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European Journal of Pharmacology 428 (2001) 315–321

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Role of $GABA_B$ receptors in the sedative/hypnotic effect of γ-hydroxybutyric acid

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Received 10 May 2001; received in revised form 9 August 2001; accepted 14 August 2001

Abstract

The present study was aimed at identifying the receptor systems involved in the mediation of the sedative/hypnotic effect of γ-hydroxybutyric acid (GHB) in DBA mice. Administration of the putative antagonist of the GHB binding site, 6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylideneacetic acid (NCS-382; 50–500 mg/kg, i.p.), significantly increased the duration of loss of righting reflex induced by GHB (1000 mg/kg, i.p.). In contrast, the $GABA_B$ receptor antagonists, (2*S*)(+)-5,5-dimethyl-2-morpholineacetic acid (SCH 50911; 25–100 mg/kg, i.p.) and (3-aminopropyl)(cyclohexylmethyl)phosphinic acid (CGP 46381; 12.5–150 mg/kg, i.p.), completely prevented the sedative/hypnotic effect of GHB. SCH 50911 (100 and 300 mg/kg, i.p.) was also capable to readily reverse the sedative/hypnotic effect of GHB (1000 mg/kg, i.p.) in mice that had lost the righting reflex. SCH 50911 (100 mg/kg, i.p.) also completely abolished the sedative/hypnotic effect of the $GABA_B$ receptor agonist, baclofen. These results indicate that the sedative/hypnotic effect of GHB is mediated by the stimulation of $GABA_B$ receptors and add further support to the hypothesis that the $GABA_B$ receptor constitutes a central site of action of GHB.

Keywords: γ-Hydroxybutyric acid (GHB); Sedative/hypnotic effect; GHB receptor antagonist; NCS-382; $GABA_B$ receptor antagonists; SCH 50911 and CGP 46381; (DBA mouse)

1. Introduction

The exogenous administration of γ-hydroxybutyric acid (GHB) has been reported to exert a number of pharmacological effects (see Maitre, 1997; Tunnicliff, 1997; Agabio and Gessa, in press), among which sedation, sleep and anesthesia are of particular interest. Accordingly, at relatively high doses GHB produces loss of righting reflex in rats (Devoto et al., 1994; Colombo et al., 1998a), hypnosis (Laborit, 1964; Yamada et al., 1967; Hoes et al., 1980) and an increase in deep slow-wave sleep (Lapierre et al., 1990) in healthy humans. These observations led to test the therapeutic efficacy of GHB in the treatment of sleep disorders; notably, open and double-blind clinical trials demonstrated (a) the ability of GHB in reducing the frequency of daytime attacks of cataplexy, sleep paralysis and hypnagogic hallucinations in narcoleptic patients (e.g., Mamelak and Webster, 1981; Scrima et al., 1990; Lammers et al., 1993; Scharf et al., 1998), and (b) its potential usefulness as an anesthetic adjuvant (Blumenfeld et al., 1962; Kleinschmidt et al., 1999).

Since GHB is an endogenous constituent of the mammalian brain and is considered to be a neuromodulator or a neurotransmitter (see Cash, 1994; Maitre, 1997), the above findings suggest that GHB may play a role in the physiology of sleep and sleep disorders. Therefore, to clarify the mechanism of these central actions of GHB is of great theoretical and practical interest.

Several lines of evidence suggest that various physiological and pharmacological actions of GHB are mediated by stimulation of specific binding sites for GHB, localized

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01334-6

in different brain areas in laboratory animals and humans (Hechler et al., 1992; Castelli et al., 2000). Accordingly, the selective GHB receptor antagonist, 6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylideneacetic acid (NCS-382), has been reported to significantly attenuate several effects of GHB, including changes in dopamine, γ-aminobutyiric acid (GABA) and glutamate release and firing activity of dopamine neurons in different rat brain regions (Maitre et al., 1990; Hechler et al., 1991; Banerjee and Snead, 1995; Godbout et al., 1995; Hu et al., 2000), sedation (Schmidt et al., 1991), catalepsy (Schmidt et al., 1991), occurrence of generalized absence seizures (Snead, 1996a), and stimulation of gastric emptying (Poggioli et al., 1999) in rats.

