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Short communication

The anxiolytic effect of γ-hydroxybutyrate in the elevated plus maze is reversed by the benzodiazepine receptor antagonist, flumazenil

Catherine Schmidt-Mutter [a,b,*], Laure Pain [b,c], Guy Sandner [c], Serge Gobaille [a], Michel Maitre [a]

[a] *Centre de Neurochimie, UPR 416 CNRS, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France*
[b] *Département d'Anesthésie-Réanimation, Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France*
[c] *Laboratoire de Psychopathologie et Pharmacologie des comportements, U 405 INSERM, 11 rue Humann, 67000 Strasbourg, France*

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Abstract

The effects of γ-hydroxybutyrate (GHB), a product of γ-aminobutyric acid (GABA) metabolism which possesses neuromodulatory properties in brain, were investigated in the elevated plus maze in rats. The number of entries and the time spent in the open arms of the maze were increased by GHB (50, 150, 250 mg/kg i.p.). This is classically considered as indicative of an anxiolytic effect of the drug. There was no sedative effect at these doses as measured by the spontaneous locomotor activity in the actimeter or the total number of arm entries. The anxiolytic properties of GHB were reversed by neither the GHB receptor antagonist, NCS-382 (6,7,8,9-tetrahydro-5(H)-5-olylidene acetic acid) (300 mg/kg i.p.), nor the opioid receptor antagonist, naloxone (10 mg/kg i.p.). However the anti-anxiety effect of GHB was antagonized by the benzodiazepine receptor antagonist, flumazenil (10 mg/kg i.p.), suggesting an interaction of GHB with the $GABA_A$ receptor complex which mediates the anti-anxiety effect of benzodiazepines. © 1998 Elsevier Science B.V.

Keywords: GHB (γ-hydroxybutyrate); Flumazenil; Anxiety; Elevated plus maze; Locomotor activity

1. Introduction

Gamma-hydroxybutyrate (GHB) is an endogenous compound of the mammalian brain synthesized primarily from γ-aminobutyric acid (GABA). A large body of evidence favors a role for GHB in central neuromodulation (for review see Maitre, 1997). When systemically administered, GHB exerts hypnotic effects in animals and man and is used as an intravenous anaesthetic agent (Laborit, 1964). Recently, GHB has been tested with success in the treatment of alcohol dependence and opiate withdrawal (Gallimberti et al., 1993), states which have been proved to include a strong anxiogenic component. There are several arguments suggesting an anxiolytic effect of GHB, the most salient being its relationship to the metabolism and the receptors of the GABAergic system. The prototypic anxiolytic drugs, benzodiazepines, are considered to produce their anxiolytic effects by facilitating the action of GABA at the $GABA_A$-benzodiazepine receptor complex and, in addition, $GABA_A$ receptor agonists like muscimol and $GABA_A$ receptor antagonists like picrotoxin and bicuculline have been shown to possess anxiolytic and anxiogenic properties, respectively, in the elevated plus maze (Shekhar, 1993). Moreover, the GABA-transaminase inhibitor, vigabatrin, which potentiates GABA neurotransmission, was also shown to induce anxiolytic-like effects in this test (Sayin et al., 1992; Sherif et al., 1994). In order to assess the hypothesis that GHB may have an effect on anxiety, low doses of GHB were tested in a fear/anxiety animal model that is widely used: the elevated plus maze based on the natural aversion of rodents for high and open spaces. The tendency of rats to enter or remain in the open elevated arms of the maze is reduced by anxiogenic drugs and increased by anxiolytic drugs (Pellow et al., 1985). However there is an obvious incompatibility between reduced locomotor activity due to sedation and the use of a maze where the animal has to move from one arm to another. Only doses of GHB below the sedation threshold may be used. Thus, a preliminary experiment was performed to determine this sedation threshold in the rat strain used for the present study. Spontaneous locomotor activity

* Corresponding author. Tel.: +33-3-8845-6638; fax: +33-3-8845-6605; e-mail: mutter@neurochem.u-strasbg.fr

of rats was examined in an activity meter system after injection of GHB. Further experiments were designed to assess the effects of GHB in the elevated plus maze and to document the pharmacological mechanism mediating the anxiolytic properties of GHB, using antagonists of three distinct receptors systems. NCS-382 (6,7,8,9-tetrahydro-5(H)-5-ol-ylidene acetic acid) is a γ-hydroxybutyrate receptor antagonist (Maitre et al., 1990) already shown to antagonize the sedative and cataleptic effects of GHB (Schmidt et al., 1991). The non-selective opioid receptor antagonist, naloxone, is also known to reverse many biochemical and pharmacological effects of GHB (Snead and Bearden, 1980) as well as the anti-anxiety effects of diazepam in the elevated plus maze (Agmo et al., 1995). Finally, the benzodiazepine receptor antagonist, flumazenil (Hunkeler et al., 1981), which blocks most of the benzodiazepine effects, was evaluated in association with GHB.

