Effects of \(\gamma\)-Hydroxybutyrate (GHB) on Schedule-Controlled Responding in Rats: Role of GHB and GABA\textsubscript{B} Receptors

Lawrence P. Carter, Huifang Wu, Weibin Chen, Christopher M. Cruz, R. J. Lamb, Wouter Koek, Andy Coop, and Charles P. France

Departments of Pharmacology (L.P.C., C.M.C., R.J.L., W.K., C.P.F.) and Psychiatry (R.J.L., W.K., C.P.F.), The University of Texas Health Science Center at San Antonio, San Antonio, Texas; and Department of Pharmaceutical Sciences (H.W., W.C., A.C.), University of Maryland, Baltimore, Maryland

Received August 20, 2003; accepted October 2, 2003

ABSTRACT
\(\gamma\)-Hydroxybutyrate (GHB), a metabolite of \(\gamma\)-aminobutyric acid (GABA), is an increasingly popular drug of abuse and was recently approved for the treatment of narcolepsy (Xyrem). GHB and GABA receptors have been implicated in mediating effects of GHB; however, the relative importance of each of these receptors is unclear. This study evaluated the effects of selective antagonists in combination with GHB and related compounds on schedule-controlled responding. Eight male Sprague-Dawley rats responded under a fixed-ratio schedule of food presentation. Cumulative dose-effect curves were generated and ED\textsubscript{50} values calculated to evaluate the relative potency at decreasing responding. The rank-order potency was as follows: diazepam = baclofen > \(\gamma\)-butyrolactone (GBL) > 1,4-butanediol (1,4-BDL) = GHB. All compounds decreased responding 20 min after administration. The duration of action of diazepam, GHB, and GBL was shorter than that of 1,4-BDL and baclofen. \(p\)-3-Aminopropyl-\(p\)-diethoxymethyl phosphinic acid (CGP 35348) antagonized the rate-decreasing effects of baclofen and not GHB; flumazenil antagonized the effects of diazepam and not GHB. The GHB receptor antagonist (2E)-[5-hydroxy-5,7,8,9-tetrahydro-6\(H\)-benzo[a][7]annulen-6-ylidene ethanoic acid (NCS-382) did not attenuate the rate-decreasing effects of GHB, baclofen, or diazepam; larger doses of NCS-382 further decreased rate of responding when given in combination with each of these compounds. These studies show that GBL, 1,4-BDL, and GHB differ significantly in potency and duration of action. The ability of CGP 35348 to antagonize the rate-decreasing effects of baclofen may be limited by the involvement of multiple GABA\textsubscript{B} receptor subtypes and the lack of antagonism of GHB by NCS-382 may be due to its own GHB-like effects.

\(\gamma\)-Hydroxybutyrate (GHB) is an endogenous metabolite of \(\gamma\)-aminobutyric acid (GABA) and a putative neurotransmitter (Roth and Giarman, 1970; Maitre, 1997). GHB binds to GABA\textsubscript{B} receptors (Mathivet et al., 1997; Lingenhoehl et al., 1999) and specific GHB sites (Benavides et al., 1982; Snead and Liu, 1984; Mehta et al., 2001) in the brain. GHB-specific binding sites (as labeled by \([^{3}H]\)NCS-382) are believed to be G protein-coupled receptors (Snead, 2000). In addition to binding at GHB and GABA\textsubscript{B} receptors, GHB is metabolized to GABA (Roth and Giarman, 1969), which may act at GABA\textsubscript{A}, GABA\textsubscript{B}, and GABA\textsubscript{C} receptors. Administration of GHB can induce sedation, amnesia, ataxia, catalepsy, absence seizures, coma, and death (Snead and Liu, 1993; Nicholson and Balster, 2001); however, the relative importance of GHB receptors and GABA receptors in mediating these effects is unclear. GHB is an emerging drug of abuse (Nicholson and Balster, 2001; Mason and Kerns, 2002); thus, a GHB antagonist could be clinically useful for treating GHB overdose and toxicity.

