

Novel Cyclic γ -Hydroxybutyrate (GHB) Analogs with High Affinity and Stereoselectivity of Binding to GHB Sites in Rat Brain[Ⓢ]

Petrine Wellendorph, Signe Høg, Jeremy R. Greenwood, Anne de Lichtenberg, Birgitte Nielsen, Bente Frølund, Lotte Brehm, Rasmus P. Clausen, and Hans Bräuner-Osborne

Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, Copenhagen, Denmark

Received June 3, 2005; accepted July 7, 2005

ABSTRACT

γ -Hydroxybutyrate (GHB) is a psychotropic compound endogenous to the brain. Despite its potentially great physiological significance, its exact molecular mechanism of action is unknown. GHB is a weak agonist at GABA_B receptors, but there is also evidence of specific GHB receptor sites, the molecular cloning of which remains a challenge. Ligands with high affinity and specificity for the reported GHB binding site are needed for pharmacological dissection of the GHB and GABA_B effects and for mapping the structural requirements of the GHB receptor-ligand interactions. For this purpose, we have synthesized and assayed three conformationally restricted GHB analogs for binding against the GHB-specific ligand [³H]NCS-382 [(*E,RS*)-(6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylidene)acetic acid] in rat brain homogenate. The cyclohexene and cyclopentene analogs, 3-hydroxycyclohex-1-enecarboxylic

acid [(*RS*)-HOCHCA] and 3-hydroxycyclopent-1-enecarboxylic acid [(*RS*)-HOCPCA], were found to be high-affinity GHB ligands, with IC₅₀ values in the nanomolar range, and had 9 and 27 times, respectively, higher affinity than GHB. The stereoselectively synthesized *R,R*-isomer of the *trans*-cyclopropyl GHB analog, HOCPrCA, proved to have 10-fold higher affinity than its enantiomer. Likewise, the *R*-enantiomers of HOCHCA and HOCPCA selectively inhibited [³H]NCS-382 binding. The best inhibitor of these, (*R*)-HOCPCA, has an affinity 39 times higher than GHB and is thus among the best GHB ligands reported to date. Neither of the cycloalkenes showed any affinity (IC₅₀ > 1 mM) for GABA_A or GABA_B receptors. These compounds show excellent potential as lead structures and novel tools for studying specific GHB receptor-mediated pharmacology.

γ -Hydroxybutyrate (GHB) is a naturally occurring substance in the mammalian brain, where it is currently believed to function as a neurotransmitter or neuromodulator (Bernasconi et al., 1999). GHB is a registered drug for the treatment of cataplexy associated with narcolepsy (Fuller and Hornfeldt, 2003) and has also displayed therapeutic potential for treating drug and alcohol dependence (Gallimberti et al., 2000). Furthermore, GHB is a drug of abuse (Wong et al., 2004), producing mild euphoria, muscle relaxation, seda-

tion, and eventually coma with increasing dosage. Despite the obvious biological and pharmacological importance of GHB, its exact mechanism of action remains elusive.

Most of the reported GHB effects are mediated through the GABA system, in particular GABA_B receptors. GHB has been shown to be a low-affinity (Mathivet et al., 1997) weak partial agonist at GABA_B receptors (Lingenhoehl et al., 1999) and may also be converted into GABA in vivo and thus affect GABA receptors indirectly (Hechler et al., 1997). However, based on recent data, it would also seem that specific GHB receptor-mediated effects exist (Castelli et al., 2003; Kemmel et al., 2003; Brancucci et al., 2004). This, in combination with the presence of high-affinity [³H]GHB binding sites in the brain (Benavides et al., 1982), suggests the existence of a distinct GHB receptor. In further support of this view, brains from GABA_{B(1)} receptor knock-out mice still exhibit [³H]GHB binding (Kaupmann et al., 2003; Wu et al., 2004), demon-

This work was supported by the Danish Medical Research Council, Apotekerfonden of 1991, European Union Grant HPAW-CT-2002-80057 (to P.W.), the Lundbeck Foundation, the computer resources of the Danish Center for Supercomputing, and the Australian Centre for Advanced Computing and Communications.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.105.090472.

[Ⓢ] The online version of this article (available at <http://jpet.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: GHB, γ -hydroxybutyrate; [³H]NCS-382, (*E,RS*)-(6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylidene)acetic acid; HOCPCA, 3-hydroxycyclopent-1-enecarboxylic acid; T-HCA, *trans*-4-hydroxycrotonic acid; HOCHCA, 3-hydroxycyclohex-1-enecarboxylic acid; HOCPrCA, *trans*-2-(hydroxymethyl)cyclopropane carboxylic acid; OxGPCA, 3-oxocyclopent-1-enecarboxylic acid; ee, enantiomeric excess.

strating that GHB and GABA binding sites are separate entities. NCS-382, a synthetic structural analog of GHB and a purported antagonist of the GHB receptor (Castelli et al., 2004), has been shown to compete with [^3H]GHB for the high-affinity sites. Radioligand binding studies have demonstrated [^3H]NCS-382 to be selective for GHB binding sites, making it a valuable tool for probing the putative GHB receptor (Mehta et al., 2001). One recent report claimed the cloning of a GHB-specific receptor yet failed to show affinity of NCS-382 for the receptor (Andriamampandry et al., 2003). Furthermore, the *in situ* hybridization expression pattern of this claimed receptor in rat brain showed labeling in the cerebellum, whereas previous autoradiography studies performed with [^3H]GHB and [^3H]NCS-382 showed negligible binding in this region of the brain (Snead, 1996; Gould et al., 2003). The dual action of GHB is a major obstacle when studying GHB receptor-mediated effects and accentuates the need for potent and selective ligands. Also, higher affinity compounds could aid the molecular cloning of GHB receptors, which remains the most prominent goal of this line of research.