2. Materials and methods

2.1. Animals

Male Long–Evans rats (Janvier, France) weighing 250–300 g were housed two per cage in a colony room maintained on a 14/10 light/dark cycle (light on at 7:00 h) with free access to food and water. An adaptation period of at least 7 d was allowed prior to the start of the behavioral experiments. During this week, the rats were handled three times so that they could get used to the experimenter.

2.2. Drugs

All drugs were prepared immediately prior to use and injected intraperitoneally (i.p.) in a volume of 2 ml/kg. Diazepam (Roche, France), naloxone (Sigma, USA), GHB, sodium salt (Sigma, USA) and NCS-382 (6,7,8,9-tetrahydro-5(H)-5-ol-ylidene acetic acid, sodium salt) synthesized as described previously (Maitre et al., 1990) were dissolved in water. Flumazenil (Hoffmann–La Roche, Basel) was dissolved in water with two drops of Tween 80. Sodium chloride 0.9% was used as a control except in the flumazenil antagonism procedure where control animals were injected with sodium chloride 0.9% to which two drops of Tween 80 had been added. The interval between injection and behavioral testing was 30 min for GHB, diazepam, NCS-382 and flumazenil, and 15 min for naloxone.

2.3. Behavioral apparatus

Spontaneous locomotor activity was measured in an activity meter system consisting of eight individual cages (45 cm length × 30 cm width × 30 cm height). The floor was covered with sawdust and a passive infrared detector (Talco, IRP124) behind a Fresnel lens was placed on the roof of each cage. This Fresnel lens sectorized the cage that the cell monitored. Movements from one part of the cage to another were detected and fed into a PC computer that was programmed to sum and store the displacements of the rats every 5 min for a period of 1 h.

The elevated plus maze was constructed of wood similarly to that described by Pellow et al. (1985). It consisted of a maze with two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) with an open roof. The arms were placed so that the two open and the two closed arms were opposite each other with a central platform of 10 × 10 cm. The entire maze was elevated to a height of 50 cm above the ground and a spotlight (40 W) provided the only room illumination and was placed 150 cm above the central area of the maze. A video camera also fixed above the maze recorded the test sessions.

2.4. Procedure

All behavioral experiments were conducted between 14:00 and 18:00 h, and each rat was used only once.

2.4.1. Preliminary experiment: Locomotor activity of GHB

Five groups of 8 rats were randomly allocated to treatment conditions (NaCl 0.9%, or GHB: 75, 150, 300 or 600 mg/kg i.p.). Each rat was injected, then placed individually in one of the activity meter compartments and its activity was recorded every 5 min for 1 h.

2.4.2. Experiment 1: Anxiolytic effects of GHB as compared to diazepam

GHB (50, 150 or 250 mg/kg), diazepam (2 mg/kg) or NaCl 0.9% was administered i.p. 30 min before testing ($n = 8$ per group). After injection, each rat was returned to its home cage and 25 min after injection it was isolated in a similar cage in the experimental room for 5 min. At the beginning of the 5 min session test, the rat was placed on the central platform of the maze with its head facing an open arm. Behavior was recorded on the videotape. The number of open- and closed-arm entries and the cumulative time spent in open arms, closed arms and central area was analyzed later using a PC computer with specific home-made software. A rat was considered to be on the central platform whenever two paws were placed there and in an arm when all four paws were on it. The ratios of open/total arm entries and open/total time were calculated for each animal and used as indices of anxiety. The total number of arm entries was used as an index of locomotor activity in the plus maze.