NCS-382 is a GHB antagonist that blocks some, but not all, of the effects of GHB. NCS-382 binds to GHB receptors in cortex and hippocampus and has no appreciable affinity at GABA\textsubscript{A} or GABA\textsubscript{B} receptors (Serra et al., 1991; Snead, 1996b; Mehta et al., 2001). Previous studies that examined the effects of GHB and NCS-382 on schedule-controlled behavior found that limited doses of NCS-382 (3.2 and 32.0 mg/kg) did not antagonize the rate-decreasing effects of GHB in rats (Lamb et al., 2003) and that in mice 56 mg/kg NCS-382 significantly reduced responding when given in combination with GHB compared with the same doses of GHB alone (Cook et al., 2002).

GABA\textsubscript{B} receptor antagonists also block some of the effects

ABBREVIATIONS: GHB, \(\gamma\)-hydroxybutyrate; 1,4-BDL, 1,4-butanediol; GBL, \(\gamma\)-butyrolactone; CGP 35348, \(p\)-3-aminopropyl-\(p\)-diethoxymethyl phosphinic acid; NCS-382, (2E)-[5-hydroxy-5,7,8,9-tetrahydro-6\(H\)-benzo[a][7]annulen-6-ylidene ethanoic acid; FR, fixed ratio; ANOVA, analysis of variance; SCH 50911, (2S)(+)-5,5-dimethyl-2-morpholineacetic acid.
of GHB. The selective, high-affinity GABA<sub>B</sub> receptor antagonist CGP 35348 attenuated discriminative stimulus effects of GHB in drug discrimination procedures in rats (Colombo et al., 1998; Carter et al., 2003) and pigeons (W. Koek, L. R. Flores, L. P. Carter, R. J. Lamb, W. Chen, H. Wu, A. Coop, and C. P. France, manuscript submitted for publication). SCH 50911 blocked GHB-induced decreases in hippocampal acetylcholine release (Nava et al., 2001) and GHB-induced increases in brain neurosteroid concentrations (Barbaccia et al., 2002). Increasing neurosteroid levels is an additional mechanism by which GHB could modulate GABA<sub>B</sub> receptors and exert some of its behavioral effects.

Schedule-controlled responding has been used extensively to study the mechanism of action of various compounds; however, few studies have evaluated the mechanism of action of GHB using this procedure (Cook et al., 2002; Lamb et al., 2003). Fixed-ratio (FR) schedules typically maintain high rates of responding which are dose dependently decreased by many classes of drugs (Dews, 1956). Given that the mechanism of action of GHB likely involves multiple receptor systems, schedule-controlled responding allows one to study drugs with different mechanisms of action using the same dependent variable.

The aim of this study was to evaluate the specific role of GHB and GABA<sub>B</sub> receptors in mediating the rate-decreasing effects of GHB. Doses of NCS-382 were given prior to GHB, the prototypical GABA<sub>B</sub> receptor agonist baclofen, and the GABA<sub>B</sub> receptor-positive modulator diazepam. Effects of NCS-382 in combination with these compounds were compared with data obtained when the selective GABA<sub>B</sub> receptor antagonist CGP 35348 was given prior to GHB or baclofen or when the GABA<sub>B</sub> receptor neutral modulator flumazenil was given prior to GHB or diazepam. To accomplish the primary goal of this study, the relative potency of GHB, GHB precursoirs 1,4-BDL and γ-butyrolactone (GBL), baclofen, and diazepam to decrease rate of responding under a food-reinforced FR schedule in rats was evaluated, and the time course of the rate-decreasing effects of these five compounds was compared across a range of doses.