So far, the structural requirements of GHB binding sites are relatively unexplored (Bourguignon et al., 2000; Macias et al., 2004). Most attempts have been to generate GHB-selective compounds that do not undergo metabolism to GABA-ergic compounds. Albeit the high value for *in vivo* studies, these compounds have at best been equipotent with GHB (Wu et al., 2003; Carter et al., 2005). In addition, the conformational flexibility of previous ligands and limited stereospecificity data associated with them would hamper any attempt to map the GHB receptor pharmacophore. Therefore, we set out to synthesize a series of conformationally restricted GHB analogs, with close attention to stereochemistry. Herein, we report the synthesis and pharmacological evaluation of the enantiomers of three highly selective GHB ligands. To our knowledge, one of these compounds, (*R*)-3-hydroxycyclopent-1-enecarboxylic acid [(*R*)-HOCPCA], is one of the most high-affinity GHB receptor ligand reported to date (structure is shown in Fig. 1).

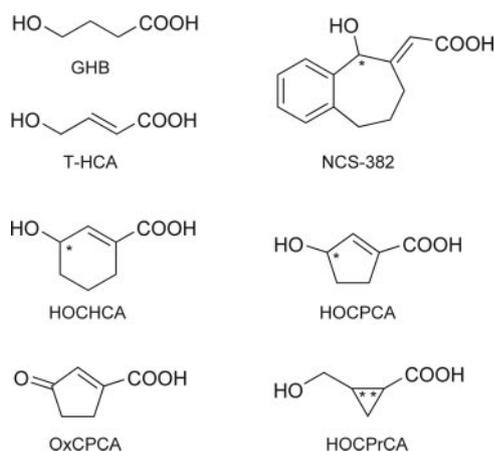


Fig. 1. GHB, the reference compounds T-HCA and NCS-382, and the novel GHB analogs HOCHCA, HOCPCA, OxCPCA, and HOCPPrCA are shown. Chiral carbon atoms are marked by asterisk.

Materials and Methods

Compounds and Radioligands

GHB sodium salt, *trans*-4-hydroxycrotonic acid (T-HCA), GABA, and (*R*)-baclofen were purchased from Sigma-Aldrich (St. Louis, MO); NCS-382 was from Tocris Cookson Ltd. (Bristol, UK). [^3H]NCS-382 (20 Ci/mmol) was obtained from ARC (St. Louis, MO). [^3H]GABA (27.6 Ci/mmol) and [^3H]muscimol (28.5 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

Synthesis

The racemic compounds (*RS*)-3-hydroxycyclopent-1-enecarboxylic acid [(*RS*)-HOCPCA] and (*RS*)-3-hydroxycyclohex-1-enecarboxylic acid [(*RS*)-HOCHCA] were synthesized in several steps (Supplemental Data, Scheme 1). Ethyl 2-oxocyclopentanecarboxylate and ethyl 2-oxocyclohexanecarboxylate were converted into the respective unsaturated esters (Beagley et al., 1989; Palaty and Abbott, 1995). Allylic oxidation of the unsaturated ethyl esters with CrO_3 by a procedure similar to that described previously for the methyl esters (Lange and Otulakowski, 1982; Lange et al., 1989) gave the respective enone esters (Mori, 1978). These were converted by selective reductions (Gemal and Luche, 1981; Cossy et al., 1995) with the aid of $\text{NaBH}_4\text{-CeCl}_3$ to the respective allylic alcohols. Treatment with aqueous Na_2CO_3 gave the unsaturated hydroxy acids. Finally, these were resolved by chiral high-performance liquid chromatography using a ChiralPak AS-H column (Daicel Chemical Industries, Tokyo, Japan) into the pure enantiomers (*S*)-HOCPCA (98.1% ee) and (*R*)-HOCPCA (97.6% ee) as well as (*S*)-HOCHCA (97.6% ee) and (*R*)-HOCHCA (96.4% ee), respectively. The absolute configurations of (+)-(*R*)-HOCPCA and (+)-(*R*)-HOCHCA were established by close agreement between the measured Electronic Circular Dichroism spectra and those predicted from first principles for the conformational ensembles in solution by the time-dependent density functional theory and supported by agreement between the signs of the measured and predicted optical rotations (Supplemental Data, Fig. 1). (1*S*,2*S*)-2-(Hydroxymethyl)cyclopropanecarboxylic acid [(*S,S*)-HOCPPrCA] and (*R,R*)-HOCPPrCA were obtained by stereoselective synthesis of (–)-menthyl (1*S*,2*S*)-2-(hydroxymethyl)cyclopropanecarboxylate and (+)-menthyl (1*R*,2*R*)-2-(hydroxymethyl)cyclopropanecarboxylate, respectively (Pajouhesh et al., 2000), followed by ester hydrolysis.