2.4.3. Experiments 2, 3 and 4: Effects of NCS-382, naloxone and flumazenil on the anxiolytic effects of GHB

NCS-382 (300 mg/kg), naloxone (10 mg/kg) or flumazenil (10 mg/kg) were evaluated on the effect of GHB (150 mg/kg) in three distinct experimental procedures (referred to experiment 2, 3 and 4 respectively). In

each of these experiments, 32 rats were randomly allocated to treatment conditions ($n = 8$ in each one) and tested following the same procedure as in experiment 1. Each rat received two injections.

In experiment 2, the two injections were administered 30 min before testing and 4 treatment conditions were assessed: saline + saline, GHB + saline, saline + NCS-382 or GHB + NCS-382.

In experiment 3, a first injection of either saline or GHB was made 30 min before testing and a second one of either naloxone or saline was given 15 min before the test, so that the following 4 conditions were used: saline + saline, saline + naloxone, GHB + saline or GHB + naloxone.

Finally in experiment 4, the same procedure as in experiment 2 was applied except that saline was replaced by Tween aqueous solution, vehicle for flumazenil and GHB, giving the 4 treatment conditions: vehicle + vehicle, vehicle + flumazenil, GHB + vehicle or GHB + flumazenil.

2.5. Statistics

The results were expressed as the means ± S.E.M. One-way analysis of variance was applied to the spontaneous activity score, to the percentage of open-arm entries and the time spent in the open arms as well as to the total number of arm entries. When a drug increased or decreased both total arm entries and the percentage of open-arm entries, analysis of covariance was performed to determine to what extent the entry in an open arm was independent of any effect on closed-arm entries. Post-hoc comparisons between individual treatment and control groups were done with the Newmann–Keuls test.

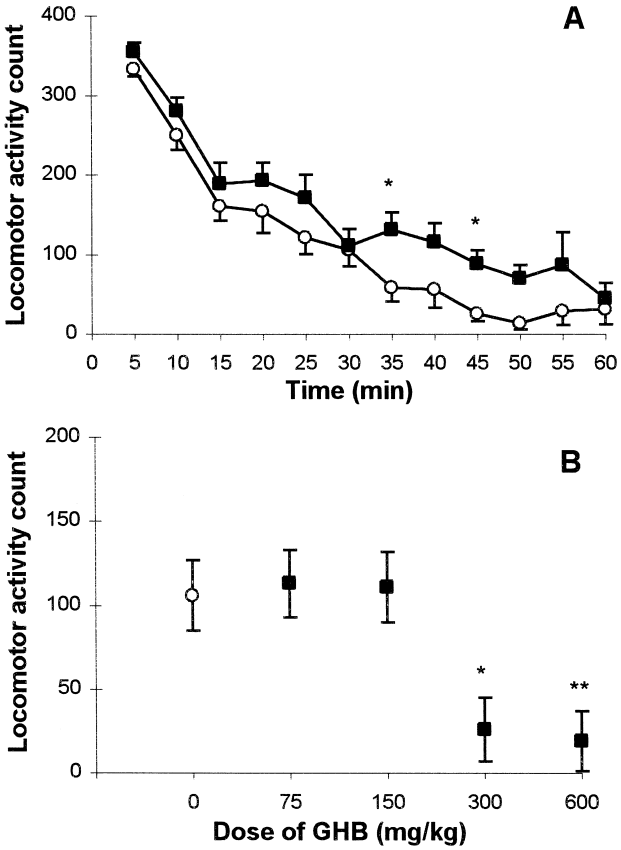

Fig. 1. Rats' spontaneous locomotor activity score (mean ± S.E.M., $n = 8$ in each group) evaluated in the activity meter system as described in Section 2. (A) measurements for each 5 min period for one hour after i.p. injection of NaCl 0.9% (open circle) or GHB (150 mg/kg, black square). (B) measurements at 30 min after i.p. injection of NaCl 0.9% (open circle) or GHB (black square) at the doses indicated. * $P < 0.05$, ** $P < 0.01$ versus control (ANOVA followed by the Newmann–Keuls test).

