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A Review of Pharmacology of NCS-382, a Putative Antagonist of γ-Hydroxybutyric Acid (GHB) Receptor

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ABSTRACT

γ-Hydroxybutyric acid (GHB), a naturally occurring metabolite of γ-aminobutyric acid (GABA), has been postulated to act as a specific agonist of GHB receptors and as well as a weak $GABA_B$ receptor agonist. To date, 6,7,8,9-tetrahydro-5-hydroxy-5H-benzocyclohept-6-ylideneacetic acid (NCS-382), a semirigid compound structurally related to GHB, is the only compound reported to be an antagonist of the GHB receptor sites. In this article we review the *in vivo* and *in vitro* pharmacological properties of NCS-382 and its interaction with GHB and $GABA_B$ receptors. Binding studies have demonstrated that NCS-382 is a stereoselective ligand for GHB-binding sites, with both, the high and the low component of population, showing the same distribution of GHB receptors. Indeed, this compound did not display affinity for $GABA_A$, $GABA_B$, or any other known receptors, while conflicting data have been reported as to its selective antagonist action at GHB receptor. Only a few studies have shown that NCS-382 antagonizes GHB-induced effect, but a re-evaluation of all data reported in the literature suggests that the antagonistic effect of this compound could be due to an indirect action at $GABA_B$ receptors. As revealed by several behavioral studies, NCS-382 fails to antagonize GHB discriminative stimuli, GHB-induced inhibition of locomotor activity and ataxia or suppression of operant responses. Moreover, it is capable of either eliciting qualitatively similar effects to those of GHB or enhancing some actions of GHB. In addition, the NCS-382-sensitive electrophysiological effects of endogenous and exogenous GHB observed *in vivo* have not been completely replicated *in vitro*. The only electrophysiological action of GHB antagonized *in vitro* by NCS-382 required a previous blockade of $GABA_B$ receptors. We concluded that NCS-382 is a good ligand but not a selective antagonist for GHB receptor.

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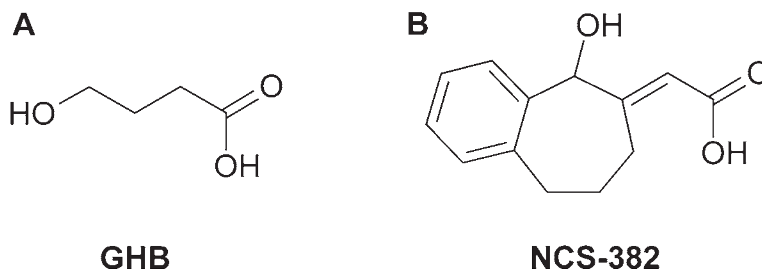

Fig. 1. Chemical structure of GHB (A) and NCS-382 (B).

INTRODUCTION

γ-Hydroxybutyric acid (GHB) is a putative neurotransmitter or neuromodulator in the mammalian brain (9,51). The chemical structure of GHB is shown in Fig. 1A. Depending on the species, anatomical structures, level of brain maturation and methods of detection, brain GHB levels range from 0.5 to 25 pmol/mg tissue (9). Specific mechanisms of synthesis, release and uptake and specific binding sites are present in discrete brain areas of the mammalian, including human, brain (19), suggesting that GHB may function as a neurotransmitter or a neuromodulator (9,51,73).

GHB, by systemic administration at low doses to either laboratory animals or humans, has been shown to induce anxiolytic and myorelaxant effects. At intermediate doses GHB increases rapid eye movement (REM) and slow-wave sleep, whilst at high doses it produces anesthesia (9). Moreover, at non-anesthetic doses GHB reduces voluntary alcohol intake in rats genetically selected for alcohol preference (1) and suppresses ethanol withdrawal syndrome in ethanol-dependent rats (25).

Clinical studies have confirmed the efficacy of GHB in the treatment of alcohol dependence and of withdrawal syndrome (25). In addition, studies in rats and mice indicate that GHB positively induces reinforcing behavior (26,54). Similarly, in humans GHB produces euphoria and may be recreationally abused (25,35). Moreover, GHB modifies the levels or action of different neurotransmitters such as dopamine, opioids, glutamate and acetylcholine (4,32,49,51). The mechanisms through which GHB induces these effects in the central nervous system (CNS) and acts as a drug of abuse are still unclear. GHB has affinity for two distinct binding sites in the brain, the GHB and the $GABA_B$ receptors (6,19,55). Snead et al. (63) reported that the GHB receptor is, similarly to the $GABA_B$ receptor, coupled to a G protein. GHB receptor differs, however, from the $GABA_B$ receptor, as it is characterized by a significantly different distribution and ontogeny (61,62). Accordingly, Andriamampandry et al. (3) cloned a putative GHB receptor that is coupled to G protein and activated by GHB. However, in contrast to the results published by Snead (63) and Andriamampandry et al. (3), other studies failed to demonstrate that GHB is a G protein coupled receptor (20,44,57).

Bourguignon et al. (11) investigated the effects of a number of structural analogues on [^{3}H]GHB binding to rat brain membranes. The most effective inhibitors were γ-methyl-GHB, γ-phenyl-GHB, γ-benzyl-GHB, γ-para-methoxy-GHB, trans-4-γ-hydroxycrotonic acid (t-HCA), and its derivatives such as γ-p-chloro-phenyl-t-HCA, γ-p-trifluoromethyl-phenyl-t-HCA, and γ-p-nitro-phenyl-t-HCA.

Although inhibitors of [^{3}H]GHB binding have been identified (11), little information is available on possible ligands with either agonist or antagonist properties at GHB receptor.

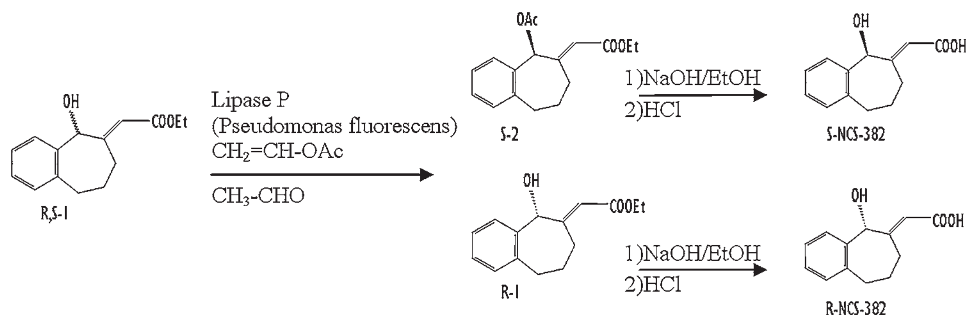

Fig. 2. Scheme of the resolution of racemic R/S-NCS-382, performed as described in Castelli et al. 2002. Reproduced from ref. 21 with permission from Elsevier B.V./ECNP.