However, accumulating evidence suggests that the $GABA_B$ receptor may be a second central site of action of GHB. Accordingly, GHB at high micro–millimolar concentrations is a $GABA_B$ agonist (Ito et al., 1995; Ishige et al., 1996; Mathivet et al., 1997; Lingenhoehl et al., 1999; see however Snead, 1996b). Further, pretreatment with the $GABA_B$ receptor antagonists (*RS*)-3-amino-2-(4-chlorophenyl)propylphosphonic acid (phaclofen), (3-aminopropyl)(diethoxymethyl)phosphinic acid (CGP 35348) and (2*S*)(+)-5,5-dimethyl-2-morpholineacetic acid (SCH 50911) has been reported to block several effects of GHB, including changes in neurotransmitter activity (Waldmeier, 1991; Banerjee and Snead, 1995; Erhardt et al., 1998; Madden and Johnson, 1998; Hu et al., 2000), generalized absence seizures (Hosford et al., 1995; Snead, 1996a) and discriminative stimulus effects (Colombo et al., 1998b) in rats, motor impairment (Nissbrandt and Engberg, 1996) and constipation (Carai et al., in preparation) in mice.

The aim of the present study was to investigate whether the sedative/hypnotic effect of GHB was mediated by the activation of $GABA_B$ and/or GHB receptors. As comparison, the sedative/hypnotic effect of the $GABA_B$ receptor agonist, baclofen, was also investigated. In this study, we used DBA mice that are particularly sensitive to the sedative action of GHB (Dudek and Fanelli, 1980).

2. Methods

2.1. Animals

Male DBA mice (Charles River, Calco, LC, Italy), weighing 25–30 g, were used. After delivery to our animal facility, mice were left undisturbed for 7 days to adapt to the new housing conditions. Mice were housed 20 per cage in standard plastic cages [55 × 33 × 19 (h) cm] with wood chip bedding under a 12-h artificial light–dark cycle (lights on at 7:00 a.m.), at a constant temperature of 22 ± 2 °C and relative humidity of approximately 60%. Tap water and standard laboratory rodent chow (MIL Morini, San Polo d'Enza, RE, Italy) were provided ad libitum.

2.2. Procedure

NCS-382 (0, 50, 100, 250 and 500 mg/kg), SCH 50911 (0, 25, 50 and 100 mg/kg), and CGP 46381 [(3-aminopropyl)(cyclohexylmethyl)phosphinic acid; 0, 12.5, 25, 50 and 150 mg/kg] were acutely administered 15 min prior to the injection of 1000 mg/kg GHB to groups of 10–12 mice. In the reversal study, GHB (1000 mg/kg) was injected first, and SCH 50911 [0 ($n = 10$), 100 ($n = 10$) and 300 ($n = 10$) mg/kg] was administered 15 min after each mouse had lost the righting reflex (see below).

In all experiments, after GHB injection each mouse was placed on its back once every 60 s until it was unable to right itself within 60 s. The time between GHB injection and the start of the 60-s interval when the mouse was unable to right itself was measured as onset of the righting reflex loss. If a mouse did not lose the righting reflex within 120 min, it was excluded from the analysis of onset. Each mouse was then left undisturbed on its back until it spontaneously regained its righting reflex (determined as having at least three paws under its body). Complete recovery of the righting reflex was defined as the mouse being able to turn itself upright twice more within 60 s. If this criterion was not fulfilled, the mouse was left undisturbed until it spontaneously regained its righting reflex. The time between loss and recovery of righting reflex was monitored in each mouse as its sleep time. In the reversal study, sleep time was defined as the interval between injection of SCH 50911 (which took place 15 min after loss of righting reflex) and the time when each mouse recovered the righting reflex.

