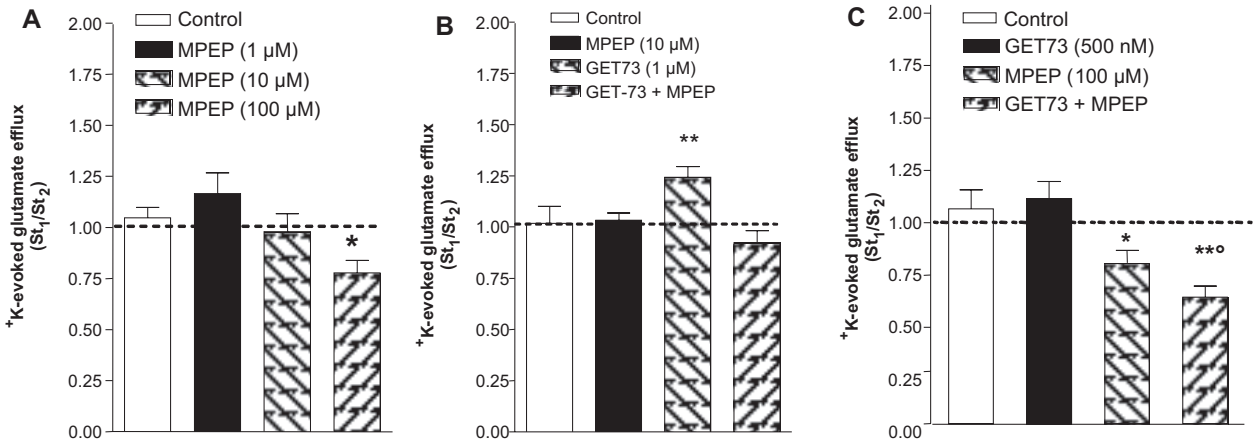

Fig. 8. Effect of MPEP, alone (Panel **A**) or in combination with 1 μM (Panel **B**) or 500 nM (Panel **C**) GET73 on K^+-evoked glutamate efflux from rat hippocampal slices. Each point represents the mean of percentage changes ± SEM of 6–9 animals. Panel **A**: MPEP was added to the perfusion medium 10 min before St_2 and maintained until the end of the experiment. * $p < 0.05$ significantly different from the other groups; Panel **B**: GET73 was added to the perfusion medium 10 min before St_2 and maintained until the end of the experiment, while MPEP was added to the perfusion medium 5 min before GET73. ** $p < 0.01$ significantly different from the other groups; Panel **C**: MPEP was added to the perfusion medium 10 min before St_2 and maintained until the end of the experiment, while GET73 was added to the perfusion medium 5 min before MPEP. * $p < 0.05$; ** $p < 0.01$ significantly different from control as well as from GET73 alone; ^{o} $p < 0.05$ significantly different from MPEP, based on ANOVA followed by the Newman-Keuls test for multiple comparisons

As shown in Figure 8C, when 500 nM GET73 was added to the perfusion medium 5 min before 100 μM MPEP, it significantly amplified the decrease in K^+-evoked glutamate efflux induced by the selective mGluR5 receptor antagonist. In contrast, coperfusion with 100 μM MPEP or 1 μM GET73 did not affect K^+-evoked glutamate efflux (102 ± 6% of basal values).

Effects of LY379268, alone and in combination with GET73, on K^+-evoked glutamate efflux

The addition of 10 μM of LY379268 to the perfusion medium 10 min before St_2 induced a significant decrease in K^+-evoked glutamate efflux. The lower concentration tested (1 μM) was ineffective (Fig. 9). The LY379268-induced decrease of K^+-evoked glutamate efflux was not affected by 500 nM GET73 (Fig. 9).