Assigned structures were in agreement with the ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra. Elemental analyses were performed at the Analytical Research Department (H. Lundbeck A/S, Copenhagen, Denmark) or by J. Theiner (Microanalytical Laboratory, Department of Physical Chemistry, University of Vienna, Austria). Further details concerning synthesis and purification are given in Supplemental Data.

Binding Assays

Membrane Preparations. All binding assays were performed using rat brain synaptic membranes of the cortex and central hemispheres from adult male Sprague-Dawley rats with tissue preparation as described earlier by Ransom and Stec (1988). On the day of the assay, the membrane preparation was quickly thawed, suspended in 40 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) (for [^3H]muscimol and [^3H]GABA binding assays) or KH_2PO_4 buffer (50 mM, pH 6.0) (for [^3H]NCS-382 binding assay) using an UltraTurrax homogenizer, and centrifuged at 48,000g for 10 min at 4°C. This washing step was repeated four times. The final pellet was resuspended in incubation buffer for the relevant binding assay.

[^3H]NCS-382, [^3H]Muscimol, and [^3H]GABA Binding Assays. The [^3H]NCS-382 binding assay described by Mehta et al. (2001) was adapted to a 96-well format filtration assay (Kaupmann et al., 2003). For saturation binding experiments, [^3H]NCS-382 concentrations ranged from 1 to 3000 nM, whereas, for competition studies, 16 nM [^3H]NCS-382 was used. Aliquots of membrane preparation (50–70

μg of protein/aliquot) in KH_2PO_4 buffer (50 mM, pH 6.0) were incubated with [^3H]NCS-382 in triplicate at 0°C for 1 h in a total volume of 200 μl . Nonspecific binding was determined using unlabeled GHB (1 mM). The binding reaction was terminated by rapid filtration through GF/C unilters (PerkinElmer Life and Analytical Sciences) using a 96-well Packard FilterMate cell harvester followed by washing with $3 \times 250 \mu\text{l}$ of ice-cold binding buffer. Microscint scintillation fluid (PerkinElmer Life and Analytical Sciences) was added to the dried filters, and the amount of filter-bound radioactivity was quantified in a Packard TopCount microplate scintillation counter (PerkinElmer Life and Analytical Sciences).

The assay of [^3H]muscimol binding to the GABA_A receptors followed a similar protocol. Membranes (100 μg of protein/aliquot) in Tris-HCl buffer (50 mM, pH 7.4) were incubated with [^3H]muscimol (5 nM) and inhibitor (1 mM) at 0°C for 60 min in a total volume of 250 μl . GABA (1 mM) was used to determine nonspecific binding. Filtration through GF/B filters, washing, and quantification was identical to that of the [^3H]NCS-382 assay.

For [^3H]GABA binding to the GABA_B receptors, membranes (200 μg protein/aliquot) were suspended in Tris-HCl buffer (50 + 2.5 mM CaCl_2 , pH 7.4) and incubated with [^3H]GABA (5 nM), isoguvacine (40 μM), and 1 mM inhibitor at 25°C for 45 min in 1-ml total volumes. Isoguvacine serves to saturate GABA_A receptors (Hill and Bowery, 1981). Nonspecific binding was determined using 0.1 mM baclofen. Binding was terminated by filtration through Whatman GF/C filters (Whatman Schleicher and Schuell, Keene, NH) using a Brandell M-48R cell harvester, filters were washed with $3 \times 3 \text{ ml}$ of ice-cold buffer, and filter-bound radioactivity was counted in a Packard Tri-carb 2100 liquid scintillation analyzer using 3 ml of Opti-fluor scintillation fluid (PerkinElmer Life and Analytical Sciences). 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, Hercules, CA).

Data Analysis. The binding data were analyzed by nonlinear regression curve-fitting using Prism 4.0b (GraphPad Software Inc., San Diego, CA). Where indicated, K_i values were calculated from IC_{50} values by means of the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Results

Inhibition of [^3H]NCS-382 Binding. For measuring the specific GHB binding sites in brain, [^3H]NCS-382 was used as a radioligand because this compound does not interfere with GABA receptors, neither directly nor indirectly, and thus discriminates between GHB and GABA sites. Under our experimental conditions, [^3H]NCS-382 displayed a single high-affinity binding site (K_d of $430 \pm 1 \text{ nM}$ and a B_{max} of $57 \pm 6 \text{ pmol/mg}$ protein; data not shown). We have previously demonstrated a single high-affinity binding site in mouse brain using the same radioligand with similar affinity (K_d of $360 \pm 60 \text{ nM}$) (Kaupmann et al., 2003). All generated inhibition curves were therefore fitted using a one-site model. GHB and known structural analogs (Fig. 1) were found to inhibit [^3H]NCS-382 binding (16 nM) in a concentration-dependent manner, and the rank order of affinity was $\text{NCS-382} > \text{T-HCA} > \text{GHB}$ (Fig. 2A; Table 1), which is in agreement with the literature (Bourguignon et al., 2000; Mehta et al., 2001). The specific amount of bound [^3H]NCS-382 inhibited by 1 mM GHB was at least 90% of total binding.

Having established our assay to be reliable and reproducible, the affinities of the three novel GHB analogs synthesized in our laboratory were measured (depicted in Fig. 1).

