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Stereoselectivity of NCS-382 binding to γ-hydroxybutyrate receptor in the rat brain

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Abstract

γ-Hydroxybutyric acid (GHB), a naturally occurring metabolite of γ-aminobutyric acid (GABA), has been postulated to act both as a specific agonist of GHB receptors and as a weak $GABA_B$ receptor agonist. The racemic compound 6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylideneacetic acid (*RS*-NCS-382), the only available antagonist of GHB receptors, has been resolved in two enantiomers, *R*- and *S*-; the potency of the latter to displace 4-hydroxy [2-3-^{3}H] butyric acid ([^{3}H]GHB) and [^{3}H]NCS-382 from GHB receptors, on one hand, and [^{3}H]baclofen from $GABA_B$ receptors on the other was compared in rat brain homogenates. *R*-NCS-382 was found to be twice and 60 times more potent than the *RS*- and *S*-forms, respectively, in displacing [^{3}H]GHB and 2 and 14 times, respectively, in displacing [^{3}H]NCS-382 from GHB binding. Neither *RS*-NCS-382 nor its enantiomers inhibited [^{3}H]baclofen binding up to a concentration of 1 mM. Our results demonstrate that *R*-NCS-382 is the enantiomer of *RS*-NCS-382 with higher affinity for GHB receptors.

Keywords: GHB (gamma-hydroxybutyrate) binding site; *RS*-NCS-382; *R*-NCS-382; *S*-NCS-382; Baclofen; $GABA_B$ receptor

1. Introduction

Gamma-hydroxybutyric acid (GHB) is an endogenous constituent of the mammalian brain, mostly deriving from γ-aminobutyric acid (GABA) metabolism (Maitre, 1997; Feigenbaum and Howard, 1996). Specific mechanisms of synthesis, release and uptake and specific binding sites are present in discrete areas of the mammalian, including human brain (Castelli et al., 2000), suggesting that GHB may function as neurotransmitter or neuromodulator (see Maitre, 1997; Bernasconi et al., 1999).

Exogenously administered GHB produces a number of effects including anxiolytic, anesthetic, sedative/hypnotic effect in humans (Laborit, 1964) and in laboratory rodents (e.g. Carai et al., 2001), generalized absence seizures in rats (Hu et al., 2000), discriminative stimulus effects in rats (Colombo et al., 1998), inhibition of intestinal motility (Carai et al., 2002), changes in the activity of different neurotransmitters (Erhardt et al., 1998; Madden and Johnson, 1998), stimulation of brain dopamine synthesis (Gessa et al., 1968), etc. (see Maitre, 1997; Bernasconi et al., 1999). While the majority of GHB effects are antagonized by the $GABA_B$ receptor antagonists, suggesting that they are mediated by $GABA_B$ receptors (see Bernasconi et al., 1999), a number of responses such as GHB self-administration in mice (Martellotta et al., 1998), GHB-induced glutamate release (Ferraro et al., 2001), inhibition of the excitatory postsynaptic potentials mediated by *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA/Kainate) receptors in rat hippocampal slices (Berton et al., 1999), increase of cGMP levels and inositol phosphate turnover in hippocampus, are selectively antagonized by the GHB receptor antagonist 6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylideneacetic acid (NCS-382) (Maitre et al., 1990), suggesting that they are specifically mediated by GHB receptors.

Finally, a number of GHB effects, such as sedation and anesthesia, stimulation of brain dopamine synthesis and absence seizures are inhibited indifferently by either NCS-382 or $GABA_B$ antagonists (Maitre et al., 1990; Bernasconi

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et al., 1992; Waldmeier, 1991; Nissbrandt and Engberg, 1996; Banarjee and Snead, 1995). Since NCS-382 does not interact directly with $GABA_B$ receptors, to explain its ability, as well as that of $GABA_B$ receptor antagonists, to mutually antagonize GHB responses, it might be suggested that GHB activates $GABA_B$ receptors both directly, at high concentrations, and indirectly via a GHB receptor and, conversely, that NCS-382 inhibits GHB effect, acting at the GHB allosteric site. Accordingly, nanomolar and micromolar concentrations of GHB displace [^{3}H]GHB from its specific high- and low-affinity binding sites in brain, respectively, while higher millimolar concentrations of GHB have been shown to displace [^{3}H]baclofen from $GABA_B$ receptors in brain membranes (Mathivet et al., 1997) and to activate recombinant $GABA_B$ receptors in *Xenopus laevis* oocytes (Lingenhoehl et al., 1999).