To date, the only compound reported to be an antagonist at the GHB receptor sites (52) is 6,7,8,9-tetrahydro-5-hydroxy-5H-benzocyclohept-6-ylideneacetic acid (NCS-382), a semirigid compound structurally related to GHB (Fig. 1B). This compound was initially reported by Maitre et al. (52) to inhibit [^{3}H]GHB binding and to antagonize several neuropharmacological effects of GHB. However, while the majority of GHB effects are antagonized by the $GABA_B$ receptor antagonists, suggesting that they are mediated by $GABA_B$ receptor, only a number of responses are selectively antagonized by the putative GHB receptor antagonist NCS-382.

The present review focuses on the pharmacological properties of NCS-382 and its interaction with both, GHB and $GABA_B$ receptors

BIOCHEMICAL PHARMACOLOGY

In Vitro Receptor Binding Studies

NCS-382 displaces [^{3}H]GHB binding from both the high- and low-affinity binding sites in the rat or human brain membranes (19,52). The IC_{50} for NCS-382 are in the nanomolar (from 42 to 200 nM) or micromolar (4 to 25 μM) ranges for the high- and the low-affinity binding sites, respectively. For both striatum and hippocampus, NCS-382 shows a lower affinity than GHB for the high affinity population of sites, but higher affinity for the low-affinity population. It displays no affinity for GABA binding site [as measured according to Enna and Snyder (31) using [^{3}H]GABA ligand (52)]. Moreover, as demonstrated by Snead (66) and Castelli et al. (21) NCS-382 does not compete for $GABA_B$ binding sites. In a recent study we demonstrated the stereoselectivity of NCS-382 binding to GHB receptor in the rat brain (21). The racemic compound R/S-NCS-382 has been resolved in two enantiomers R- and S- (Fig. 2); the potency of the latter to displace [^{3}H]GHB and the radiolabeled NCS-382 from GHB receptors has been compared in rat brain homogenate. The displacement ability of NCS-382 is stereoselective regardless of the radioligand used to label GHB binding sites. As shown in Table 1, R-NCS-382 (0.21 μM) is twice as potent as the racemic form (0.42 μM) and 62-fold more potent than the S enantiomer (13 μM) in displacing [^{3}H]GHB from GHB binding sites. On the other hand, the R-enantiomer is 2- (1.04 μM) and 13-fold more potent than the racemic (2.04 μM) and S- forms (13.90 μM), respectively, when using [^{3}H]NCS-382 as a radio-

ligand. The fact that R- and R/S-NCS-382 were approximately 5-fold more potent in displacing [^{3}H]GHB than [^{3}H]NCS-382 from the GHB receptors might be explained by the fact that [^{3}H]GHB and [^{3}H]NCS-382, at the concentrations used in displacement experiments, differentially label the high- and the low-affinity site of the GHB receptor, respectively. Indeed, while the high specific activity of [^{3}H]GHB (60 Ci/mmol) allows the selective labeling of the high-affinity site of the GHB receptor, the lower specific activity of [^{3}H]NCS-382 is insufficient to detect the high-affinity site, which constitutes less than 10% of the total binding site. Therefore, at 16 nM concentration used in the displacement experiments, [^{3}H]NCS-382 should have labeled the predominant low-affinity site of the GHB receptors, as previously reported by Mehta et al. (56).

Despite the latter finding, it is noteworthy that NCS-382 does not bind to the recently cloned GHB receptor even at 200 μM concentration (3), suggesting that the GHB receptor system might include several subtypes.

The properties of [^{3}H]NCS-382 as a radioligand for GHB receptors *in vitro* have been evaluated and compared with the radioligand [^{3}H]GHB by Mehta et al. (56). The authors revealed specific binding of [^{3}H]NCS-382 to the rat cerebral cortex and hippocampus, while very little binding was observed in the rat cerebellum, heart, kidney, liver and lung membranes. These results regarding the regional analysis of specific binding in rat brain are consistent with studies performed using [^{3}H]GHB as radioligand (6,67). Scatchard analysis of saturation isotherm revealed two different populations of binding sites in the rat cerebral cortex (K_{d1}, 795 nM, B_{max1} 25.4 pmol/mg protein; K_{d2}, 21 μM, B_{max2} 178 pmol/mg protein) as well as in the rat hippocampus (K_{d1}, 441 nM, B_{max1} 16.2 pmol/mg protein; K_{d2}, 9.8 μM, B_{max2} 255 pmol/mg protein) (Fig. 3). These data confirm the presence of two binding sites for GHB receptors, as previously shown using [^{3}H]GHB as radioligand. However, no comparison is possible between these K_d values of [^{3}H]NCS-382 binding with the K_d values of [^{3}H]GHB reported in several studies since different experimental conditions, such as pH, were used to perform Scatchard analysis for the two radioligands. In fact, since the [^{3}H]GHB binding is pH dependent, with a maximum binding at pH 5.0–6.5, binding analyses using this radioligand were carried out at acidic pH, while [^{3}H]NCS-382 binding was investigated at pH 7.4, in view of the fact that a substantial degree of binding occurred at this physiological pH.

GHB and NCS-382 completely inhibited [^{3}H]NCS-382 binding in the rat cerebrocortical and hippocampal membranes, and NCS-382 (IC_{50} = 1.8 and 2.3 μM, respectively) was approximately 10 times more potent compared with GHB (IC_{50} = 25 and 23 μM, respectively). Displacement curves showed only one population of binding site, probably due to

TABLE 1. Inhibition of [^{3}H]GHB and [^{3}H]NCS-382 binding by R/S-NCS-382, R and S enantiomers

Compounds	[^{3}H]GHB (IC_{50} μM)	[^{3}H]NCS-382 (IC_{50} μM)
R/S-NCS-382	0.42 ± 0.03	2.38 ± 0.09
R-NCS-382	0.21 ± 0.01	1.04 ± 0.05
S-NCS-382	12.93 ± 2.33	13.90 ± 3.67

The IC_{50} values were calculated from displacement curves using either 10 nM of [^{3}H]GHB (60 Ci/mmol) or 16 nM of [^{3}H]NCS-382 (20 Ci/mmol). IC_{50} values are expressed as means ± S.E.M. of at least three determinations in duplicate. Reprinted from ref. 21 with permission from Elsevier B.V./ECNP.