In the baclofen study, 0 ($n = 12$) and 100 ($n = 12$) mg/kg SCH 50911 were administered 15 min before the injection of 60 mg/kg baclofen. Onset and duration of the loss of righting reflex induced by baclofen were recorded as described above for the GHB studies.

The experimental procedure employed in the present study was approved by the Ethical Committee of the University of Cagliari.

2.3. Drugs

GHB (sodium salt; donated by Laboratorio Farmaceutico C.T., Sanremo, IM, Italy) was dissolved in distilled water and injected in a 29.4 ml/kg volume [this large injection volume was chosen to minimize tissue irritation at the injection site and avoid animal distress, as indicated by the lack of any effect on spontaneous locomotor activity (this laboratory, unpublished results)]. NCS-382 (sodium salt; synthesized by G. Ci. as previously described by Maitre et al., 1990) was dissolved in 12.5 ml/kg distilled water. SCH 50911 (synthesized by S.M., G.M., A.M.P. and C.S. as previously described by Blythin et al., 1996), CGP 46381 (Tocris, Ballwin, MO, USA), and baclofen (RBI, Natick, MA, USA) were dissolved in 12.5 ml/kg saline. All drugs were injected i.p.

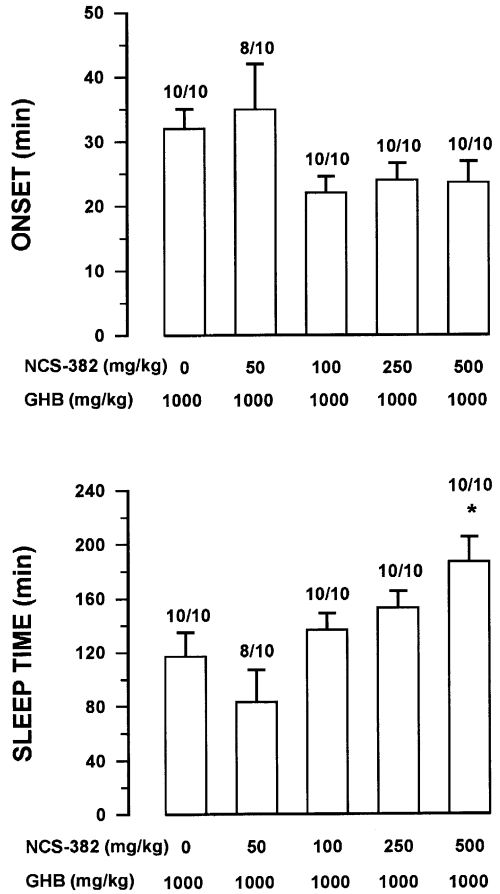

Fig. 1. Potentiation of the sedative/hypnotic effect of γ-hydroxybutyric acid (GHB) by the putative GHB receptor antagonist, NCS-382, in DBA mice. Top and bottom panels illustrate, respectively, the time to lose (onset) and regain (sleep time) the righting reflex after administration of NCS-382 and GHB. NCS-382 was administered i.p. 15 min prior to the i.p. injection of GHB. Figure on top of each bar indicate the number of mice which lost the righting reflex over the total number of mice tested. In the top panel, each bar is the mean ± S.E.M. of the onset of mice which lost the righting reflex; in the bottom panel, each bar is the mean ± S.E.M. of the sleep time of 10 mice (mice that did not lose the righting reflex were included, and assigned the value zero). * $P < 0.05$ in comparison to 0 mg/kg NCS-382 plus 1000 mg/kg GHB group.