At present, NCS-382, a structural analog of GHB, is the only compound reported to be an antagonist of the GHB receptor sites (Maitre et al., 1990). NCS-382 has been shown to displace [^{3}H]GHB from both the high- and low-affinity binding sites (Maitre et al., 1990; Castelli et al., 2000). A recent study (Mehta et al., 2001) using radiolabeled NCS-382 has revealed the presence in the rat cerebral cortex and hippocampus of specific binding sites, which are completely inhibited by GHB and NCS-382, but are not influenced by a variety of ligands for other receptors. NCS-382 is a racemic mixture of two enantiomers, *R*- and *S*-NCS-382, that have been recently separated in our laboratory. With the aim of determining the active form of NCS-382 (*RS*-NCS-382), with respect to GHB receptor binding, the interaction of the racemic compound and its two enantiomers *R*- and *S*-NCS-382 with GHB receptors has been investigated. The interaction with $GABA_B$ receptors has also been studied. This study was carried out in rat cortical membranes using [^{3}H]GHB and [^{3}H]NCS-382 as radioligands for GHB receptors and [^{3}H]baclofen for $GABA_B$ ones.

2. Materials and method

2.1. Drugs

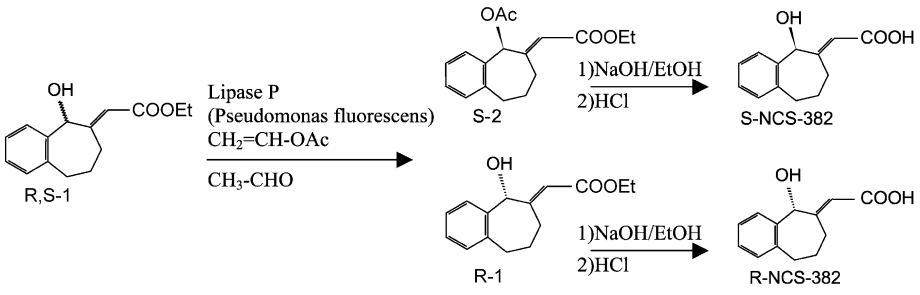
GHB sodium salt was purchased from Sigma (St. Louis, MO, USA); *R*-(−)-baclofen and the $GABA_B$ antagonist SCH 50,911 from Tocris (Bristol, UK).

[^{3}H]GHB sodium salt (60 Ci/mmol), [^{3}H]NCS-382 (20 Ci/mmol) and [^{3}H]baclofen (38.7 Ci/mmol) were obtained from ARC (St. Louis, MO, USA) and New England Nuclear (NEN), Boston, respectively. The synthesis of *RS*-NCS-382 and its *R*- and *S*-enantiomers was performed by Giorgio Cignarella, Daniela Berta and Arianna Gelain. The resolution of racemic NCS-382 (Scheme 1) was performed by the enzymatic enantioselective acetylation of the hydroxyl group in 5-position. Lipase P "Amano" from *Pseudomonas fluorescens* turned out to be the only effective enzyme among other lipases in the transesterification of vinyl acetate with racemic NCS-382 ethyl ester (Burgess et al., 1991). The conversion of NCS-382 into the ethyl ester 1 was necessary because the acid as such was not reactive toward the enzyme in the same conditions. The reaction is irreversible because the enol, produced by vinyl acetate, is immediately transformed into acetaldehyde. The enzyme recognized the *S*-enantiomer of NCS-382 ester and the mixture of the diester *S*-2 and the monoester *R*-1 was separated by flash chromatography. Thus, hydrolysis of each ester gave the two enantiomers of NCS-382 with an enantiomeric excess higher than 98% measured by HPLC analysis. The absolute configuration of *S*-NCS-382 and indirectly of *R*-NCS-382 was assigned by crystallographic analysis.

2.2. Tissue preparation

Male Sprague–Dawley rats (Charles River, Como, Italy), weighing 200–250 g, were used in all experiments and were maintained on ad libitum food and water. Rats were killed by decapitation, their brains rapidly removed and cerebral cortices dissected on ice.