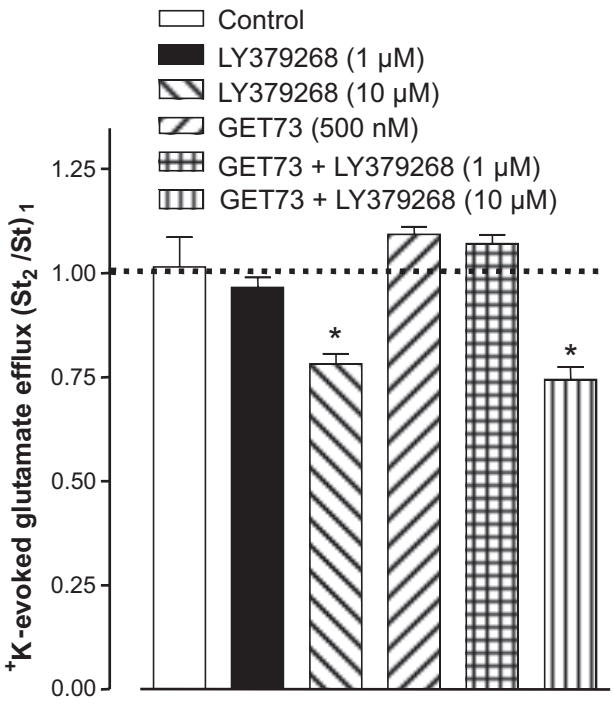

Fig. 9. Effect of LY379268, alone or in combination with 500 nM GET73 on K^+-evoked glutamate efflux from rat hippocampal slices. The selective mGluR2/3 receptor agonist was added to the perfusion medium 10 min before St_2 and maintained until the end of the experiment, while GET73 was added 5 min before LY379268. Each point represents the mean of percentage changes ± SEM of 4–7 animals. * $p < 0.05$ significantly different from the other groups, based on ANOVA followed by the Newman-Keuls test for multiple comparisons

Discussion

The present study combined *in vivo* (microdialysis) and *in vitro* (tissue slices) experiments and clearly demonstrated that the GHB-analog GET73 significantly affects glutamate transmission in the rat hippocampus. The *in vivo* microdialysis results indicate that

the profile of action of GET73 on hippocampal glutamate levels is different from that displayed by the parent compound GHB. In fact, intra-CA1 perfusion with GET73 induced a concentration-dependent increase in extracellular glutamate levels, while GHB, under the same experimental conditions, displayed a biphasic action on extracellular CA1 glutamate levels [22]. Specifically, GHB enhanced extracellular glutamate levels at nanomolar concentrations, whereas at higher millimolar concentrations, it caused a reduction in the levels of the excitatory amino acid; the intermediate micromolar concentrations were ineffective [22]. The GHB-induced increase in extracellular glutamate levels has been ascribed to the activation of local GHB receptors because it was suppressed by the GHB receptor antagonist NCS-328. In contrast, the reduction of glutamate levels observed following the perfusion of millimolar concentrations of GHB was completely abolished by treatment with the $GABA_B$ receptor antagonist CGP35348, thus suggesting the involvement of $GABA_B$ receptors in this effect [15, 22]. Based on these findings, it seems reasonable to suggest that GET73, at least at the concentrations tested, does not significantly interact with $GABA_B$ receptors given that no inhibition of glutamate levels was observed after its intra-CA1 perfusion. Accordingly, *in vitro* binding experiments indicate that GET73 has no affinity for $GABA_B$ receptors or for GHB receptors [Loche et al., unpublished data]. Thus, these results suggest that GET73 affects hippocampal glutamate transmission *via* a mechanism that differs from those that mediate the GHB-induced effects. In view of this finding and in order to provide useful information for the understanding of the neurochemical profile of action of the new compound, in the second part of the present research, the effect of GET73 on glutamate transmission was pharmacologically characterized in rat hippocampal slices. This methodological approach represents a less integrated preparation than experiments using the whole brain, thus allowing us to easily characterize the neurochemical properties of the compound. Using this preparation, it was demonstrated that GET73 did not affect L-[^{3}H]glutamate uptake, thus excluding the possibility that the increase in dialysate extracellular glutamate levels induced by the compound could be due to an effect on glutamate reuptake mechanism(s). On the basis of this result, the effects of GET73 on spontaneous and K^+-evoked glutamate efflux from hippocampal slices were evaluated. The evidence that, under the present *in vitro* experimental conditions, GET73 affected K^+-evoked, but not spontaneous, glutamate efflux, suggests that the compound acts preferentially by interfering with the neurosecretory coupling mechanisms rather than affecting glutamate leakage from nerve terminals. In line with this view, the observation that *in vivo*, but not *in vitro*, GET73 affects spontaneous glutamate levels could be explained by a higher firing rate of glutamate neurons in the whole brain compared to tissue slices, possibly due to a loss of excitatory inputs in the latter preparation.