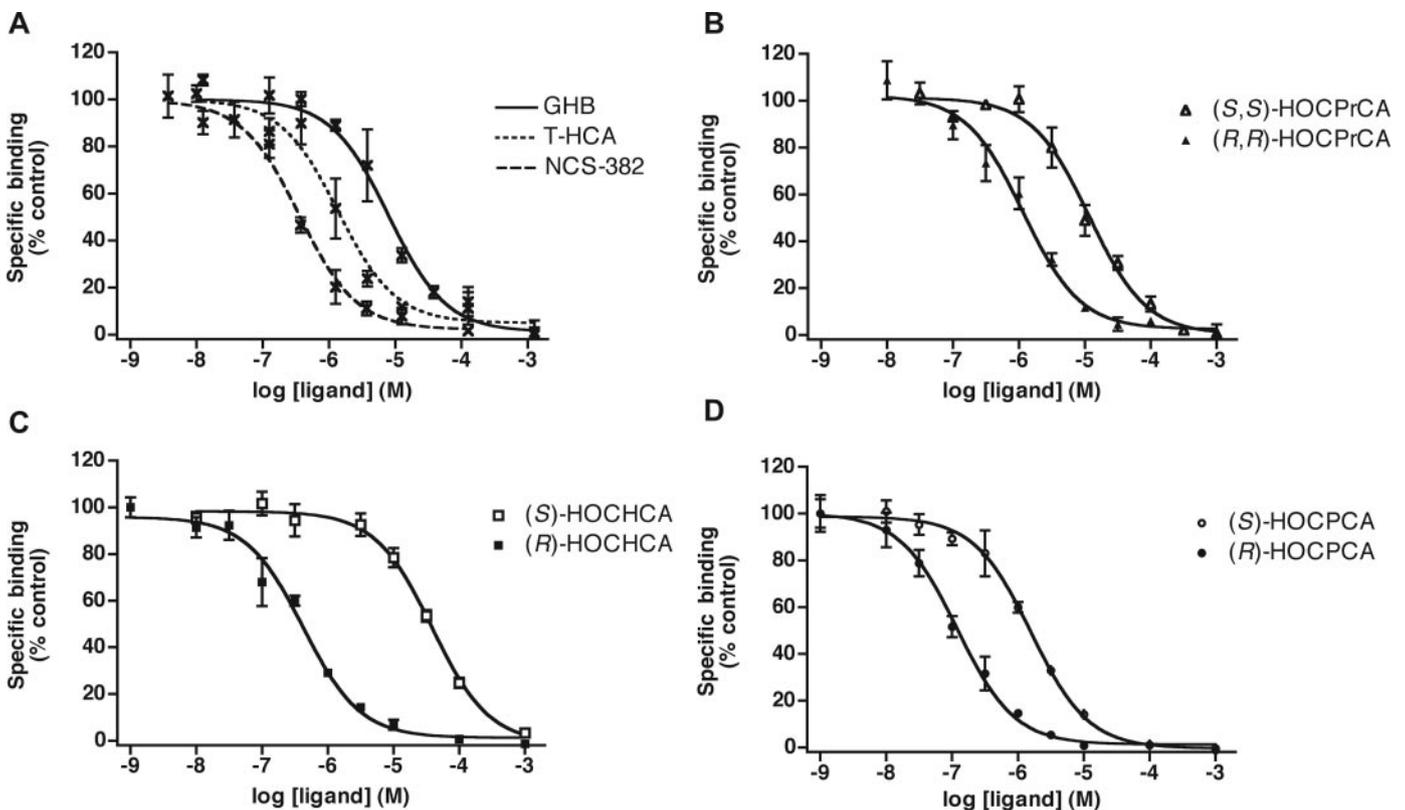


Fig. 2. Concentration-dependent inhibition of [^3H]NCS-382 binding to rat synaptic membranes by the established compounds GHB, T-HCA, and NCS-382 (A) and by the novel cyclic analogs (*S,S*)- and (*R,R*)-HOCPPrCA (B), (*S*)- and (*R*)-HOCHCA (C), and (*S*)- and (*R*)-HOCPCA (D). Data shown are mean \pm S.D. of a single representative experiment performed in triplicate. At least three individual experiments were carried out to calculate the average values summarized in Table 1.

TABLE 1

Comparative inhibitory affinities of GHB and analogs on specific [^3H]NCS-382 (16 nM) binding in rat brain synaptic membranes. IC_{50} values were calculated from inhibition curves and converted to K_i values. Each value is mean ($\text{p}K_i \pm \text{S.E.M.}$) of at least three independent experiments carried out in triplicate.

Compound	K_i ($\text{p}K_i \pm \text{S.E.M.}$)	Affinity Relative to GHB
	μM	
GHB	4.3 (5.4 ± 0.041)	1
T-HCA	1.1 (6.0 ± 0.02)	4.0
NCS-382	0.3 (6.5 ± 0.02)	15
(<i>R,R</i>)-HOCPrCA	1.1 (6.0 ± 0.03)	3.9
(<i>S,S</i>)-HOCPrCA	11 (5.0 ± 0.02)	0.41
(<i>RS</i>)-HOCHCA	0.48 (6.3 ± 0.08)	8.9
(<i>R</i>)-HOCHCA	0.48 (6.3 ± 0.0)	8.9
(<i>S</i>)-HOCHCA	34 (4.5 ± 0.05)	0.13
(<i>RS</i>)-HOCPCA	0.16 (6.8 ± 0.04)	27
(<i>R</i>)-HOCPCA	0.11 (6.9 ± 0.02)	39
(<i>S</i>)-HOCPCA	1.4 (5.8 ± 0.01)	3.0
OxCPCA	>100	