Cortical tissue was homogenized using an homogenizer system (Glas-Col, Terre Haute, IN, USA) in 20 volumes (v/w) tissue of ice-cold 0.32 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant collected and recentrifuged at 20,000 × *g* for 20 min. The pellet was resuspended in 20 volumes (v/w) of ice-cold water, homogenized using a Polytron homogenizer and centrifuged at 8000 × *g* for 20 min. The supernatant together with the buffy layer on the pellet was then centrifuged at 45,000 × *g* for 20 min. The resulting pellet was resuspended in ice-cold distilled water and once more centrifuged at 45,000 × *g* for 30 min. The



Scheme 1.

final pellet was frozen and stored at −80 °C for at least 18 h before use for binding assay.

2.3. [^{3}H]NCS-382 binding assay

Membranes were prepared as previously described (Mehta et al., 2001). For binding, aliquots of (150–200 μg of protein) membranes preparation in Tris buffer (50 mM, pH 7.4) were incubated with [^{3}H]NCS-382 (16 nM) in triplicate at 4 °C for 20 min in 1 ml of volume. Nonspecific binding was determined using NCS-382 (1 mM). The reaction was terminated by rapid filtration through Whatmann GF/B glass filters using a Brandell 96-sample harvester (Gaithesburg, MD, USA). Filters were then rinsed twice with ice-cold 50 mM Tris–HCl buffer (pH 7.4).

[^{3}H]NCS-382 displacement curves were carried out using serial dilutions ranging from 10^{-9} to 10^{-3} M of the unlabelled compounds and [^{3}H]NCS-382 at 16 nM (20 Ci/mmol).

2.4. [^{3}H]baclofen and [^{3}H]GHB binding assay

For binding assay, membrane pellets were allowed to thaw at 4 °C before resuspension in 20 volumes (v/w) of both 50 mM KH_2PO_4 buffer (pH 6.5) containing 1 mM EDTA and 50 mM Tris–HCl, pH 7.4, for [^{3}H]GHB and [^{3}H]baclofen, respectively. The suspension was incubated for 20 min at 20 °C before centrifugation at 7000 × *g* for 10 min. The washing step was repeated three more times allowing 15-min incubation with each addition of the same buffer to remove the endogenous ligand GABA or GHB. The final pellet was then resuspended in appropriate binding buffer to a final concentration of 200–300 μg of membrane protein for both [^{3}H]GHB and [^{3}H]baclofen, respectively. The incubation buffer used was in (mM): 50 Tris–HCl, pH 7.4, 2.5 $CaCl_2$, or 50 KH_2PO_4, pH 6.5, 1 EDTA for [^{3}H]baclofen and [^{3}H]GHB, respectively. [^{3}H]GHB binding assay was performed in triplicate in a volume of 0.6 ml at 4 °C for 30 min. Nonspecific binding was estimated in the presence of 1 mM unlabelled GHB. In both binding assays, free ligand was separated from bound ligand by rapid filtration through Whatmann GF/B glass filters using a Brandell 96-sample harvester. Filters were then rinsed twice with either ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 2.5 mM $CaCl_2$, or 50 mM KH_2PO_4 buffer (pH 6.5) for [^{3}H]baclofen and [^{3}H]GHB, respectively. Filter-bound radioactivity was counted in a liquid scintillation counter (Packard Tricarb 1600), using 3 ml of scintillation fluid (Packard Ultima Gold MV).

Table 1
Inhibition of [^{3}H]GHB and [^{3}H]NCS-382 binding by *RS*-NCS-382, *R* and *S* enantiomers

Compounds	[^{3}H]GHB (IC_{50} μM)	[^{3}H]NCS-382 (IC_{50} μM)
RS-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.

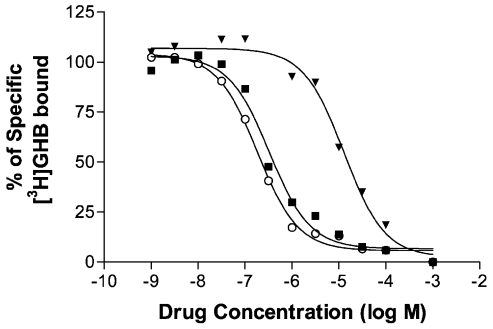


Fig. 1. Displacement curves of [^{3}H]GHB by *RS*-NCS-382 and its enantiomers *R* and *S*. Binding experiments were performed as described in Section 2. The amount of radioactive ligand ([^{3}H]GHB; 10 nM, sp. act. 60 Ci/mmol) was chosen in order to label almost exclusively the high-affinity GHB binding site. *RS*-NCS-382 IC_{50}: 0.40 μM (■); *R*-NCS-382 IC_{50}: 0.20 μM (○); *S*-NCS-382 IC_{50}: 15.14 μM (▲). Data represent a typical experiment out of three independent experiments. Statistical fitting was obtained by nonlinear regression using Graphpad Prism program.