**Materials and Methods**

**Animals.** Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN; n = 8) were housed individually in 45 × 20 × 20 cm plastic cages containing rodent bedding (Sani-chips, Harlan Teklad, Madison, WI) in a colony room maintained on a 12:12 light/dark cycle (experiments conducted in the light period). Rats were fed 5 to 16 g of chow (rat sterilizable diet, Harlan Teklad) after daily experimental sessions to maintain body weights. Rats weighed between 240 and 260 g at the beginning of the study and were allowed to increase up to 350 g according to normal growth curves established for Sprague-Dawley rats. Water was available continuously in the home cage. All rats were experimentally naive prior to training. Two animals died during the course of these studies due to causes unrelated to the experiment. Animals were maintained, and experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and with the 1996 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences).

**Operant Chambers.** Experimental sessions were conducted in operant chambers that were located within sound-attenuating, ventilated chambers (model nos. ENV-018M and ENV-008CT; MED Associates Inc., St. Albans, VT). Two stimulus lights (2.5 cm in diameter) were located on the response panel of the operant chamber. A lever was located below each stimulus light on the response panel and a 5 × 5 cm opening was located equidistant between the two levers for food pellet delivery (45 mg, PJA-0045, Noyes Precision Pellets; Research Diets Inc., New Brunswick, NJ). Data were collected using MED-PC IV software (MED Associates Inc.) and a PC interface.

**Experimental Sessions.** Rats were initially trained to press a lever to receive a food pellet under a schedule of continuous reinforcement. Initial training sessions were 60 min in duration; lights above both levers were illuminated, and a single response on either lever resulted in food pellet delivery. Over subsequent sessions the schedule of reinforcement was modified to a FR schedule; the FR was systematically increased by one, and only responding on the left lever occasioned food pellet delivery (both lights were illuminated during the response period). The period of food availability was decreased to 45 min, and a pretreatment period of 15 min was introduced; during the pretreatment period the operant chamber was dark, and responses were recorded but had no programmed consequence. When rats received 100 food pellets under these conditions (median 19 days from the first session; range: 19–22) the response period was shortened to 5 min so that the total cycle time was 20 min (15-min pretreatment, 5-min response period). Final training conditions consisted of several (2–6) 20-min cycles and a FR10 schedule of food pellet delivery. Saline, drug, or sham injections were given during the first minute of each cycle (sham injections consisted of brief pressure applied to the abdomen with a capped needle).

Rate was measured as responses per second during the 5-min response period of each cycle. Initial criteria for testing were that the rate of responding for a session (average rate across cycles) varied by less than ±20% for 10 consecutive or 11 of 12 sessions. Experimental sessions were conducted 5 to 7 days per week. Test sessions were identical to training sessions, except that cumulative dosing was used to generate dose-effect curves, and test sessions ended if fewer than 10 responses occurred on the active lever in a single cycle. Tests sessions were conducted two to three times per week with at least two intervening training (saline/sham) sessions.

Cumulative dose-effect curves were generated for GHB (100, 178, 320, and 560 mg/kg), GBL (17.8, 32, 56, 100, 178 and 320 mg/kg), 1,4-BDL (56, 100, 178, and 320 mg/kg), baclofen (1.0, 1.78, 3.2, and 5.6 mg/kg), and diazepam (0.1, 0.32, 1.0, 3.2, and 10.0 mg/kg). The time course of selected acute doses of these compounds was determined over six cycles (120 min). Baclofen and 1,4-BDL exhibited longer durations of action and, therefore, were on separate occasions studied for six cycles (120–240 min) following a 100-min pretreatment in the home cage. All antagonists were administered 10 min prior to the start of the first cycle, resulting in a 25-min pretreatment time before the first response period (Carai et al., 2001; Carter et al., 2003). Doses of NCS-382 (10, 32, 56, and 100 mg/kg) were studied alone and in combination with GHB, baclofen, and diazepam. CGP 35348 (56, 100, and 178 mg/kg) was studied in combination with GHB and baclofen, whereas flumazenil (1.0, 3.2, and 5.6 mg/kg) was studied in combination with GHB and diazepam (selection of doses was based on previous studies; see Carter et al., 2003).