HOCHCA and HOCPCA were initially tested as racemic mixtures, whereas both the (*R,R*) and (*S,S*) forms were tested for HOCPrCA, which was synthesized from asymmetric precursors. Table 1 shows that the racemic mixtures of HOCHCA and HOCPCA inhibit [^3H]NCS-382 binding with K_i values of 0.48 and 0.16 μM , respectively, indicating that these cyclic GHB analogs have correspondingly 9 and 27 times higher affinity than GHB itself. It is interesting that, for the cyclopropyl analog HOCPrCA, the (*R,R*) form (K_i of 1.1 μM) was approximately 10-fold better than the (*S,S*) form (Fig. 2B; Table 1). This prompted us to resolve the racemic mixtures of HOCHCA and HOCPCA into (*R*) and (*S*) forms and measure their affinities. As illustrated in Fig. 2, C and D, respectively, (*R*)-HOCHCA (K_i of 0.48 μM) has 70-fold higher affinity than (*S*)-HOCHCA for GHB binding sites. Likewise, (*R*)-HOCPCA (K_i of 0.11 μM) has 13-fold higher affinity than (*S*)-HOCPCA. We also analyzed the importance of the hydroxyl group at the 3-position of HOCPCA by testing the corresponding ketone, 3-oxocyclopent-1-enecarboxylic acid (OxCPCA) (Fig. 1). The presence of an oxo group instead of a hydroxyl group greatly attenuated the ability to compete for [^3H]NCS-382 binding (Table 1). This demonstrates that the GHB binding site interacts stereospecifically with its substrates and that the hydroxyl group of HOCPCA is essential for the high affinity of this compound.

Selectivity Studies. The selectivity of HOCHCA and HOCPCA for GHB binding sites over GABA_B and GABA_A receptors was investigated in binding assays using tritiated GABA and muscimol for respective labeling of the receptors. In each case, GABA inhibited binding as expected. Figure 3 and Table 2 show that GHB binds to GABA_B receptors with low affinity (IC_{50} of 230 μM) as reported by others (Mathivet et al., 1997). However, IC_{50} values of the racemic mixtures of HOCHCA and HOCPCA are not reached at concentrations of 1 mM (changes in specific binding of -17 ± 3 and $-14 \pm 1\%$, respectively) (Fig. 3; Table 2), nor do the two compounds possess any affinity for the GABA site of the GABA_A receptor at 1 mM concentrations, as measured by [^3H]muscimol binding (no significant decrease in specific binding) (Table 2). These results demonstrate that HOCHCA and HOCPCA are selective for GHB binding sites/GHB receptors over GABA receptors.

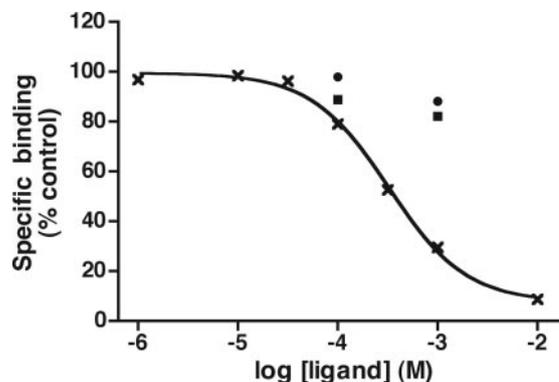


Fig. 3. Comparison of the ability of GHB (\times), (*R,S*)-HOCHCA (\blacksquare), and (*R,S*)-HOCPCA (\bullet) to inhibit [^3H]GABA binding from GABA_B receptors in rat cerebrocortical membranes is shown. Isoguvacine was added to saturate GABA_A receptors. Data shown are from a single representative experiment performed in triplicate, which was repeated three times. Means \pm S.D. were less than 5% and have been omitted for clarity. Average values are summarized in Table 2.

TABLE 2

Effects of GABA, GHB, and cyclic analogs (1 mM) on [^3H]GABA (5 nM) binding to GABA_B receptors and [^3H]muscimol (5 nM) binding to GABA_A receptors in rat brain synaptic membranes

Each value is mean IC_{50} (micromolar) ($\text{pIC}_{50} \pm \text{S.E.M.}$) for GABA_B binding or mean K_i (micromolar) ($\text{p}K_i \pm \text{S.E.M.}$) for GABA_A binding of at least three independent experiments in triplicate.

Compound	[^3H]GABA; IC_{50} ($\text{pIC}_{50} \pm \text{S.E.M.}$); GABA_B Receptor	[^3H]Muscimol; K_i ($\text{p}K_i \pm \text{S.E.M.}$); GABA_A Receptor
GABA	0.013 (7.9 ± 0.05)	0.049 (7.3 ± 0.06)
GHB	230 (3.7 ± 0.11)	N.A.
(<i>RS</i>)-HOCHCA	N.A.	N.A.
(<i>RS</i>)-HOCPCA	N.A.	N.A.

N.A., no affinity, meaning $\text{IC}_{50} > 1 \text{ mM}$.