3. Results

Spontaneous locomotor activity of the rats placed in a new environment is usually high; in the present experiment, it lasted more than 30 min and then decreased to a plateau. Fig. 1A shows this decreasing spontaneous locomotor activity of the rats after the dose of 150 mg/kg GHB because it is the main dose used in the subsequent pharmacological experiments. Fig. 1B presents the effect of four doses of GHB at the corresponding latency (30 min). For pharmacokinetic reasons, the delay of 30 min after GHB i.p. administration is used in most of the pharmacological experiments carried out with GHB. Locomotor activity of rats treated with either 300 or 600 mg/kg of GHB was rapidly decreased and remained less than that of the controls until the fortieth minute or the end of the session, respectively, indicating a sedative effect (not shown). Doses of 75 and 150 mg/kg of GHB did not reduce spontaneous activity as compared to the controls and slight hyperactivity was even observed 35 min after injection. These results indicate that, at least up to 150 mg/kg, there was no GHB-induced decrease in locomotor activity, thus no sedation was believed to occur. Both non-sedative doses and a delay of 30 min after injection, for which no difference in locomotor activity occurred, were chosen to determine the anxiolytic effect of GHB.

In experiment 1, GHB 50, 150 and 250 mg/kg increased the percentage of entries into and the percentage of time spent in the open arms as shown in Fig. 2A, but this proved to be statistically significant only for 150 and 250 mg/kg. At the dose of 250 mg/kg, GHB also significantly increased the total number of arm entries (Fig. 3A), indicating that no sedative effect occurred at this dose, but the analysis of covariance of the number of arm entries (open arm entries as the dependent variable and closed arm entries as the covariate) still did reveal a significant effect of GHB to increase the open arm entries. As a positive reference, diazepam (2 mg/kg i.p.) was tested in the same experiment and was shown to significantly increase the percentage of open-arm entries as well as the time spent in open arms under our conditions.