2.4. Data analysis

Onset of the loss of righting reflex and sleep time after GHB administration were expressed in min and used as measures of GHB-induced sedative/hypnotic effect. Data on onset and sleep time from each prevention experiment, as well as data on sleep time from the reversal experiment with SCH 50911, were evaluated by a one-way analysis of variance (ANOVA), followed by the Newman–Keuls test for post hoc comparisons. In the experiments testing NCS-382, SCH 50911 (prevention) and CGP 46381, ANOVAs for onset were limited to the data from mice which lost the righting reflex, while ANOVAs for sleep time also included data from mice that did not lose the righting reflex, to which the value zero was assigned. Data from the baclofen study on the number of mice losing the righting reflex after drug treatment were analyzed by the Fisher's exact test for a 2×2 table [treatment (saline, SCH 50911) $\times$ loss of righting reflex (presence, absence)].

3. Results

Mice treated with the combination of NCS-382 with GHB lost the righting reflex, with the sole exception of two (out of ten) mice in the 50 mg/kg NCS-382 group. ANOVA failed to reveal any significant effect of treatment with NCS-382 on sleep onset [$F(4,47) = 2.03$, $P > 0.05$], but indicated a significant effect of NCS-382 administration on sleep time [$F(4,47) = 3.29$, $P < 0.05$]. Post hoc

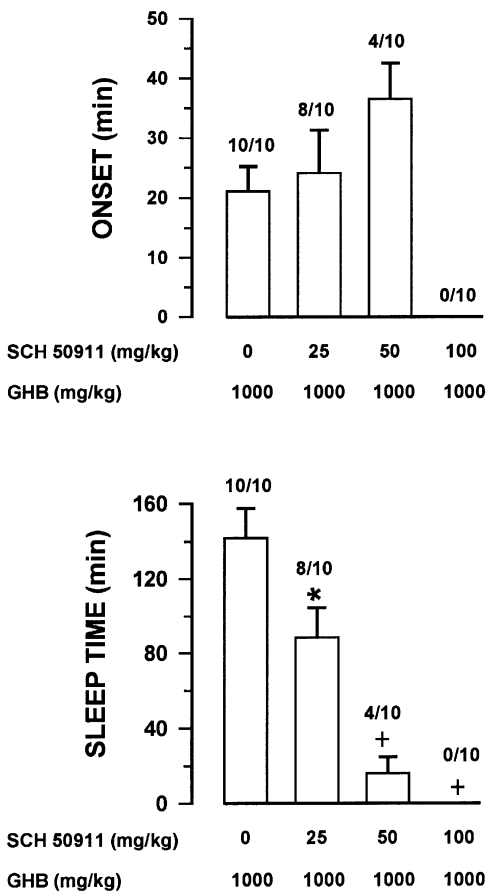

Fig. 2. Prevention of the sedative/hypnotic effect of γ-hydroxybutyric acid (GHB) by the $GABA_B$ receptor antagonist, SCH 50911, in DBA mice. Top and bottom panels illustrate, respectively, the time to lose (onset) and regain (sleep time) the righting reflex after administration of SCH 50911 and GHB. SCH 50911 was administered i.p. 15 min prior to the i.p. injection of GHB. Figure on top of each bar indicate the number of mice which lost the righting reflex over the total number of mice tested. In the top panel, each bar is the mean ± S.E.M. of the onset of mice which lost the righting reflex; in the bottom panel, each bar is the mean ± S.E.M. of the sleep time of 10 mice (mice that did not lose the righting reflex were included, and assigned the value zero). *: $P < 0.005$ in comparison to 0 mg/kg SCH 50911 plus 1000 mg/kg GHB group. +: $P < 0.0005$ in comparison to 0 mg/kg SCH 50911 plus 1000 mg/kg GHB group.