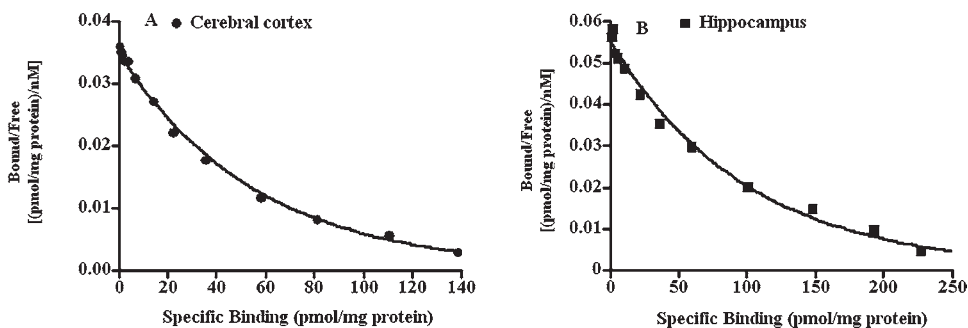

Fig. 3. Representative Scatchard analysis of [^{3}H]NCS-382 binding to the rat cerebrocortical (A) and hippocampal membranes (B). Reproduced from ref. 56 with permission of ASPET.

the low specific activity of the radioligand NCS-382 utilized. GABA (100 µM) and the $GABA_B$ receptor agonist baclofen (500 µM) partially (23–30%) inhibited [^{3}H]NCS-382 binding. This partial inhibition by high concentrations of GABA and baclofen is probably not mediated through $GABA_B$ receptors, since GABA and baclofen are both reported to inhibit the [^{3}H]baclofen binding to $GABA_B$ receptors with IC_{50} values of 22 to 84 nM (13). Indeed, bicuculline, muscimol, picrotoxin and phaclofen did not modify the [^{3}H]NCS-382 binding. Moreover, a variety of ligands for other receptors did not inhibit this binding, suggesting selectivity of this radioligand.

[^{3}H]NCS-382 was used to investigate the distribution of GHB receptors by means of quantitative autoradiography (40). The concentrations of radioactive and the specific activity of the commercially available racemic [^{3}H]NCS-382 implied that the low component of GHB binding was evaluated. The K_d and B_{max} values, determined from one-site curve fit in CA1-CA2/CA3 regions of hippocampus and cortex were 1.0–4.5 µM and 67–38 pmol/mg protein, respectively. As shown in Fig. 4, maximal high affinity binding occurs in CA1 (12.6 pmol/mg protein), CA2/CA3 (10.9 pmol/mg protein), dentate gyrus (11.0 pmol/mg protein) and layers of the parietal cortex (11.4 pmol/mg protein). Areas such as caudate putamen and nucleus accumbens exhibited low to intermediate levels of binding, while locus coeruleus, cerebellum and ventral hypothalamus showed negligible levels of binding. Thus, the distribution of GHB binding sites using [^{3}H]NCS-382 as radioligand is consistent to that obtained with [^{3}H]GHB. Comparison of the distribution of [^{3}H]NCS-382 with both $GABA_A$ and $GABA_B$ receptors revealed a degree of overlap, although some differences are revealed in high receptor/binding site densities in different brain areas. Moreover, although [^{3}H]NCS-382 labeled only the low-affinity population of GHB binding, the use of [^{3}H]NCS-382 appears to offer some advantage over [^{3}H]GHB as a radioligand for quantitative autoradiography, due to the fact that it seems to be more potent than GHB, and unlike GHB, does not interact with $GABA_A$ or $GABA_B$ receptors.

Second Messengers Studies

Second messengers, linked to GHB receptors and intracellular events subsequent to their activation, have not yet been fully established and few conflicting results have been reported. It is, therefore, difficult to assess the ability of NCS-382 to antagonize the GHB-induced effect on its second messenger.