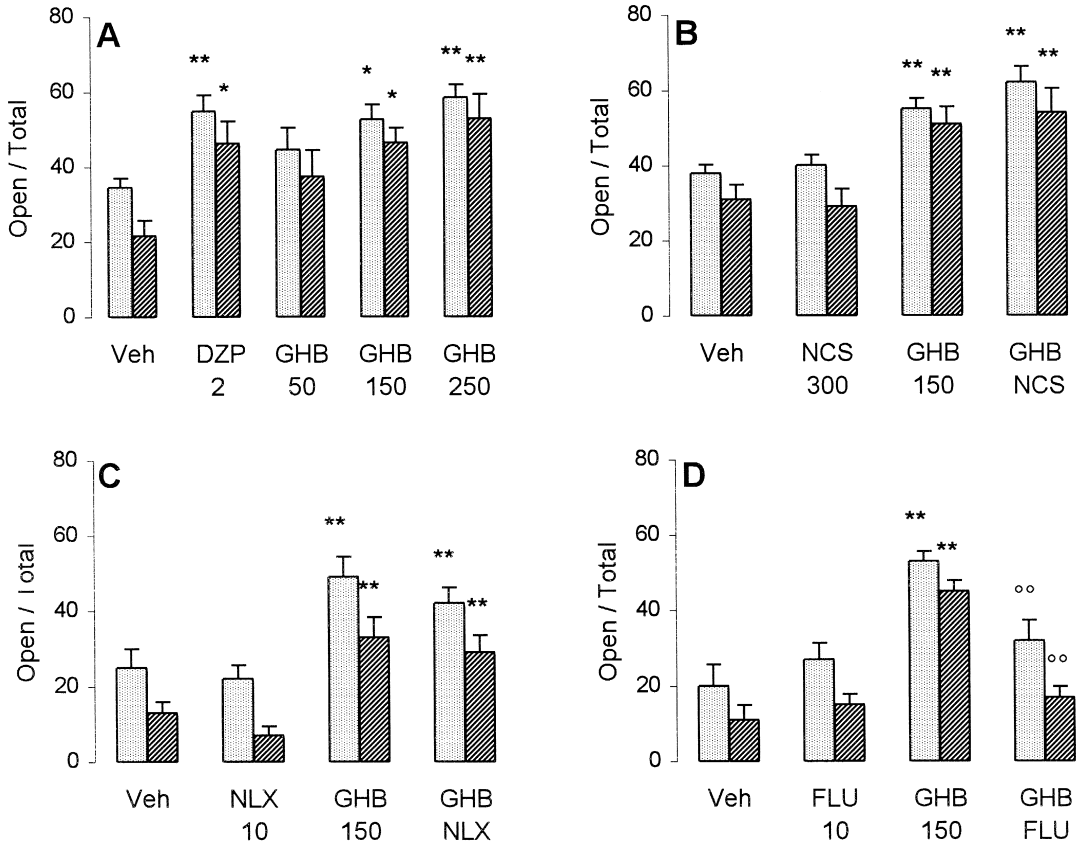

Fig. 2. Percentage of open-arm entries (grey bars) and of time spent (hatched bars) in open arms (mean ± S.E.M., $n = 8$ in each group) in rats given a 5 min test in the elevated plus maze after i.p. injection with: (A) diazepam (DZP, 2 mg/kg) or GHB (50, 150 or 250 mg/kg), (B) GHB (150 mg/kg) and/or NCS-382 (NCS, 300 mg/kg), (C) GHB (150 mg/kg) and/or naloxone (NLX, 10 mg/kg), (D) GHB (150 mg/kg) and/or flumazenil (FLU, 10 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated group; $^{oo}P < 0.01$ versus GHB alone (ANOVA followed by the Newmann–Keuls test).

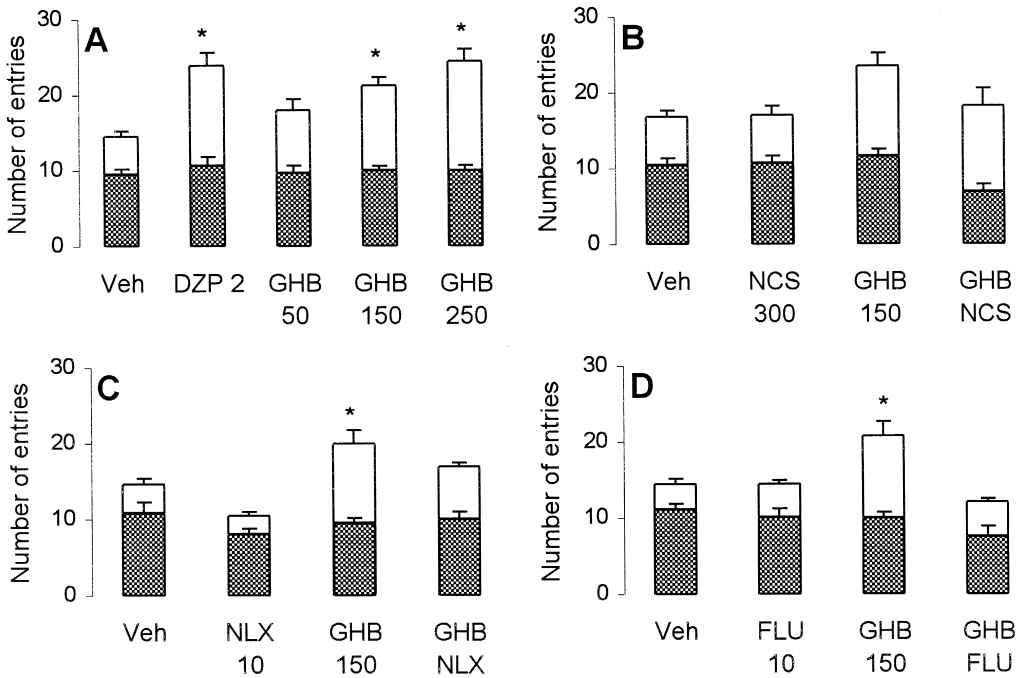

Fig. 3. Number of closed-arm entries (grey bars, mean ± S.E.M.), open-arm entries (white part of bars, mean ± S.E.M.) and total number of entries (entire column) in rats given a 5 min test in the elevated plus maze after i.p. injection as described in Fig. 2. ANOVA followed by the Newmann–Keuls test was applied to the total number of arm entries ($n = 8$ in each group), $^{*}P < 0.05$ versus control.

In experiment 2, the GHB receptor antagonist NCS-382 (300 mg/kg i.p.), had a significant effect on neither the percentage of entries and the percentage of time spent in open arms when injected alone (Fig. 2B), nor on the total number of arm entries (Fig. 3B). When NCS-382 was coadministered with GHB, it was unable to antagonize the anxiolytic effect of GHB at the dose used. The percentage of open-arm entries and of time spent in open arms still increased in a significant way with post-hoc comparisons showing no differences between GHB/vehicle and GHB/NCS-382 groups.