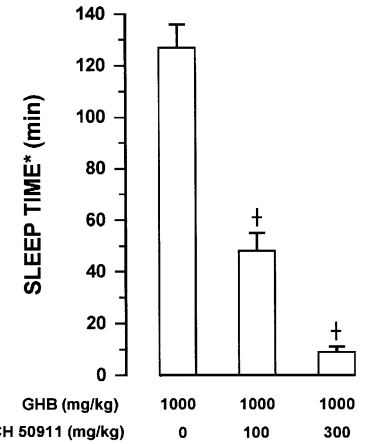

Fig. 3. Reversal of the sedative/hypnotic effect of γ-hydroxybutyric acid (GHB) by the $GABA_B$ receptor antagonist, SCH 50911, in DBA mice. This picture illustrates the time to regain (sleep time) the righting reflex after administration of GHB and SCH 50911. SCH 50911 was administered i.p. 15 min after each mouse had lost the righting reflex. Each bar is the mean ± S.E.M. of the sleep time of 10 mice. +: $P < 0.0005$ in comparison to 1000 mg/kg GHB plus 0 mg/kg SCH 50911 group. * Sleep time was regarded as the interval between injection of SCH 50911 (which took place 15 min after loss of righting reflex) and the time when each mouse recovered the righting reflex.

analysis revealed that the combination of GHB with 500 mg/kg NCS-382 resulted in a significantly longer sleep time than in GHB alone-treated mice (Fig. 1).

Pretreatment with SCH 50911 resulted in a dose-dependent antagonism of GHB effect; GHB-induced loss of righting reflex was completely suppressed by the dose of 100 mg/kg SCH 50911. Thus, the number of mice which lost the righting reflex after the combination of 0, 25, 50 and 100 mg/kg SCH 50911 plus 1000 mg/kg GHB were 10/10, 8/10, 4/10 and 0/10, respectively. ANOVA for onset was limited to the data from mice which lost the righting reflex (consequently, the 100 mg/kg SCH 50911 mouse group was excluded from the analysis) and failed to show a significant effect of SCH 50911 treatment [$F(2,19) = 1.34$, $P > 0.05$]. In contrast, ANOVA for sleep time (including data from mice that did not lose the righting reflex, to which the value zero was assigned) showed a highly significant effect of SCH 50911 treatment [$F(3,36) = 29.94$, $P < 0.000001$]. At each dose of SCH 50911, sleep time was significantly shorter than in saline-treated group (Fig. 2).

In the reversal study, administration of 1000 mg/kg GHB resulted in loss of righting reflex in all mice tested, with comparable onset among the three groups subsequently tested with saline or SCH 50911. Administration of 100 and 300 mg/kg SCH 50911 to mice once they had lost the righting reflex resulted in a significant, dose-dependent reduction of GHB-induced sleep time [$F(2,27) = 76.08$, $P < 0.000001$]. Indeed, the time elapsed between SCH 50911 injection and recovery of the righting reflex was 62% and 93% lower in mice treated with 100 and 300 mg/kg SCH 50911, respectively, than in saline-dosed mice (Fig. 3).

Similar results were obtained with CGP 46381. This compound dose-dependently prevented the sedative/hypnotic effect of GHB, with a complete protection at the dose of 150 mg/kg. Indeed, the number of mice which lost the righting reflex after the combination of 0, 12.5, 25, 50 and 150 mg/kg CGP 46381 plus 1000 mg/kg GHB were 12/12, 11/12, 8/12, 4/12 and 0/12, respectively. ANOVA for onset (limited to the data from mice which lost the righting reflex) showed a significant effect of treatment with CGP 46381 [$F(3,31) = 12.92$, $P < 0.0001$]. Post hoc analysis revealed significantly longer onset in 25 and 50 mg/kg CGP 46381-dosed mice than in vehicle-treated mice (Fig. 4, top panel). ANOVA for sleep time (which included data from mice that did not lose the righting reflex, to which the value zero was assigned) showed a highly significant effect of treatment with CGP