Previously obtained behavioral data led to the hypothesis that some GET73-induced effects could be mediated by an interaction with glutamate receptors [Loche et al., unpublished data]. It is well known that glutamate activates the following two categories of receptors: ligand-gated ion channels (ionotropic glutamate receptors, or iGluRs), which mediate fast excitatory neurotransmission, and G-protein coupled receptors (metabotropic glutamate receptors, or mGluRs), which mediate slower modulatory neurotransmission [13, 16]. Numerous animal studies have shown that NMDA receptors are one of the primary targets of ethanol, and iGluRs antagonists counteract the reward induced by the abuse of the drug, although their serious side effects in humans have prevented their development into commercially available treatments. Moreover, an alternative target in the pharmacotherapeutic approach to treating alcohol addiction is the metabotropic glutamate receptors (mGluRs) of Group I and II [41]. The hypothetical influence of GET73 on both types of receptors was therefore been tested in the present study. The results obtained demonstrated that GET73 did not modify the NMDA-induced increase of spontaneous glutamate efflux in hippocampal slices. Thus, it seems unlikely that GET73 interferes with NMDA receptors in modulating glutamatergic transmission in the hippocampus. This finding further confirms that the profile of action of GET73 is different from that of GHB. In fact, it has been reported that GHB plays a key role in the regulation of NMDA receptor function. In particular, behavioral studies clearly demonstrated that NMDA antagonists (MK-801, ketamine and phencyclidine) enhance the cataleptic effects of GHB, thus suggesting the existence of a synergic functional interaction between GHB and NMDA receptor antagonists [49, 50]. Koek et al. [32] demonstrated that ketamine and phencyclidine specifically potentiate the discriminatory stimulus effects of low doses of GHB. In addition, the same

study further confirmed the relevance of the interactions between GHB and NMDA antagonists in enhancing the cataleptic effects of high doses of GHB. In view of these results, the authors suggest that NMDA antagonists might potentiate the subjective effects of GHB in humans.