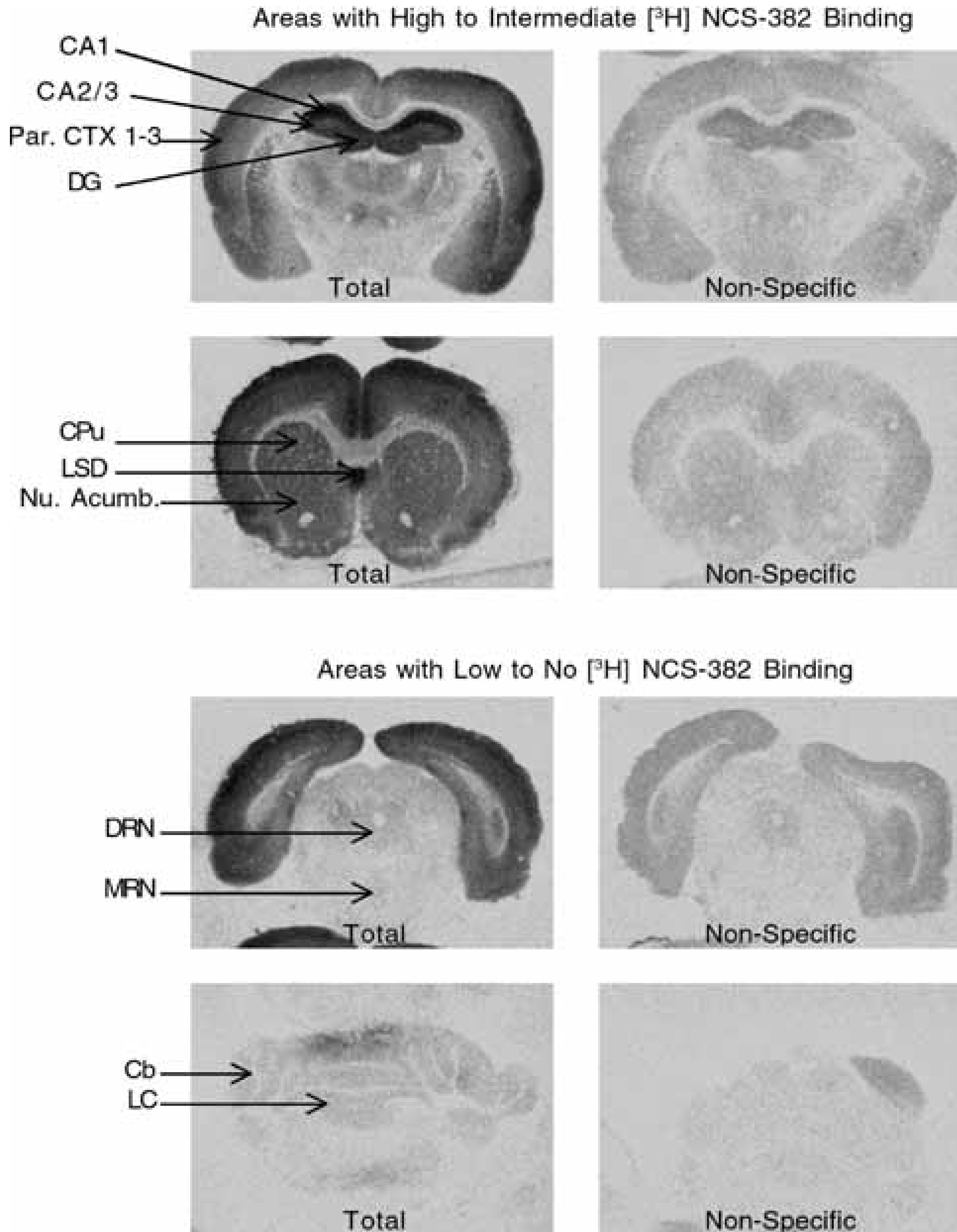

Fig. 4. Distribution of [^{3}H]NCS-382 binding sites in the rat brain. CA1, CA1 area of hippocampus; CA2/3, CA2 and CA3 areas of hippocampus; Par. CTX 1–3, parietal cortex layers I-III; DG, dentate gyrus; LSD, lateral septal nucleus, dorsal region; CPu, caudate putamen; Nu.Acumb., accumbens nucleus; DRN, dorsal raphe nucleus; MRN, medial raphe nucleus; Cb, cerebellum; LC, locus coeruleus. Reproduced from ref. 40 with permission from Elsevier.

Earlier studies (69,70) have demonstrated that GHB *in vivo* and *in vitro* increases cGMP levels and inositol phosphate turnover in rat hippocampus. Subsequently, Maitre (52) showed that the GHB-induced increase of cGMP levels and inositol phosphate turnover in rat hippocampus were both blocked by NCS-382, indicating the antagonistic properties of this compound. However, Bernasconi et. al (7,8) demonstrated that the administration of the precursor of GHB, γ-butyrolactone (GBL) in the rat brain induced a decrease in cGMP levels. Since the cerebellum is devoid of GHB receptors this decrease could not have been due to GHB receptor stimulation. Surprisingly, the authors showed that NCS-382 alone significantly decreased cGMP levels, as well as GBL itself, and when administered prior to GBL exerted an additive effect. NCS-382, as previously described (see section of binding study), does not interact with $GABA_B$ receptors. This finding strongly suggests that the action of NCS-382 in the cerebellum is unspecific and cannot be explained in terms of mechanism implicating GHB or $GABA_B$ receptors; therefore, this compound may not be a selective antagonist for GHB binding sites.

Recently, Snead (63) demonstrated that GHB decreased forskolin-stimulated cyclic AMP levels in rat cortical and hippocampal membranes with concurrent stimulatory effects on high affinity GTPase activity and guanosine 5′-O-(3-[^{35}S]GTPγS) binding in these brain regions. GHB-stimulated [^{35}S]GTPγS and GTPase activity were observed in cortex and hippocampus but not in thalamus or in cerebellum. Since there is no binding in the cerebellum it is not unexpected that GHB has no effect on GTPγS assay in this region; on the contrary, it is not clear why GHB does not exert any effect in the thalamus. The author explains this discrepancy with a lower sensitivity of the method employed to detect cAMP levels or, for GTPγS binding, with the low density of low-affinity binding sites in this region. Alternatively, the same author speculated that GHB might couple to a different second messenger in the thalamus. The stimulation of GHB on [^{35}S]GTPγS and GTPase activity were blocked by NCS-382 but not by a specific $GABA_B$ antagonist, suggesting that these effects are GHB receptor-mediated. Subsequently, three further studies failed to demonstrate either the stimulation of GTPγS binding by GHB or its inhibition by NCS-382. Results from our laboratory, performed with GTPγS assay either in cortex membrane homogenate or by autoradiography, confirmed Snead's (63) observation that GHB shares with baclofen the ability to stimulate G protein activity. However, GHB effect was found to be rather modest; a maximal stimulation of about 40 and 30% of GTPγS binding produced by GHB at 1 mM. This effect, in contrast with Snead's (63) data, was suppressed by the $GABA_B$ antagonist CGP 35348, but not by NCS-382, indicating that GHB weakly activates a G protein coupled to $GABA_B$ and not to GHB receptor (20). In both studies NCS-382 alone did not affect [^{35}S]GTPγS binding. Moreover, Kaupman et al. (44) demonstrated that GHB (≥1 mM) was able to stimulate [^{35}S]GTPγS binding in wild type mice, while this effect was completely abolished in $GABA_{B1}$ knockout mice ($GABA_{B1}$ deficient –/–). In the wild type mice, in the presence of the positive modulator $GABA_B$ receptor CGP7930, GHB induced a substantial GTPγS binding signal starting at a concentration of 1 mM. The $GABA_B$ receptor antagonist blocked this effect, while NCS-382 did not antagonize GHB-induced GTPγS binding. Finally, Odagaky and Yamauchi (57) failed to demonstrate the activation of both high affinity GTPase activity and GTPγS binding induced by GHB in any of the investigated brain regions. We have no convincing explanation for the discrepancy between these latter findings and Snead's (63) data. The discordant results might be attributed to the different animals (rats or mice) or different strains of rats used in the various laboratories. Taken together, these data do not support the hypothesis that high-affinity [^{3}H]GHB reflects functional G protein coupled

receptor ligand binding site. Moreover, the putative antagonist action of NCS-382 could not have been established.

In Vivo Microdialysis Studies

Release of dopamine, GABA, and glutamate

Earlier *in vivo* microdialysis studies reported an increase in dopamine (DA) release in rat striatum following GHB administration (41,52). Pretreatment of rats subjected to *in vivo* microdialysis by i.p. injection of 500 mg/kg of NCS-382 totally abolished the dopaminergic response induced 60 min later by local application of 120 μM GHB. Given alone, NCS-382 had no effect on DA release. Later, Maitre (51) showed that by systemic administration at low (100–300 mg/kg) or high (500 mg/kg) doses GHB decreased and increased DA release, respectively. However, despite this compelling evidence, several pharmacological, biochemical and behavioural studies indicate that GHB inhibits central DA release *in vitro* and *in vivo* (for a review see 33). Indeed, a research paper and a review (33,43), both of which critically examined these conflicting data, indicate that GHB inhibits rather than stimulates DA release. The use of either an anesthetic or Ca^{2+} in the dialysis fluid exerts considerable influence on DA release, thus indicating that possible differences in the procedure and in experimental design could explain the discrepancy between the various studies. Furthermore, up to date NCS-382 has not been used to assess the GHB-induced decrease in dopaminergic release occurring at low doses of GHB.