[^{3}H]GHB and [^{3}H]baclofen displacement curves were carried out using serial dilutions ranging from 10^{-9} to 10^{-3} M of the unlabelled compounds and either [^{3}H]GHB (10 or 60 nM) or [^{3}H]baclofen (20 nM). Independent experiments were repeated on membrane preparations from at least three different brains.

The Bradford (1976) protein assay was used for protein determination using bovine serum albumin as a standard according to the protocol of the supplier (Bio-Rad, Milan, Italy).

The calculation of IC_{50} (concentration which inhibits 50% of specific radioligand binding) was performed by nonlinear curve fitting of the concentration–effect curves using GraphPad Prism Program, San Diego, CA. The *F*-test was used to determine the best approximation of a nonlinear curve fitting to one or two site model ($P < 0.05$).

3. Results

In agreement with previous studies (Castelli et al., 2000; Maitre et al., 1990), by using a 60-nM concentration of [^{3}H]GHB as radioligand, *RS*-NCS-382 displaced two populations of binding sites, one at high and the other at low affinity, with an IC_{50} of 400 nM and 4 μM, respectively (data not shown). The displacing potency of *RS*-NCS-382 and its enantiomers against [^{3}H]GHB and [^{3}H]NCS-382 was compared (Table 1). A concentration of 10 nM of [^{3}H]GHB (60 Ci/mmol) was used to selectively bind the high-affinity site of the GHB receptor. Displacement curves indicated that *RS*-, *R*- and *S*-NCS-382 displaced [^{3}H]GHB from the high-

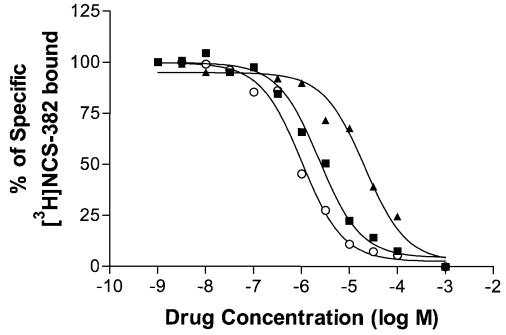


Fig. 2. Displacement curves of [^{3}H]NCS-382 by *RS*-NCS-382 and its enantiomers *R* and *S*. Binding experiments were performed as described in Section 2. The IC_{50} values were calculated from displacement curves using 16 nM of [^{3}H]NCS-382 (sp. act. 20 Ci/mmol). Data represent a typical experiment out of three independent experiments. *RS*-NCS-382 IC_{50}: 2.35 μM (■); *R*-NCS-382 IC_{50}: 0.99 μM (○); *S*-NCS-382 IC_{50}: 21 μM (▲). Statistical fitting was obtained by nonlinear regression using Graphpad Prism program.

affinity site with an IC_{50} of 0.4, 0.2 and 15.14 μM, respectively (Fig. 1). On the other hand, the specific activity of the commercially available [^{3}H]NCS-382 (20 Ci/mmol of the racemic compound) was insufficient to selectively detect the high-affinity binding site of the GHB receptor, which represents only 6–10% of total binding. Therefore, in the displacement studies, a 16-nM concentration of [^{3}H]NCS-382 was used to label mostly the predominant low-affinity site. Accordingly, *RS*-, *R*- and *S*-NCS-382 displaced [^{3}H]NCS-382 with an IC_{50} of 2.35, 0.99 and 21 μM, respectively (Fig. 2).

As shown in Table 2, neither *RS*-NCS-382 nor its enantiomers displaced [^{3}H]baclofen binding up to a concentration of 1 mM (Table 2).

As expected, (2*S*)(+)-5,5-dimethyl-2-morpholineacetic acid (SCH 50,911), a specific $GABA_B$ receptor antagonist, inhibited [^{3}H]baclofen with an IC_{50} of 2 μM, as previously reported (Bolser et al., 1995) (data not shown).