Discussion

Although the pharmacological effects of GHB have been the subject of many investigations in recent years, the role of specific GHB receptors remains unclear. It has been demonstrated in numerous studies that GHB causes stimulation of GABA_B receptors, thus complicating and possibly masking the measurement of native GHB receptor-mediated effects (Carai et al., 2001; Jensen and Mody, 2001; Nava et al., 2001; Barbaccia et al., 2002; Kaupmann et al., 2003; Ren and Mody, 2003). As a consequence, the biological significance and mere existence of a GHB receptor have been questioned. The fact that the unique overlapping [^3H]GHB and [^3H]NCS-382 high-affinity binding sites exist in mammalian brain (Mehta et al., 2001; Gould et al., 2003) and that these remain intact in mice lacking functional GABA_B receptors (Kaupmann et al., 2003; Wu et al., 2004), however, stand in favor of a distinct GHB binding site or receptor. This has already been suggested based on the differential expression profile and ontogeny of GABA_B and specific GHB binding sites (Snead, 1996). Therefore, in the GHB field, there is a clear need for the development of potent and selective compounds.

In this work, we have explored the affinity and stereoselectivity of three cyclic ligands for the putative GHB receptor with a view to obtaining new lead structures and pinning down the precise geometry required for activity. For the first time, we have separately examined the *trans*-enantiomers of HOCPrCA (Bourguignon et al., 2000). The cyclopentene and cyclohexene compounds are entirely novel as GHB ligands

and were designed to lock in place as far as possible the hydroxyl and carboxylate moieties, as distinct from the flexibility of the carbon chain of GHB, greatly reducing the conformational degrees of freedom and providing more precise information on the topography of the binding site. Our findings confirm prior observations that the partially conformationally restricted analogs, T-HCA and NCS-382, possess higher affinity than GHB for the receptor sites labeled by either [³H]NCS-382 or [³H]GHB (Bourguignon et al., 2000; Mehta et al., 2001). Compared with GHB, T-HCA contains a double bond between the α - and β -carbons, eliminating folded conformations and increasing the affinity by a factor 4. NCS-382 is somewhat more constrained by a semirigid seven-membered ring system and has approximately 15 times higher affinity than GHB, part of which may be attributed to the incorporation of a benzene ring. Nonetheless, NCS-382 still leaves a number of possibilities open for the optimal geometric relationship between the main pharmacophore elements, the hydroxyl and carboxylate moieties. In keeping with this theme of conformational restriction, the newly characterized analog offering the greatest spatial repertoire for these groups, HOCPrCA, is also the weakest inhibitor, but the introduction of two stereocenters and observation of 10-fold stereoselectivity further narrows down the pharmacophore. In the two novel GHB structures, the α and γ carbons of GHB are locked in a ring for the first time, and the *R*-isomer of the cyclopentene derivative HOCPCA has as much as 39 times higher affinity than GHB, indicating that this structure closely mimics the bioactive conformation of the endogenous ligand. Similar to HOCPrCA and in accordance with the observations made by Castelli et al. (2002) for NCS-382, the new analogs bind to the GHB receptor in a stereoselective manner. Thus, the alignment of GHB at its specific recognition sites is essentially solved and the structural preferences of the receptor in this vicinity are largely mapped out, with the corollary that GHB most likely takes on a different conformation when binding to GABA_B receptors.

The only major feature not pinpointed by these analogs is the hydroxyl proton. Because conversion of the hydroxyl group of HOCPCA to a carbonyl group greatly abolishes the affinity of this scaffold, it is tempting to conclude that the hydroxyl group donates an important hydrogen bond to the GHB receptor. However, it was recently shown that the less polar 3-chloropropanoic acid inhibits [³H]NCS-382 binding with only slightly less efficiency than GHB, challenging the concept that hydrogen bond donation is essential for binding (Macias et al., 2004). Conversely, the oxo group in OxCPCA forces the distal hydrogen bond acceptor into the plane of the ring and the carboxylate group, a distinctly different arrangement from that of the active analogs, leaving the precise role of the hydroxyl proton yet to be determined.

With the demonstration that, unlike GHB, the two best binding inhibitors HOCPCA and HOCHCA are devoid of affinity for the GABA receptors, these compounds represent exciting new tools for studying GHB pharmacology both in vivo and in vitro. An obvious advantage of HOCPCA and HOCHCA is their low molecular weights, which make them potential blood-brain barrier penetrants, although this has not been tested as of yet. In this context, it will be necessary to determine the intrinsic activity of the compounds. Although isolated reports of functional assays have been described in the literature (Castelli et al., 2003; Kemmel et al.,

2003; Brancucci et al., 2004), these remain to be validated by other laboratories. The compounds are also ideal lead structures, presenting ample opportunity for further substitution and structural elaboration, again with the advantage of low molecular weight and high binding efficiency of the scaffolds, bearing in mind that the synthesis of a radioligand with high specific activity could greatly aid in the molecular cloning of the GHB receptor or lay the way for tomography studies. In addition, combining the stereospecificity data obtained here with the structure-activity relationships from this and previous studies paves the way for a more complete pharmacophore model of the extended GHB binding site. Therefore, this work offers a platform for further efforts in both pharmacology and medicinal chemistry toward the final goal of characterizing and understanding the physiological role of the GHB receptor.