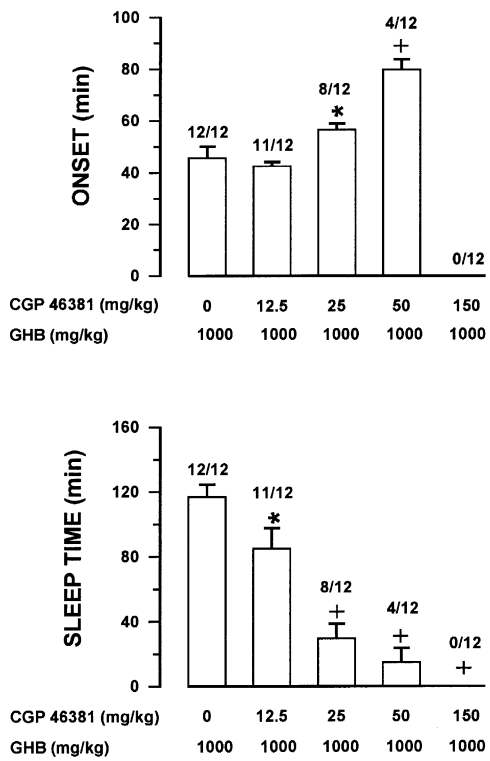

Fig. 4. Prevention of the sedative/hypnotic effect of γ-hydroxybutyric acid (GHB) by the $GABA_B$ receptor antagonist, CGP 46381, in DBA mice. Top and bottom panels illustrate, respectively, the time to lose (onset) and regain (sleep time) the righting reflex after administration of CGP 46381 and GHB. CGP 46381 was administered i.p. 15 min prior to the i.p. injection of GHB. Figure on top of each bar indicate the number of mice which lost the righting reflex over the total number of mice tested. In the top panel, each bar is the mean ± S.E.M. of the onset of mice which lost the righting reflex; in the bottom panel, each bar is the mean ± S.E.M. of the sleep time of 12 mice (mice that did not lose the righting reflex were included, and assigned the value zero). *: $P < 0.05$ in comparison to 0 mg/kg CGP 46381 plus 1000 mg/kg GHB group. +: $P < 0.0005$ in comparison to 0 mg/kg CGP 46381 plus 1000 mg/kg GHB group.

46381 [$F(4,55) = 32.25$, $P < 0.0000001$]. Sleep time induced by each dose of CGP 46381 was significantly shorter than in vehicle-treated group (Fig. 4, bottom panel).

As predictable, pretreatment with SCH 50911 resulted in a complete antagonism of the sedative/hypnotic effects of baclofen; indeed, while all mice of the control group (saline plus 60 mg/kg baclofen) lost their righting reflex (onset: 15.2 ± 0.9 min; sleep time: 117.7 ± 7.3 min), no mouse in the SCH 50911 plus baclofen group lost the righting reflex ($P < 0.00001$, Fisher's exact test).

4. Discussion

The results of the present study indicate that the sedative/hypnotic effect induced by GHB is due to the activation of $GABA_B$ receptors. Indeed the behavioral depression of GHB was similar to that produced by baclofen, a specific $GABA_B$ receptor agonist. Moreover, the sedative/hypnotic effect of both drugs was completely prevented by the specific $GABA_B$ receptor antagonists, SCH 50911 and CGP 46381. In addition, the administration of SCH 50911 to mice that had lost the righting reflex readily and completely reversed this condition. These results are in agreement with previous observations showing that several pharmacological effects of GHB, including motor impairment, absence seizures, suppression of firing of dopaminergic neurons, inhibition of dopamine, GABA and glutamate release and discriminative stimulus effects, are suppressed by $GABA_B$ receptor antagonists (see Introduction for references).

The molecular basis of the GHB–$GABA_B$ interaction is not completely understood at present. GHB has been reported to act as a weak agonist at the $GABA_B$ binding site (Ito et al., 1995; Ishige et al., 1996; Mathivet et al., 1997; Lingenhoehl et al., 1999; see, however, Snead, 1996b), and the dose of GHB tested in the present study (1000 mg/kg, i.p.) should elicit brain levels of GHB comparable to those reported to displace $GABA_B$ receptor agonists in in vitro assays. $GABA_B$ receptor-mediated effects of GHB have been hypothesized to be secondary to (a) conversion of GHB into GABA or (b) GHB-induced stimulation of GABA release, both actions resulting in an increase in synaptic levels of GABA, which in turn binds to $GABA_B$ receptors (Hechler et al., 1997; Gobaille et al., 1999). Third, GHB might activate a GHB recognition site related to a $GABA_B$ receptor, forming a presynaptic $GABA_B$/GHB receptor complex (Snead, 1996b).