Due to the above negative results concerning the modulation of NMDA function by GET73 and the evidence indicating that metabotropic glutamate receptor 5 (mGluR5) is implicated in ethanol- and drug-seeking behaviors in rodent, a further set of experiments were performed to evaluate the possible effects of GET73 on mGluR5 function. Treatment with 100 μM of the mGluR5 agonist CHPG induced a significant increase in K^+-evoked glutamate efflux. This finding is in line with previous studies demonstrating that CHPG increased K^+-evoked or electrically-evoked efflux of pre-accumulated D-[^{3}H]aspartate from rat cerebrocortical minislices or hippocampal slices [19, 47]. Thus, it seems likely that mGluR5 activation exerts an excitatory control on hippocampal glutamate efflux; this hypothesis is strengthened by the observation that the mGluR5 antagonist MPEP significantly reduced K^+-evoked glutamate efflux from hippocampal slices. The results obtained in the present study also demonstrate that GET73 is able to functionally interact with mGluR5 in a very complex way. In fact, the CHPG-induced increase of K^+-evoked glutamate efflux was partially counteracted by the presence of a threshold concentration of GET73 (500 nM) in the perfusion medium but not at the higher (1 μM) concentration. In addition, the low concentration of 500 nM GET73 significantly amplified the reduction of glutamate efflux induced by perfusion with 100 μM MPEP. Conversely, pretreatment with a concentration of MPEP that by itself is ineffective significantly counteracted the increase of K^+-evoked glutamate efflux from rat hippocampal slices induced by 1 μM GET73. At present, it remains difficult to explain this complex profile of action of GET73 on mGluR5-mediated modulation of hippocampal glutamate efflux, but some hypotheses can be suggested. It is well known that a major breakthrough in the area of mGluR5 biology came with the discovery of selective positive or negative allosteric modulators of this receptor, called PAMs and NAMs, respectively [46]. For instance, MPEP is classified as a NAM [24, 25]. PAMs and NAMs do not interact with the orthosteric glutamate binding site but instead bind to allosteric sites in the seven transmembrane-spanning domains of mGluR5 to favor or inhibit coupling of the receptor to GTP binding proteins [28, 30]. Recently, the existence of different allosteric sites in mGluR5 was proposed [28]. On the basis of these properties, it could be hypothesized that, at least under the present experimental conditions, GET73 at a nanomolar concentration, which alone has no effect on glutamate efflux, can preferentially bind to a negative allosteric site on mGluR5 and consequently partially counteract the CHPG-induced increase of K^+-evoked glutamate efflux from rat hippocampal slices. The observation that, at this concentration, GET73 amplifies the inhibitory effect of MPEP suggests that these compounds probably bind to different sites on mGluR5, thus exerting a synergic action. On the other hand, at higher concentrations, GET73 (1 μM), like CHPG, increases K^+-evoked glutamate efflux from rat hippocampal slices, and this effect is counteracted by MPEP. One possible explanation could be that, at these concentrations, GET73 binds to a positive allosteric site on mGluR5, and this action supersedes the negative modulatory activity. When 1 μM GET73 was coperfused with 100 μM MPEP, any significant effect on K^+-evoked glutamate efflux was observed, probably because of the opposing actions of the two compounds on mGluR5 at these concentrations. Obviously, this hypothesis remains to be confirmed, and other possibilities, such as the involvement of intracellular signaling and the interaction with other neurotransmitters, cannot be ruled out. The evidence that GET73 did not affect the reduction of the K^+-evoked glutamate efflux induced by the mGluR2/3 receptor agonist LY379268 suggests that the action of GET73 seems to be specific to the mGluR5 subtype.

As previously mentioned, unpublished data [42; Colombo et al.] demonstrate that GET73 produces an inhibition of alcohol intake in Sardinian alcohol-preferring rats, thus leading to its possible use in the treatment of alcoholism. If confirmed, the neurochemical properties of GET73 could assume a particular relevance in view of the role that mGluR5 plays in several aspects of alcohol abuse [4, 7, 10]. In fact, it has been reported that mGluR5 antagonism may provide a promising avenue for the amelioration of alcoholic behavior [5, 48] and thus the ability of a nanomolar concentration GET73 to preferentially bind to a negative allosteric site on mGluR5 could, at least partially, explain some behavioral effects of the compound. In addition, the complex neurochemical profile of action of GET73 in regulating glutamatergic transmission might represent one of the possible

mechanisms underlying its ability to reduce the consumption and reinforcing effect of sucrose as well as other highly palatable foods. In this context, it has recently been reported that mGluR5 antagonists reduce the consumption of highly palatable food in rat and baboon models of binge eating [6, 45]. These hypotheses, however, remain to be verified.

In conclusion, the present study demonstrates that the GHB analog GET73 significantly affects hippocampal glutamate transmission and that its profile of action differs from that of the parent compound. In particular, the present findings lead us to hypothesize a possible interaction between GET73 and mGluR5-mediated regulation of glutamate transmission, an effect which may be relevant to the ability of GET73 to reduce alcohol intake in an alcohol-preferring rat strain.

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