Indeed, the effect of NCS-382 on both, GHB-induced decrease and increase of basal and K^+ evoked release of GABA, has been investigated using *in vivo* microdialysis. GHB at doses of ≤2.0 mmol/kg (producing maximal concentration of GHB in rat brain lower than 400–500 μM) inhibited GABA release in the rat thalamus (4) and frontal cortex (37). At higher doses of GHB (4.0 mmol/kg, about 800–1000 μM in brain) or of the GHB selective ligand NCS-356, a marked increase of extracellular levels of glutamate was observed. Both effects were inhibited by the peripheral administration of a large dose (2.0 mmol/kg) of NCS-382 (37). Recently, Ferraro et al. (34) and Castelli et al. (20) demonstrated that NCS-382 blocked the GHB-induced increase of extracellular hippocampal CA1 levels, measured by microdialysis in freely moving rats, and the *in vitro* K^+ evoked release of glutamate from rat hippocampal synaptosomes. Furthermore, the stimulating effect of trans-4-γ-hydroxycrotonic acid (t-HCA) and NCS-435, selective ligands of GHB receptors, on extracellular levels of hippocampal CA1 glutamate was abolished by NCS-382. Alone, the compound did not modify either the extracellular glutamate levels or the basal and K^+ evoked efflux from rat hippocampal synaptosomes *in vitro*. Conversely, GHB at high concentration (1 mM) reduced extracellular glutamate levels via $GABA_B$ receptors (34). This effect was abolished by the $GABA_B$ receptor antagonist CGP 35348.

ELECTROPHYSIOLOGICAL STUDIES

In Vivo and *in Vitro* Effects of NCS-382

The results of electrophysiological studies on the effect of GHB and NCS-382 are contradictory (for a review see 28); *in vivo* investigations show GHB responses that are mediated by both GHB and $GABA_B$ receptors, while most of the *in vitro* investigations highlighted $GABA_B$ receptor responses. So far, only two studies (10,14) have been able to

detect a GHB receptor-mediated electrophysiological response to endogenous or exogenous GHB (defined as a response antagonized by NCS-382) in any type of CNS cells under normal conditions.

A GHB receptor-mediated effect of GHB has been observed by Berton et al. (10), but only after blockade of $GABA_B$ receptors with a $GABA_B$ antagonist. In the same study, the effect of low doses of GHB on the Schaffer collateral excitatory postsynaptic potentials (EPSPs) in rat CA1 pyramidal neurons was investigated. The NMDA and AMPA components of these EPSPs were differentiated by adding 6,7-dinitroquinoxaline-2,3-dione (DNQX) and DL-7-amino-5-phosphonovalerate (d-APV), respectively, to the perfusion medium, while $GABA_A$ and $GABA_B$ receptors were blocked by the presence of bicuculline (30 μM) and CGP 35348 (500 μM), respectively. The reduction of GHB-induced NMDA EPSPs was fully antagonized by NCS-382 (500 μM), which had no effect on its own. In addition, GHB (600 μM) decreased the AMPA EPSPs, this effect being, once again, antagonized by NCS-382 (500 μM). The latter study of Cammalleri et al. (14) reported that NCS-382, in the presence of CGP 35348 (500 μM) or CGP 55845 (1 μM) abolished the decrease of $GABA_A$ inhibitory postsynaptic potentials (IPSPs) in the CA1 region of the hippocampus. In contrast, recently Gervasi et al. (36) were not able to detect any effect of NCS-382 on thalamic EPSPs and IPSPs induced by GHB. NCS-382 given alone at a concentration of ≥300 μM induced an increase in the EPSPs amplitude. The discrepancy between these studies may reflect a real difference between thalamus and hippocampus or may imply that GHB is still acting on presynaptic $GABA_B$ receptors in the hippocampal studies, 1 mM being necessary to fully block presynaptic $GABA_B$ receptors in this area (29).

Accordingly, Xie and Smart (74) showed a hyperpolarization and a small membrane conductance increase when GHB was applied to hippocampal CA1 pyramidal neurons; this effect being completely abolished by the $GABA_B$ antagonist, CGP 35348. Moreover, King et al. (46) revealed that GHB dose-dependently depresses the field potential (FP) *in vitro*. This action of GHB was depressed by a large concentration of 2-OH-saclofen, but not by NCS-382 at concentrations up to 5 mM. NCS-382 produces a concentration-dependent increase in the FP slope when applied alone. In addition, removal of NCS-382 brings about a relative fast, though partial, recovery of this effect, as even after 60 min washout of NCS-382 the FP remains elevated to about 40% of the control value. From this result the author claims that either NCS-382 is an inverse agonist at hippocampal GHB receptors or there is a tonic activation of GHB receptors in this area.

Only one study (2) has focused on the *in vivo* electrophysiological action of GHB and NCS-382 in the hippocampus. In this study NCS-382 (50 mg/kg i.p.) appears to slightly potentiate the GBL-induced decrease in the population spike (PS) in the CA1 region following electrical stimulation of the CA3 area in urethane-anesthetized mice. However, the action of the compound alone was not tested.

The effect of NCS-382 was also evaluated on thalamocortical (TC) neurons, the cells that receive sensory information from the periphery and provide the thalamic output to the cortex. This compound was unable to block a) the postsynaptic GHB hyperpolarization of rat and cat neurons (71) that, depending on the concentration, produces either an increase or a decrease in excitability (28) and b) a reduction of sensory EPSPs, cortical EPSPs and intrathalamic IPSPs. Both GHB-induced effects were blocked by $GABA_B$ antagonists. NCS-382 slightly potentiates these GHB-electrophysiological actions, showing effects that are consistent with it being a partial agonist of $GABA_B$ receptors. It is unlikely that this partial agonist action is the result of a direct action of NCS-382 on the pre- or

postsynaptic $GABA_B$ receptors, as NCS-382 does not bind to $GABA_B$ receptors (see section on binding studies). Alternatively, this additive action could be explained with its ability to inhibit competitively GHB-dehydrogenase and the subsequent availability of GHB.

In vivo studies revealed the ability of NCS-382 to block the GHB-induced spontaneous firing rate of prefrontal cortex neurons recorded in urethane-anesthetized rats (38). Conversely, NCS-382 failed to block electrophysiological GHB-induced effects in ventral tegmental area (49), and substantia nigra (32). Maitre's group has recently investigated the presence of a functional GHB system in differentiated clonal neurohybridoma NCB-20 cells (45, 50). In these cells, together with the presence of specific GHB binding sites, the authors described a GHB-mediated inhibition of Ca^{2+} currents that was fully reversed by NCS-382.