**Drugs.** Drugs were administered i.p. (pH 6–8) and dissolved or diluted (GBL and 1,4-BDL) in sterile water or saline unless otherwise noted. Injection volumes were between 0.1 and 1.0 ml. Compounds studied included GHB (sodium salt), (+)-baclofen, GBL, and 1,4-BDL (Sigma-Aldrich Corp., St. Louis, MO), diazepam (Sigma/ RBI, Natick, MA), flumazenil (a gift from F. Hoffmann-La Roche Ltd., Basel, Switzerland), NCS-382 (sodium salt), and CGP 35348 (sodium salt). NCS-382 and CGP 35348 were synthesized as previously described (Maitre et al., 1990; Froestl et al., 1995). Diazepam was dissolved in vehicle consisting of 70% emulphor, 20% sterile water, and 10% ethanol (by volume). Flumazenil was dissolved in vehicle consisting of 50% saline, 40% propylene glycol, and 10%
Results

Rate-Decreasing Effects. Rate of responding was stable over multiple cycles for 10 consecutive or 11 of 12 sessions after a median of 33 training sessions (range: 32–41). The rank-order potency of the rate-decreasing effects of the compounds studied alone was as follows: diazepam = baclofen > GBL > 1,4-BDL = GHB (Fig. 1; Table 1). Sensitivity to GHB remained stable over the course of these studies; average ED$_{50}$ values for four cumulative GHB dose-effect curves determined over 6 months were not significantly different from one another ($F_{(3,29)} = 0.11, p > 0.5$; Table 1).

Time Course Studies. The onset of action of all compounds was rapid with maximal rate-decreasing effects occurring 20 min after injection for each compound except 1,4-BDL, which exhibited its greatest rate-decreasing effects 40 min following administration (Fig. 2). All compounds dose dependently decreased rate of responding; significant interactions between dose and time were observed for all compounds except for baclofen ($F_{(10,143)} = 1.06, p > 0.3$), which exhibited the longest duration of action of all compounds studied (Fig. 2, panel E). The duration of maximum effect varied markedly across compounds. Diazepam (5.6 mg/kg) suppressed responding up to 40 min following administration; rate-decreasing effects of 5.6 mg/kg diazepam began to wane at 60 min, and rate of responding was not different from control 120 min after administration (Fig. 2, panel A). Equi-effective doses of GHB (320 mg/kg) and GBL (178 mg/kg) suppressed responding up to 60 min after administration; following these doses, rate of responding returned to control values 100 min after administration (Fig. 2, panels B and C). 1,4-BDL exhibited a longer duration of action than GHB, GBL, or diazepam, suppressing responding up to 120 min after administration; rate of responding returned to control values 160 min after administration of 320 mg/kg 1,4-BDL (Fig. 2, panel D). Baclofen (5.6 mg/kg) exhibited the longest duration of action with responding being suppressed up to 160 min after administration; marked rate-decreasing effects of baclofen were observed up to 240 min following administration of 5.6 mg/kg (Fig. 2, panel E).

Drug Interaction Studies. Neither NCS-382 nor CGP 35348 significantly antagonized the rate-decreasing effects of GHB (Fig. 3, panels A and B; Table 2). NCS-382 failed to antagonize the rate-decreasing effects of GHB or baclofen at doses of 10 and 32 mg/kg and further decreased responding when 56 mg/kg was given alone or in combination with these compounds (Fig. 3, panels B and D). In contrast, CGP 35348 attenuated the rate-decreasing effects of baclofen as evidenced by significantly larger ED$_{50}$ values ($F_{(4,38)} = 30.06, p < 0.001$; Tukey-Kramer MCT, $a = 0.05$).