In experiment 3, the opioid receptor antagonist, naloxone (10 mg/kg i.p.), had no significant effect when injected alone as shown in Fig. 2C. When GHB (150 mg/kg) was combined with naloxone (10 mg/kg), the percentage of open-arm entries and of time spent in open arms still increased in a significant way, with post-hoc comparisons by the Newmann–Keuls test showing no differences between GHB/vehicle and GHB/naloxone groups.

In experiment 4 (Fig. 2D), the benzodiazepine receptor antagonist, flumazenil (10 mg/kg i.p.), that showed no significant effect by itself under our conditions, significantly reduced the percentage of open-arm entries ($P < 0.01$) and the percentage of time spent in open arms ($P < 0.01$); post-hoc comparisons showed no significant differences between GHB/flumazenil and vehicle/vehicle groups.

4. Discussion

Low doses of GHB exhibited anxiolytic properties in rats as tested in the elevated plus maze. There was no indication of a sedative effect at the doses used, either regarding measurements of spontaneous locomotor activity or the total number of entries in the open and closed arms of the plus maze. Although the effective doses of 150 and 250 mg/kg of GHB increased locomotor activity, subsequent analysis of covariance of the number of arm entries indicated that there still was a significant open- versus closed-arm effect. The anxiolytic effect of GHB was not reversed by an equimolecular dose of the GHB receptor antagonist, NCS-382, or by a high dose of the opioid receptor antagonist, naloxone. The anxiolytic properties of GHB were significantly antagonized by an intermediate dose of the benzodiazepine receptor antagonist, flumazenil.

Non-sedative doses of GHB were chosen in accordance with the results of a preliminary experiment as there are no reports regarding behavioral and locomotor activity after low doses of GHB in the rat. In mice, the spontaneous locomotor activity after GHB administration was evaluated by Zerbib et al. (1992). The locomotor activity of mice treated with 2 mmol/kg i.p. (250 mg/kg), as either acute or chronic injections, was significantly lowered as early as after 30 min, this phase being followed by increased activity that the authors considered to be renewed exploratory behavioral activity rather than hyperactivity. However, we found that, in the rat, slight hyperactivity as compared to the control was observed even in the absence of a preceding period of lower activity.

NCS-382 is a synthetic structural analogue of GHB exhibiting antagonistic properties at GHB binding sites (Maitre et al., 1990); it possesses high affinity for GHB receptors and blocks GHB-induced cell responses in vivo and in vitro (Hechler et al., 1991). Moreover, this compound completely reverses both the EEG abnormalities induced by GHB in the Wistar rat (Maitre et al., 1990) and the sedative and cataleptic properties of GHB (Schmidt et al., 1991), suggesting that GHB receptors are implicated directly in these effects of GHB. Only one dose of NCS-382 (300 mg/kg) was used, corresponding to an equimolecular dose of GHB, and this procedure (i.e. co-injection of equimolecular or lower doses of NCS-382 thirty minutes before testing) was the one used to demonstrate the previously described antagonistic properties of this compound (Maitre et al., 1990; Schmidt et al., 1991). At this dose, NCS-382 was found unable to antagonize the anti-anxiety effects of GHB in the elevated plus maze. Thus, it could be assumed that the anxiolytic properties of GHB do not involve GHB receptors and that distinct mechanisms are possibly implicated in sedative/cataleptic effects and anxiolytic effects of GHB as already described for tracazolate (File and Pellow, 1985).

To explore the mechanism of anti-anxiety effects of GHB in more detail, we studied the action of naloxone in association with GHB in the elevated plus maze. This opioid receptor antagonist has already been shown to reverse many biochemical and pharmacological modifications induced by GHB (for review see Maitre, 1997). Naloxone in particular was demonstrated to overcome dopaminergic, EEG and cataleptic effects of GHB in rats (Snead and Bearden, 1980), but not GHB-induced sleep in mice (Devoto et al., 1994), reinforcing the hypothesis that there are different mechanisms for sedative and anxiolytic effects of GHB. It has previously been shown that large doses of naloxone (5–10 mg/kg i.p.) are required to inhibit the anticonflict effects of benzodiazepines and pentobarbital and the anti-anxiety effects of diazepam in the elevated plus maze (Agmo et al., 1995). Naloxone did not significantly reduce the anxiolytic effects of GHB under our conditions even at the dose of 10 mg/kg i.p. Agmo et al. (1995) suggested that the release of endogenous opioids associated with a variety of stresses may play an important role in the control of anxiety by benzodiazepines. It has been shown that GHB induces an increased release of striatal substance displacing [^{3}H]naloxone binding (Hechler et al., 1985) but a reduction in the release of striatal methionine-enkephalin (Gobaille et al., 1994). Therefore it could be argued that if the release of endogenous opioid peptides is important in the anxiolytic response of diazepam and benzodiazepines, it should not