In contrast, pretreatment with NCS-382 potentiated, instead of antagonizing, GHB-induced sedative/hypnotic effect. These results were quite unexpected, since NCS-382 was reported to antagonize different effects of GHB (see Introduction for references) and, to our knowledge, no augmentation of any GHB effect after NCS-382 administration has ever been described. Interestingly, and apparently in contrast with the results of the present study, Schmidt et al. (1991) reported the ability of NCS-382 (administered at the dose of 2.08 mmol/kg, corresponding to 500 mg/kg) to completely antagonize GHB-induced sedation, hypomotility and catalepsy in rats. The reason for the discrepancy between the results reported by Schmidt et al. (1991) and those from the present study escapes our understanding; at present, we can only identify a few differences in the experimental procedures of the two studies. First, Schmidt et al. (1991) used Wistar rats while the present investigation employed mice of the DBA strain (which were found to be particularly sensitive, at least in comparison to the C57BL strain, to the sedative/hypnotic effect of GHB (this laboratory, unpublished results) and γ-butyrolactone (GBL); Dudek and Fanelli, 1980). Second, the dose of GHB used in the study by Schmidt et al. (1991) (3.97 mmol/kg, corresponding to 500 mg/kg) was half that tested in the present study, resulting therefore in a different intensity of sedation (accordingly, Schmidt et al., 1991 measured mostly hypomotility while the present study assessed the loss of righting reflex, an index of more severe sedation). In good agreement with the results of the present study, Maitre et al. (1990) mentioned the results of preliminary experiments suggesting that "the sedative/hypnotic effect of GHB obtained at a high dosage (7–20 mmol/kg i.p.) does not appear to be significantly attenuated by NCS-382 pretreatment (2–4 mmol/kg i.p.)". The doses of GHB tested by Maitre et al. (1990) (882–2520 mg/kg) were similar or even higher than that used in the present study (1000 mg/kg), and at least twofold higher than that tested in the investigation by Schmidt et al. (1991). Whether also the NCS-382-induced potentiation of the sedative/hypnotic effect of GHB, observed in the present study, is secondary to stimulation of $GABA_B$ receptors is not known at present; however, although NCS-382 has been reported to lack any affinity for the $GABA_B$ receptor (Maitre et al., 1990; Snead, 1996b), a recent investigation from this lab found that SCH 50911 antagonized the constipating effect of NCS-382 in the mouse small intestine (Carai et al., unpublished results), suggesting a $GABA_B$ receptor mediation in this effect of NCS-382.

In summary, the results of the present study indicate that combination of the $GABA_B$ receptor antagonists, SCH 50911 or CGP 46381, with GHB resulted in a complete abolishment of the sedative/hypnotic effect of GHB. These results add further support to the hypothesis that the $GABA_B$ receptor constitutes a central site of action of GHB. The interest of these findings resides in the fact that GHB is a putative neurotransmitter or neuromodulator. Therefore, the endogenous GHB, via interaction with the $GABA_B$ receptor, might play a role in the physiology of sleep and sleep disorders.

Finally, the results of the reversal study with SCH 50911 suggest that $GABA_B$ receptor antagonists might have a potential usefulness in reversing the episodes of coma, respiratory depression and loss of consciousness reported to accidentally occur in an increasing number of

individuals overdosing GHB for its euphoric and mood-enhancing properties (e.g., Thomas et al., 1997; Ryan and Stell, 1997; Li et al., 1998; Ingels et al., 2000).

Acknowledgements

The authors are grateful to Mrs. Anne Farmer for language editing of the manuscript. The present study was partially supported by the European Community, the Italian Government and the Regione Autonoma della Sardegna through "Programma Operativo Plurifondo—Sardegna".

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