Acknowledgments

We thank Shahrokh Padrah for technical assistance.

References

- Andriampandry C, Taleb O, Viry S, Muller C, Humbert JP, Gobaille S, Aunis D, and Maitre M (2003) Cloning and characterization of a rat brain receptor that binds the endogenous neuromodulator γ -hydroxybutyrate (GHB). *FASEB J* **17**: 1691–1693.
- Barbaccia ML, Colombo G, Affricano D, Carai MA, Vacca G, Melis S, Purdy RH, and Gessa GL (2002) GABA_B receptor-mediated increase of neurosteroids by γ -hydroxybutyric acid. *Neuropharmacology* **42**:782–791.
- Beagley B, Larsen DS, Pritchard RG, Stoodley RJ, and Whiting A (1989) An oxidative rearrangement of *t*-butyl (3*RS*, 5*SR*)-2-ethoxycarbonylcarbapen-1-em-3-carboxylate to *t*-butyl (1*RS*, 5*SR*, 7*RS*, 8*SR*)-8-ethoxycarbonyl-8-hydroxy-3-oxo-2-oxa-6-azabicyclo[3.3.0]octane-7-carboxylate. *J Chem Soc Perkin Trans 1* **6**: 1127–1137.
- Benavides J, Rumigny JF, Bourguignon JJ, Cash C, Wermuth CG, Mandel P, Vincendon G, and Maitre M (1982) High affinity binding sites for γ -hydroxybutyric acid in rat brain. *Life Sci* **30**:953–961.
- Bernasconi R, Mathivet P, Bischoff S, and Marescaux C (1999) Gamma-hydroxybutyric acid: an endogenous neuromodulator with abuse potential? *Trends Pharmacol Sci* **20**:135–141.
- Bourguignon JJ, Schmitt M, and Didier B (2000) Design and structure-activity relationship analysis of ligands of γ -hydroxybutyric acid receptors. *Alcohol* **20**: 227–236.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254.
- Brancucci A, Berretta N, Mercuri NB, and Francesconi W (2004) Presynaptic modulation of spontaneous inhibitory postsynaptic currents by gamma-hydroxybutyrate in the substantia nigra pars compacta. *Neuropsychopharmacology* **29**:537–543.
- Carai MA, Colombo G, Brunetti G, Melis S, Serra S, Vacca G, Mastinu S, Pistuddi AM, Solinas C, Cignarella G, et al. (2001) Role of GABA_B receptors in the sedative/hypnotic effect of γ -hydroxybutyric acid. *Eur J Pharmacol* **428**:315–321.
- Carter LP, Wu H, Chen W, Matthews MM, Mehta AK, Hernandez RJ, Thomson JA, Ticku MK, Coop A, Koek W, et al. (2005) Novel γ -hydroxybutyric acid (GHB) analogs share some, but not all, of the behavioral effects of GHB and GABA_B receptor agonists. *J Pharmacol Exp Ther* **313**:1314–1323.
- Castelli MP, Ferraro L, Mocchi I, Carta F, Carai MA, Antonelli T, Tanganelli S, Cignarella G, and Gessa GL (2003) Selective γ -hydroxybutyric acid receptor ligands increase extracellular glutamate in the hippocampus, but fail to activate G protein and to produce the sedative/hypnotic effect of γ -hydroxybutyric acid. *J Neurochem* **87**:722–732.
- Castelli MP, Mocchi I, Pistis M, Peis M, Berta D, Gelain A, Gessa GL, and Cignarella G (2002) Stereoselectivity of NCS-382 binding to γ -hydroxybutyrate receptor in the rat brain. *Eur J Pharmacol* **446**:1–5.
- Castelli MP, Pibiri F, Carboni G, and Piras AP (2004) A review of pharmacology of NCS-382, a putative antagonist of γ -hydroxybutyric acid (GHB) receptor. *CNS Drug Rev* **10**:243–260.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.
- Cossy J, Furet N, and BouzBouz S (1995) Ring opening of cyclopropylketones induced by photochemical electron transfer. *Tetrahedron* **51**:11751–11764.
- Fuller DE and Hornfeldt CS (2003) From club drug to orphan drug: sodium oxybate (Xyrem) for the treatment of cataplexy. *Pharmacotherapy* **23**:1205–1209.
- Gallimberti L, Spella MR, Soncini CA, and Gessa GL (2000) Gamma-hydroxybutyric acid in the treatment of alcohol and heroin dependence. *Alcohol* **20**:257–262.
- Gemal AL and Luche J-L (1981) Lanthanoids in organic synthesis: VI. The reduction of α -enones by sodium borohydride in the presence of lanthanoid chlorides: synthetic and mechanistic aspects. *J Am Chem Soc* **103**:5454–5459.
- Gould GG, Mehta AK, Frazer A, and Ticku MK (2003) Quantitative autoradiographic analysis of the new radioligand [³H](2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6*H*-