be the same with GHB unless dynorphin acting at κ-, rather than μ- or δ-opioid receptors was involved but this needs further investigation. In support of this idea, some recent reports demonstrated that κ-opioid agonists produce an anxiolytic effect on rats in the elevated plus maze (Privette and Terrian, 1995) and are involved in the anxiolytic effects of diazepam in mice (Tsuda et al., 1996).

The observed reversal effect of flumazenil on GHB anxiolytic properties favors the possibility of GHB acting directly or indirectly on the $GABA_A$-benzodiazepine receptor complex. A direct interaction of GHB at $GABA_A$ receptors and on Cl^- conductance has been suspected but remains a matter of dispute. Some previous studies suggest an interaction between the GHBergic and the GABAergic systems (Hösli et al., 1983; Snead and Nichols, 1987; Snead et al., 1992). However GHB fails to alter directly the function of the $GABA_A$ receptor complex since in vitro and in vivo binding studies have shown that GHB has no effect on the binding of [^{3}H]muscimol, [^{3}H]flunitrazepam and [^{35}S]*t*-butylbicyclophosphorothionate or on the uptake of $^{36}Cl^-$ (Serra et al., 1991; Snead and Liu, 1993). Alternatively, the hypothesis of an indirect effect of GHB at $GABA_A$ receptors seems to be plausible. Indeed, GHB modulates the GABAergic system either via the metabolism of GHB to GABA which has been demonstrated both in vivo (De Feudis and Collier, 1970) and in vitro (Vayer et al., 1985), or by regulating GABA release in some specific brain regions. Barnejee and Snead (1995) demonstrated that GHB decreases the basal extracellular release of GABA in rat thalamic ventrobasal nucleus, a finding inconsistent with an anxiolytic effect but associated with enhanced GABAergic transmission. However investigations in our laboratory clearly showed a GHB-induced increase of GABA release in the frontal cortex (unpublished results).

Flumazenil is a selective benzodiazepine receptor antagonist (Hunkeler et al., 1981) that has not yet been shown to have any affinity for GHB receptors, but has been demonstrated to antagonize the GHB action on growth hormone secretion (Gerra et al., 1994) while naloxone did not (Gerra et al., 1995). Flumazenil has not yet been assessed in association with $GABA_A$ receptor ligands or GABA-transaminase inhibitors in the evaluation of anxiety. However, this benzodiazepine receptor antagonist is able to reverse the anxiolytic effects of non-benzodiazepine compounds such as tracazolate (File and Pellow, 1985) or F2692, a pyridazine derivative (Assié et al., 1993) which both display potent anxiolytic effects but exhibit negligible affinity for benzodiazepine binding sites in vivo and in vitro. Similarly, the effects of some 5-HT_{1A} receptor agonists, 5-HT_2 receptor antagonists and CCK-B receptor agonists and antagonists on anxiety can also be antagonized by flumazenil (Engel et al., 1989; Nagatani et al., 1991; Chopin and Briley, 1993), suggesting that the $GABA_A$-benzodiazepine complex may be a common downstream component of various neurochemical systems controlling anxiety states (Assié et al., 1993). The hypothesis that GHB may act indirectly via a putative endogenous ligand for the benzodiazepine receptor (Costa and Guidotti, 1991) is also conceivable and has already been proposed for other compounds exhibiting anxiolytic activity (Assié et al., 1993; Chopin and Briley, 1993).

In summary, low doses of GHB display anxiolytic effects in the elevated plus maze in the rat. This action seems to involve an indirect interaction at the $GABA_A$-benzodiazepine receptor complex as evidenced by the inhibition observed with flumazenil rather than mediation by opioid or GHB receptors. Thus, further investigation with GHB receptor agonists that are not converted into GABA would be of interest to support this indirect action of GHB. The sedative/cataleptic effects and the anxiolytic action of GHB may proceed by distinct mechanisms and the interest of GHB as a potential anxiolytic in opiate or alcohol withdrawal would thus be increasingly justified.

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