4. Discussion

A recent study (Mehta et al., 2001) has reported that the radiolabeled *RS*-NCS-382 binds to the same binding sites as GHB and has the same tissue distribution of [^{3}H]GHB. The present study shows that *R*-NCS-382 is the active enantiomer of NCS-382 with regard to GHB receptor binding. Indeed the displacement ability of NCS-382 is stereoselective regardless of the radioligand used to label GHB-binding sites. Thus, *R*-NCS-382 is twice as potent as the racemic form and 62-fold more potent than the *S*-enantiomer in displacing [^{3}H]GHB from GHB binding sites. On the other hand, the *R*-enantiomer is 2- and 13-fold more potent than the racemic and *S*-form, respectively, when using [^{3}H]NCS-382 as radioligand. The fact that *R*- and *RS*-NCS-382 were about 5-fold more potent in displacing [^{3}H]GHB than [^{3}H]NCS-382 from the GHB receptor might be explained with the fact that [^{3}H]GHB and [^{3}H]NCS-382, at the concentrations used in the 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 (20 Ci/mmol of the racemic compound) is insufficient to detect the high -affinity site, which constitutes less than 10% of the total binding site. Therefore, at the 16 nM concentration used in the displacement experiments, [^{3}H]NCS-382 should have labeled the predominant low-affinity site of the GHB receptor, as previously reported by Mehta et al. (2001). According to this interpretation, Scatchard analysis of saturation isotherms of [^{3}H]NCS-382 binding has indicated two populations of binding sites (Mehta et al., 2001), similarly to observations made in displacement experiments using [^{3}H]GHB as radioligand and NCS-382 as a displacer (Castelli et al., 2000; Maitre et al., 1990). While GHB has been shown to displace, with low-affinity, both [^{3}H]baclofen, and [^{3}H] 3-aminopropylphosphinic acid ([^{3}H]CGP 27,492) from $GABA_B$ receptors in cortical, thalamic and hippocampal membranes (Mathivet et al., 1997; Bernasconi et al., 1992), the present results show that none of the NCS-382 enantiomers are effective in displacing [^{3}H]baclofen from $GABA_B$ binding, up to a concentration of 1 mM. These results are in accordance with the observations of Lingenhoehl et al. (1999) that NCS-382 has no effect on the K^+ inward currents produced by GHB via recombinant $GABA_B$ receptors expressed in *X. laevis* oocytes.

The fact that GHB affinity for $GABA_B$ receptors is within the range of brain concentrations obtained after administration of pharmacologically active doses of the drug, and the majority of effects produced by systemically administered GHB are inhibited by $GABA_B$ receptor antagonists, indicates that the GHB effects are mediated by $GABA_B$ receptors. However, since a number of GHB effects such as sedation and anesthesia (Schmidt et al., 1991; Carai et al., 2001), increase in dopamine synthesis rate (Waldmeier, 1991; Maitre et al., 1990), and induction of absence-like seizures (Bernasconi et al., 1992; Hu et al., 2000) are inhibited interchangeably by NCS-382 and $GABA_B$ receptor antagonists, while NCS-382 does not directly interact

Table 2
Effect of *RS*-NCS-382, *R* and *S* enantiomers on [^{3}H]baclofen binding

Compounds	*n*	Percent change
RS-NCS-382, 0.1 mM	4	3.00 ± 0.45
RS-NCS-382, 1 mM	4	−4.25 ± 0.33
R-NCS-382, 0.1 mM	5	2.26 ± 0.41
R-NCS-382, 1 mM	4	−4.63 ± 0.45
S-NCS-382, 0.1 mM	5	2.63 ± 0.23
S-NCS-382, 1 mM	3	3.43 ± 0.39

Each value is means ± S.E.M. of number of individual experiments indicated (*n*), and each experiment was performed in triplicate. None of the compounds had a significant effect on [^{3}H]baclofen binding (20 nM, sp. act. 38.7 Ci/mmol) in the rat cerebral cortex membranes.

with $GABA_B$ receptors, it might be suggested that NCS-382 may inhibit GHB effect at an allosteric binding site where GHB acts as a positive modulator of $GABA_B$ receptors. The use of the active and inactive enantiomers of NCS-382 might help to further clarify this problem, to determine which effects of GHB are stereospecifically inhibited by NCS-382 and, conversely, to determine which effects of GHB are specifically mediated by the GHB receptor.

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