- benzo[a][7]annulen-6-ylidene) ethanoic acid (^3H NCS-382) at γ -hydroxybutyric acid (GHB) binding sites in rat brain. *Brain Res* **979**:51–56.
- Hechler V, Ratomponirina C, and Maitre M (1997) γ -Hydroxybutyrate conversion into GABA induces displacement of GABA_B binding that is blocked by valproate and ethosuximide. *J Pharmacol Exp Ther* **281**:753–760.
- Hill DR and Bowery NG (1981) ^3H -baclofen and ^3H -GABA bind to bicuculline-insensitive GABA_B sites in rat brain. *Nature (Lond)* **290**:149–152.
- Jensen K and Mody I (2001) GHB depresses fast excitatory and inhibitory synaptic transmission via GABA_B receptors in mouse neocortical neurons. *Cereb Cortex* **11**:424–429.
- Kaupmann K, Cryan JF, Wellendorph P, Mombereau C, Sansig G, Klebs K, Schmutz M, Froestl W, van der Putten H, Mosbacher J, et al. (2003) Specific γ -hydroxybutyrate-binding sites but loss of pharmacological effects of γ -hydroxybutyrate in $\text{GABA}_{B(1)}$ -deficient mice. *Eur J Neurosci* **18**:2722–2730.
- Kemmel V, Taleb O, Andriamampandry C, Aunis D, and Maitre M (2003) γ -Hydroxybutyrate receptor function determined by stimulation of rubidium and calcium movements from NCB-20 neurons. *Neuroscience* **116**:1021–1031.
- Lange GL, Decicco CP, Willson J, and Strickland LA (1989) Ring expansions of [2 + 2] photoadducts: potential applications in the synthesis of triquinane and taxane skeletons. *J Org Chem* **54**:1805–1810.
- Lange GL and Otulakowski JA (1982) Improved preparation of methyl 3-oxo-1-cyclohexene-1-carboxylate and its use in the synthesis of substituted 1,5-cyclodecadienes. *J Org Chem* **47**:5093–5096.
- Lingenhoehl K, Brom R, Heid J, Beck P, Froestl W, Kaupmann K, Bettler B, and Mosbacher J (1999) γ -Hydroxybutyrate is a weak agonist at recombinant GABA_B receptors. *Neuropharmacology* **38**:1667–1673.
- Macias AT, Hernandez RJ, Mehta AK, MacKerell AD Jr, Ticku MK, and Coop A (2004) 3-chloropropanoic acid (UMB66): a ligand for the gamma-hydroxybutyric acid receptor lacking a 4-hydroxyl group. *Bioorg Med Chem* **12**:1643–1647.
- Mathivet P, Bernasconi R, De Barry J, Marescaux C, and Bittiger H (1997) Binding characteristics of γ -hydroxybutyric acid as a weak but selective GABA_B receptor agonist. *Eur J Pharmacol* **321**:67–75.
- Mehta AK, Muschawack NM, Maeda DY, Coop A, and Ticku MK (2001) Binding characteristics of the γ -hydroxybutyric acid receptor antagonist [^3H](2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6*H*-benzo[a][7]annulen-6-ylidene) ethanoic acid in the rat brain. *J Pharmacol Exp Ther* **299**:1148–1153.
- Mori K (1978) Synthesis of the both enantiomers of grandisol, the boll weevil pheromone. *Tetrahedron* **34**:915–920.
- Nava F, Carta G, Bortolato M, and Gessa GL (2001) γ -Hydroxybutyric acid and baclofen decrease extracellular acetylcholine levels in the hippocampus via GABA_B receptors. *Eur J Pharmacol* **430**:261–263.
- Pajouhesh H, Chen J, and Pajouhesh SH (2000) An efficient and stereoselective synthesis of (2*S*,1'*S*,2'*S*)-2-(carboxycyclopropyl) glycine (LCCG-I): conformationally constrained L-glutamate analogues. *Tetrahedron: Asymmetry* **11**:4537–4541.
- Palaty J and Abbott FS (1995) Structure-activity relationships of unsaturated analogues of valproic acid. *J Med Chem* **38**:3398–3406.
- Ransom RW and Stec NL (1988) Cooperative modulation of [^3H]MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine and polyamines. *J Neurochem* **51**:830–836.
- Ren X and Mody I (2003) γ -Hydroxybutyrate reduces mitogen-activated protein kinase phosphorylation via GABA_B receptor activation in mouse frontal cortex and hippocampus. *J Biol Chem* **278**:42006–42011.
- Snead OC III (1996) Relation of the [^3H]gamma-hydroxybutyric acid (GHB) binding site to the gamma-aminobutyric acid (GABA_B) receptor in rat brain. *Biochem Pharmacol* **52**:1235–1243.
- Wong CG, Gibson KM, and Snead OCI (2004) From the street to the brain: neurobiology of the recreational drug γ -hydroxybutyric acid. *Trends Pharmacol Sci* **25**:29–34.
- Wu H, Zink N, Carter LP, Mehta AK, Hernandez RJ, Ticku MK, Lamb R, France CP, and Coop A (2003) A tertiary alcohol analog of γ -hydroxybutyric acid as a specific γ -hydroxybutyric acid receptor ligand. *J Pharmacol Exp Ther* **305**:675–679.
- Wu Y, Ali S, Ahmadian G, Liu CC, Wang YT, Gibson KM, Calver AR, Francis J, Pangalos MN, and Carter Snead OI (2004) γ -Hydroxybutyric acid (GHB) and γ -aminobutyric acid_B receptor (GABA_BR) binding sites are distinctive from one another: molecular evidence. *Neuropharmacology* **47**:1146–1156.

Address correspondence to: Dr. Rasmus P. Clausen, Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark. E-mail: rac@dfuni.dk