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>$n$</th>
<th>ED$_{50}$ (mg/kg)</th>
<th>95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>8</td>
<td>2.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Baclofen 1</td>
<td>8</td>
<td>2.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Baclofen 2</td>
<td>8</td>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>GBL</td>
<td>7</td>
<td>110.7</td>
<td>66.9</td>
</tr>
<tr>
<td>1,4-BDL</td>
<td>8</td>
<td>147.1</td>
<td>94.6</td>
</tr>
<tr>
<td>GHB 1</td>
<td>8</td>
<td>217.3</td>
<td>169.7</td>
</tr>
<tr>
<td>GHB 2</td>
<td>8</td>
<td>213.4</td>
<td>183.1</td>
</tr>
<tr>
<td>GHB 3</td>
<td>8</td>
<td>218.4</td>
<td>173.8</td>
</tr>
<tr>
<td>GHB 4</td>
<td>6</td>
<td>215.0</td>
<td>167.7</td>
</tr>
</tbody>
</table>

Rank order potency: diazepam = baclofen > GBL > 1,4-BDL = GHB ($F_{(4,38)} = 30.06, p < 0.001$; Tukey-Kramer MCT, $a = 0.05$).

GHB, or diazepam, suppressing responding up to 120 min after administration; rate of responding returned to control values 160 min after administration of 320 mg/kg 1,4-BDL (Fig. 2, panel D). Baclofen (5.6 mg/kg) exhibited the longest duration of action with responding being suppressed up to 160 min after administration; marked rate-decreasing effects of baclofen were observed up to 240 min following administration of 5.6 mg/kg (Fig. 2, panel E).
The largest dose of flumazenil (5.6 mg/kg) did not significantly alter the ED50 value of the GHB dose-effect curve ($F_{1,11} = 0.36, p = 0.57$; Table 2). NCS-382 did not attenuate the rate-decreasing effects of diazepam (Table 2); rate of responding was lower when NCS-382 was administered in combination with diazepam than when diazepam was given alone (Fig. 3, panel F).

Discussion

The mechanism of action of GHB is not fully understood; however, the effects of GHB appear to involve GABA$_A$, GABA$_B$, and GHB receptors. Previous work has shown that over a relatively narrow dose range, NCS-382 does not reliably attenuate the rate-decreasing effects of GHB on operant responding (Cook et al., 2002; Lamb et al., 2003). The current study provides additional evidence that, across a larger range of doses, NCS-382 is ineffective at antagonizing decreases in responding by GHB, agonism at GABA$_B$ receptors (baclofen), or positive modulation of GABA$_B$ receptors (diazepam; see Barbaccia et al., 2002). Antagonism at GABA$_B$ receptors (CGP 35348) attenuated the rate-decreasing effects of baclofen and not GHB, whereas flumazenil antagonized the rate-decreasing effects of diazepam. Relative potency and time course were similar for all compounds to those reported in previous drug discrimination studies (Carter et al., 2003).

GHB, 1,4-BDL, GBL, baclofen, and diazepam all decreased responding under an FR schedule of food delivery in a dose-dependent manner. The rank-order potency was as follows: diazepam > baclofen > GBL > 1,4-BDL = GHB. The finding that the GHB precursor GBL (and to a lesser extent 1,4-BDL) is more potent than GHB in suppressing simple schedule-controlled behavior is in agreement with other studies that have reported greater potency of GHB precursors than GHB when sleep time (Carai et al., 2002b) and discriminative stimulus effects (Carter et al., 2003) were measured. Greater potency of GHB precursors compared with GHB may be due to more complete penetration of the brain by the precursors; GBL and 1,4-BDL are less polar than GHB, do not bind to GHB or GABA receptors (Carter et al., 2002), and are stochiometrically converted to GHB in vivo.