Finally, the electrophysiological response, induced by NCS-382 and GHB was recorded by patch clamp in CHO cells, expressing the cloned GHB receptor (3). As expected from the binding experiments carried out in these transfected cells, NCS-382 did not inhibit the GHB-induced response, indicating the existence of an NCS-382-sensitive and -insensitive GHB receptor.

Based on these data, it appears hazardous to establish with certaintly whether NCS-382 acts at GHB receptor as antagonist, inverse agonist or partial agonist.

Effects on GHB-Induced Absence Seizures

GHB and its prodrug GBL have the ability to induce absence-seizures in a number of species (64). GBL produces exactly the same EEG and behavioral effect as GHB and is used known for consistency and rapidity of onset of its effects. The mechanism by which GHB induces absence seizures is still a matter of debate. Although both GHB and $GABA_B$ receptors appear to be involved in the GHB-induced absence seizures, the role of GHB receptor is still unclear.

$GABA_B$ antagonists and the specific $GABA_B$ agonist baclofen abolish and induce absence-like seizures, respectively. Systemic injection of NCS-382 blocks the behavioral and EEG expression of absence seizures in GHB (62) and other models of absence epilepsy (2,52,62), though the effect of intrathalamic injection has not been investigated. However, some considerations argue against the effective antagonistic effect of NCS-382 at GHB receptors. First, NCS-382 is a competitive inhibitor of GHB-dehydrogenase, and the possibility remains that this compound blocks elicited absence seizures via inhibition of this enzyme and not via its presumed selective antagonism of GHB receptors. Second, t-HCA, a selective GHB ligand which is not a substrate for GHB-dehydrogenase (51) and does not bind to $GABA_B$ receptors (8,20), is unable to evoke absence-like seizures in control animals or to aggravate them in Genetic Absence Epilepsy Rats from Strasbourg (GAERS) when injected i.p. at doses even four-fold higher than GHB (30,65). Moreover, barbiturates, valproate, ethosuximide, trimethadione, inhibitors of GHB-dehydrogenase, block the conversion of GHB to GABA, causing the accumulation of GHB in the brain after *in vivo* GHB administration. Despite this accumulation, all these compounds showed antiepileptic effects, indicating that the anti-absence seizure properties of NCS-382 are due to induced inhibition of GHB dehydrogenase. Alos, GBL failed to induce EEG alterations typical of absence seizures induced by GHB $GABA_{B1}$ in subunit knockout mice (44).

Altogether these data suggest that the antagonistic effect of NCS-382 is not due to its direct antagonism at the GHB receptor.

BEHAVIORAL PHARMACOLOGY

Drug Discrimination Studies

Largely due to its pharmacological selectivity (39,68), the drug discrimination (DD) paradigm represents a widely used approach for the study of drugs with atypical mechanisms of action. A large number of studies have shown that a) drugs acting in a similar manner at a specific receptor possess similar discriminative stimulus (DS) effects and b) antagonists at a specific receptor block the discriminative stimulus effect of receptor agonists (39,58). Rats can discriminate GHB from saline, with GHB effects supposed to be selective, as pharmacologically unrelated drugs (e.g., phencyclidine, ketamine, ethanol, d-amphetamine) do not generalize with GHB (5,17,22,24,72). To date, only few reports have investigated the ability of the putative antagonist NCS-382 to antagonize the DS effects of GHB. Colombo et al. (22) demonstrated that this compound is able to block the DS effect of GHB in a T-maze, food–reinforced DD procedure. Two groups of rats were trained to run the left arm of the maze, 30 min later GHB, 300 or 700 mg/kg, was administered i.g., followed by water.

Once discrimination was acquired, a combination of different doses of NCS-382 and GHB training doses were tested for blockade of GHB discrimination. NCS-382 at doses of 25 and 50 mg/kg i.p. blocked GHB-appropriate responding in rats receiving both 300 and 700 mg/kg GHB. These data suggest that stimulation of GHB receptors constitutes a salient component of the GHB cue. However, in a subsequent study (23) the same author found that NCS-382 dramatically reduced alcohol absorption from the gastrointestinal system and, therefore, discrimination of i.g. administered alcohol. Thus, further studies are needed to verify whether blockade of the DS of GHB, administered i.g., by NCS-382 might be due to the reducing effect of NCS-382 on GHB absorption from the gastrointestinal tract. Recently, NCS-382 effect on the DS of GHB was investigated in rats and pigeons (17,47) using two-lever and two peck keys response, respectively, under a fixed ratio (FR) schedule of responding for food. Carter et al. (17) showed that in the rat small doses (10 to 32 mg/kg) of this compound partially attenuated GHB-lever responding; while larger doses (56 to 100 mg/kg) failed to antagonize the DS effects of GHB and, when administered alone, these doses elicited considerable responding on the GHB-associated lever. In a second study (47) pigeons were able to reliably discriminate GHB from vehicle, and their sensitivity to the DS and rate-decreasing effects of GHB did not markedly differ from that of rats. Indeed, in pigeons trained to discriminate 100 mg/kg GHB from saline, GHB, its precursors GBL and 1,4-butandiol (1,4-BD) produced 80 to 100% GHB-appropriate responding, while other compounds such as morphine, naltrexone, cocaine and haloperidol produced no more than 34%. NCS-382 did not block or attenuate such effects, but when given alone produced 70% GHB-appropriate responding at a dose (320 mg/kg) that also significantly decreased response rate (No. response/second). The GHB-like DS effects of NCS-382 were completely blocked by the $GABA_B$ antagonist CGP 35348, suggesting that $GABA_B$ receptors may be involved in this GHB-induced enhancement of DS.

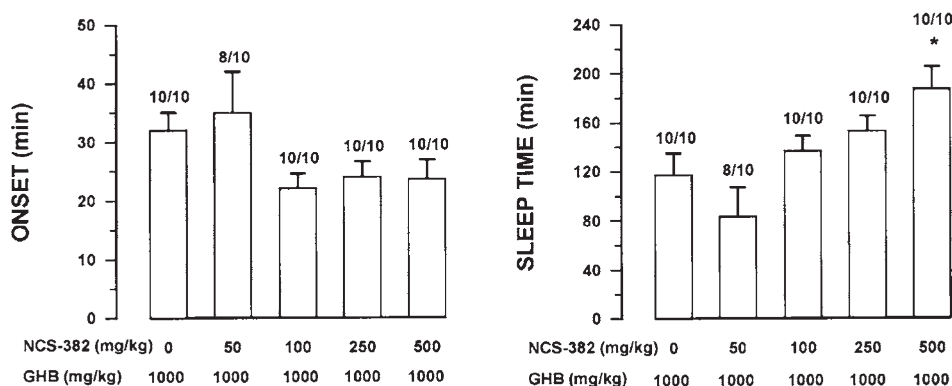

Fig. 5. Potentiation of the sedative/hypnotic effect of γ-hydroxybutyric acid (GHB) by the putative GHB receptor antagonist, NCS-382, in DBA mice. Left and right panels illustrate, respectively, the onset (time to lose the righting reflex) and sleep time (monitored as the time between loss and recovery of 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 left panel, each bar is the mean ± S.E.M. of the onset time required for mice to lose the righting reflex; in the right panel, each bar is the mean ± S.E.M. for the duration of the righting reflex of 10 mice. *$P < 0.05$ in comparison to 0 mg/kg NCS-382 plus 1000 mg/kg GHB group. Reproduced from ref. 16 with permission from Elsevier.

Effect on GHB-induced depressant behavioral effects

The first study performed to analyze the effects of NCS-382 on both sedation/anesthesia and catalepsy induced by GHB showed that this compound diminished, in a dose-dependent manner, such behavioral effects (60). In fact, NCS-382 completely abolished the GHB sedative/hypnotic action measured by a variety of sensimotor tests such as grasping, swimming, chimney, and cork tests. When injected alone, the compound produced no significant effect in any of the tests performed. However, recent studies aimed at investigating the ability of NCS-382 to antagonize the depressant behavioral effect of GHB were carried out using different procedures such as functional observation battery (FOB), schedule-controlled response and loss of righting reflex, and conflicting results were reported. The sedative/hypnotic effect of GHB and the ability of NCS-382 or $GABA_B$ receptor antagonists to revert this action were investigated in DBA mice by measuring onset of the loss of righting reflex and sleep time (16). The $GABA_B$ receptor antagonists completely prevented the GHB-induced sedative-hypnotic effect. In contrast, pretreatment with NCS-382 potentiated, instead of antagonizing, GHB-induced sedative/hypnotic effect (Fig. 5). Moreover, Cook et al. (27) showed that NCS-382 failed to convincingly antagonize in mice the depressant-like effect on learned and unlearned behavior. In this study, the behavioral action of GHB and the ability of NCS-382 to block this effect was examined a) in an FOB, a multi-variable, observational series of tests (i.e., forelimb grip strength, inverted screen, hind-limb splay and mean rearing in an open field); b) on locomotor activity; and c) as in a fixed-ratio food-maintained schedule of reinforcement. The FOB results obtained with GHB are similar to those of the depressants, pentobarbital and ethanol (12), suppressing locomotor activity and operant responses at doses >0.2 g/kg. In the FOB tests, with the exception of forelimb grip, NCS-382

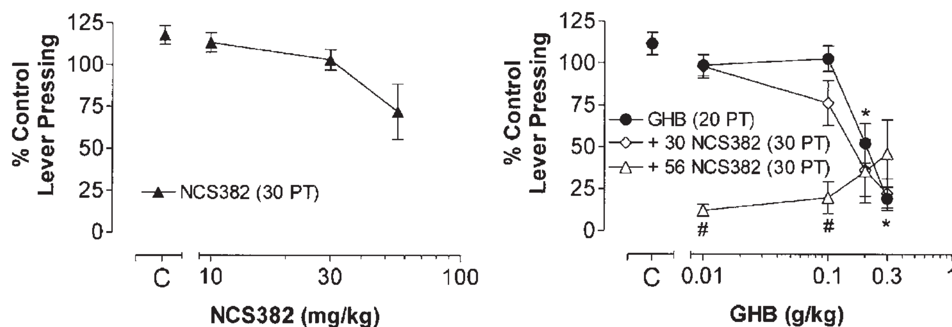

Fig. 6. Effects of NCS-382 alone (top panel, $n = 9$) and in combination with gammahydroxybutyrate (GHB; bottom panel, $n = 6–9$) on rate lever pressing. *Significant difference between vehicle and GHB; #significant difference between GHB alone and GHB plus NCS-382. Reproduced from ref. 27 with permission from Springer-Verlag GmbH.

(30 mg/kg) was ineffective in reversing the suppressant effect of GHB. Similarly, NCS-382 failed to block the GHB-induced depressant locomotor effect in the locomotor activity test. Only a dose of 30 mg/kg i.p. reversed this effect, indicating that the compound is not likely to be a competitive antagonist. In contrast, NCS-382 alone exhibits locomotor depressant activity. In the operant conditioning experiments there was no evidence of antagonism by NCS-382. As shown in Fig. 6, given alone the compound did not alter responding to saline control, while the co-administration of NCS-382 (30 and 56 mg/kg i.p.) enhanced the rate-suppressing effect of GHB (0.01–0.1 mg/kg) that alone had little effect. Accordingly, a subsequent study using schedule-controlled responding in rats revealed that NCS-382 did not attenuate the rate-decreasing effect of GHB; although larger doses of this compound further decreased the rate of responding when given in combination with GHB (18). The discrepancies between the results reported by Schmidt et al. (60) and those from the more recent studies are difficult to explain. These conflicting results might be attributed to different animals (rats or mice), different NCS-382 doses and/or different types of sensory-motor tasks employed. Furthermore, as revealed by all these behavioral data, NCS-382 not only fails to antagonize GHB-induced discriminative stimulus, inhibition of locomotor activity, ataxia and suppression of operant responding, but is also capable of eliciting qualitatively similar effects to those of GHB or enhancing some actions of GHB.

Effect on GHB-self-administration

To date, the only study investigating the effect of NCS-382 on GHB intravenous self-administration (SA) was carried out by Martellotta et al. (53). Drug-naïve mice were allowed to self-administer GHB (0.01–0.5 mg/kg/injection) for 30 min, under a continuous (FR1) schedule of reinforcement and nose-poking as operandum. As shown in Fig. 7 GHB is acutely self-administered by mice according to a concentration-dependent bell-shaped curve. More specifically, concentrations of 0.05 and 0.1 mg/kg/injection maintained a significant self-administration behavior and were considered to possess reinforcing properties, the lowest (0.01 mg/kg/injection) and the highest (0.5 mg/kg/injection) doses of GHB being unable to sustain responding. It is important to point out that