Time course data from this study show that the smallest doses of GHB and GBL that suppressed responding exhibited an onset and duration of action that were very similar (responding was suppressed for 60 min and returned to control by 100 min), whereas equieffective doses of 1,4-BDL and...
baclofen suppressed responding for approximately twice as long (120 and 160 min, respectively). These data are in agreement with drug discrimination work in which 1,4-BDL exhibited a longer duration of action than GHB or GBL (Carter et al., 2003). The finding that cumulative doses of GHB (320 mg/kg; Fig. 1, triangles) and GBL (178 mg/kg; Fig. 1, squares) decreased responding to a lesser extent than the same doses administered acutely (Fig. 2, panels B and C) provides additional evidence that the relatively rapid metabolism of these compounds (in rats) may contribute to their shorter duration of action. In contrast, a dose of 320 mg/kg 1,4-BDL given in a cumulative fashion (Fig. 1, diamonds) decreased responding to a greater extent than the same dose administered acutely (Fig. 2, panel D) supporting the notion that 1,4-BDL requires more time to reach its peak effect. These data indicate that the potency and pharmacokinetics of GHB, GBL, and 1,4-BDL vary markedly. To the extent that these compounds are knowingly or unknowingly used interchangeably (presumably due to their shared mechanism of action and the illicit status of GHB), the greater potency of GBL or the longer duration of action of 1,4-BDL may account for some of the cases of apparent GHB overdose and toxicity.

NCS-382 is a selective GHB receptor ligand in rat brain (Maitre et al., 1990; Mehta et al., 2001); however, antagonism of GHB by NCS-382 in vivo appears to depend on the behavioral endpoint that is studied. Some effects of GHB, such as discriminative stimulus effects (Colombo et al., 1995), reinforcing effects (Martellotta et al., 1998), sedative and cataleptic effects (Schmidt et al., 1991), and induction of absence seizures (Snead, 1996a), are attenuated or antagonized by NCS-382, whereas other effects of GHB, such as anxiolytic effects (Schmidt-Mutter et al., 1998), sedative/hypnotic effects (Carai et al., 2001), inhibition of intestinal motility (Carai et al., 2002a), inhibition of locomotor activity, ataxia,
that measured GHB- and baclofen-induced increases in neurotetratheres is provided by studies that show baclofen participates in the GABAB receptor via its conversion into \( \beta \)-hydroxybutyric acid (GHB) by the GHB receptor antagonist NCS-382. Baclofen and GHB bind to GABAB receptors and appear to exert some behavioral effects through this mechanism. Administration of GHB 35348, a selective GABAB receptor antagonist, attenuated the rate-decreasing effects of baclofen and not GHB. The GHB receptor antagonist NCS-382 was ineffective at blocking the rate-decreasing effects of GHB, baclofen, or diazepam. The additional decreases in rate that were observed when these compounds were administered in combination with NCS-382 may be due to the GHB-like effects of NCS-382 that have been reported in other studies. These data indicate that NCS-382 and GHB 35348 would likely have limited potential for treating GHB overdose and toxicity. More selective pharmacological tools are needed to better understand the actions of GHB at GHB receptors and to effectively treat the signs and symptoms associated with GHB intoxication, overdose, and withdrawal.

### Acknowledgments
We thank Daniel Mojica, Ginger Phillips, and Henry Renteria for excellent technical assistance.

### References


Mehta AK, Muschawek NM, Maeda DY, Coop A, and Ticku MK (2001) Binding characteristics of the \(\gamma\)-hydroxybutyric acid receptor antagonist \(\left[3H\right]\left(5\text{-hydroxy-5,7,8,9-tetrahydro-6H-benzo[a][7]annulen-6-yldiene ethanoic acid}\right)\) in the rat brain. *J Pharmacol Exp Ther* 299:1148–1153.


Snead OC 3rd (1996b) Relation of the \(\left[3H\right]\gamma\)-hydroxybutyric acid (GHB) binding site to the \(\gamma\)-aminobutyric acid\(_B\) (GABA\(_B\)) receptor in rat brain. *Biochem Pharmacol* 52:1235–1243.


**Address correspondence to:** Dr. Charles P. France, Department of Pharmacology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. E-mail: france@uthscsa.edu