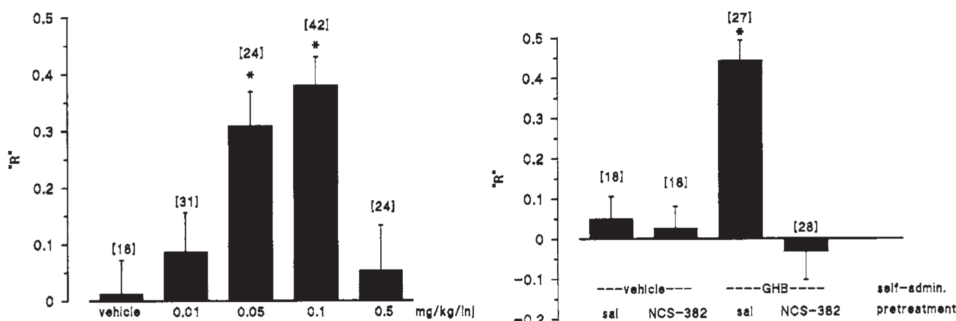

Fig. 7. Concentration-dependent GHB self-administration and antagonism of GHB self-administration by NCS-382. Left: each bar represents $R \pm$ S.E.M. for each GHB concentration. Number of pairs are indicated in parentheses. $^*P < 0.01$, the Newman–Keuls test. Right: each bar represents $R \pm$ S.E.M. for each group self-administering either vehicle or GHB (0.1 mg/kg/injection). Mice were pretreated either with saline or NCS-382 (12.5 mg/kg i.p. 10 min prior to the i.v. self-administration test). $^*P < 0.01$, the Newman–Keuls test. R, index of reinforcing as described by Martellotta et al. (53) and Kuzmin et al. (48). Reproduced from ref. 53.

NCS-382, at 12.5 mg/kg, did not affect spontaneous motor activity in these animals, but completely prevented GHB (0.1 mg/kg/injection) self-administration (Fig. 7 right).

GENERAL PHARMACOLOGY

Effect on Gastric and Intestinal Motility

Recently, the influence of GHB and NCS-382 on gastric emptying and intestinal mobility was investigated in rats and in mice, respectively (15,59). Poggioli et al. (59) found that GHB at 100 mg/kg p.o. stimulated gastric emptying in rats. NCS-382 not only completely prevented the effect of GHB, but also displayed a strong, dose-dependent inhibitory effect on gastric emptying. These data suggest that GHB and NCS-382 effects are mediated either by the stimulation or blockade of GHB receptors. In the publication by Carai et al. (15) the effect of acutely administered GHB and NCS-382 on the propulsive activity in the mouse small intestine was assessed by measuring the transit of an orally administered non-absorbable marker (carmine). Administration of 25 to 300 mg/kg i.p. GHB resulted in a dose-dependent reduction of the propulsive activity in the mouse small intestine in comparison to saline-treated mice. As shown in Fig. 8, administration of 25 to 75 mg/kg of NCS-382 i.p. elicited a similar effect, dose-dependently decreasing the propulsive activity in the small intestine. Indeed, pretreatment with the $GABA_B$ antagonist SCH 50,911 resulted in a blockade of the inhibitory effects of both, GHB and NCS-382. Thus, NCS-382 appears to act through a different mechanism on different areas of the gastrointestinal tract; a) as an antagonist at GHB receptors in the stomach and b) through a $GABA_B$ receptor-mediated mechanism in the small intestine.

SUMMARY AND CONCLUSIONS

In conclusion, NCS-382 is a stereoselective ligand for GHB-binding sites, with both the high and the low component of population showing the same distribution of GHB re-

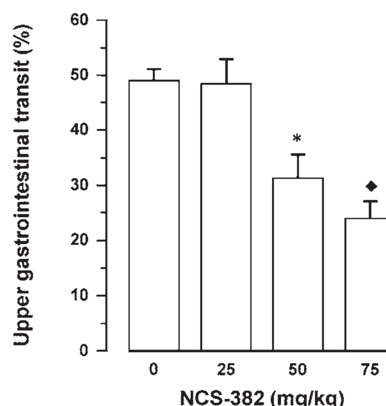

Fig. 8. Effect of the γ-hydroxybutyric acid (GHB) receptor antagonist, NCS-382, on propulsive activity in the small intestine. NCS-382 was administered i.p. 20 min prior to the i.g. administration of the non-absorbable marker, carmine. Twenty minutes later, mice were killed and the distance travelled by the head of the marker, between the pylorus and the cecum, was measured and expressed as percent of total length of the small intestine. Each bar is the mean ± S.E.M. of 10–15 mice. $^*P < 0.005$ with respect to saline-treated mice (the Newman–Keuls test). Reproduced from ref. 15 with permission from Elsevier.

ceptors. Indeed, this compound *in vitro* did not display affinity for $GABA_A$, $GABA_B$, or any other known receptors. However, although NCS-382 does not bind to $GABA_B$ receptors (21,51,66), a number of unresolved issues relate to its "selectivity": a) the antagonism of GHB-dehydrogenase by NCS-382 is still a matter of controversy (8,42,55), the possibility of a GHB-derived GABA pool should be considered when evaluating the pharmacological properties of NCS-382; b) in the cerebellum, where no GHB receptors are present (19,51), doses of NCS-382 similar to those that block other *in vivo* pharmacological actions of GHB, markedly decrease cGMP by an action additive to GBL; and c) NCS-382 showed partial/inverse agonism both in electrophysiological (46,71) and in behavioral studies. Furthermore, NCS-382 not only fails to antagonize the depressant and discriminative stimulus induced by GHB but, depending on the doses employed, enhances these effects. The likelihood of obtaining antagonism of GHB with NCS-382 appears to depend on doses as well as behavioral endpoints, due to the fact that NCS-382 might be an antagonist at GHB receptor and an agonist at $GABA_B$ receptor. Thus, NCS-382-induced GHB-like DS (47) and GHB-like effect on intestinal mobility in mice are blocked by $GABA_B$ receptors (15). These actions of NCS-382 at $GABA_B$ receptor might be due to a) indirect action, such as inhibition of GHB-dehydrogenase and subsequent accumulation of GHB, b) the formation *in vivo* of NCS-382 metabolites that could be active at $GABA_B$ receptors. Finally, since NCS-382 fails to bind to the recently cloned GHB receptor (3), the incomplete nature of the antagonism of NCS-382 might be due to different classes of GHB receptors, both sensitive and insensitive to NCS-382. In conclusion, NCS-382 is a good ligand, but not a selective antagonist, for GHB receptor.

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ADDENDUM. Chemical names

CGP 35348, (3-Aminopropyl)(diethoxymethyl) phosphinic acid;

CGP 55845, (2S)-3-[(1S)-1-(3,4–dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl) phosphinic acid;

SCH 50911, (2S)-(+)-5,5-dimethyl-2-morpholineacetic acid;

CGP 7930, 3,5-bis-(1,1-dimethylethyl)-4-hydroxy-β,β-dimethyl-benzenepropanol;

NCS-435, (γ-(p-methoxybenzyl)-γ-hydroxybutyric acid;
NCS-356, γ-p-chlorophenyl-trans-4-hydroxycrotonic acid.

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