The Role of GABA<sub>B</sub> Receptors in the Discriminative Stimulus Effects of γ-Hydroxybutyrate in Rats: Time Course and Antagonism Studies

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ABSTRACT

γ-Hydroxybutyrate (GHB) is a neurotransmitter in brain and an emerging drug of abuse, although its mechanism of action is poorly understood. This study characterized the role of GABA<sub>B</sub>, GABA<sub>A</sub>, and other receptors in the discriminative stimulus effects of GHB. Eight rats reliably discriminated 200 mg/kg GHB from saline after a median of 35 (range: 23–41) training sessions. GHB, a metabolic precursor 1,4-butanediol (1,4-BDL), and the GABA<sub>B</sub> agonist (±)baclofen all occasioned greater than 83% responding on the GHB lever. The onset of action was similar for GHB and 1,4-BDL; however, 1,4-BDL exhibited a longer duration of action than GHB. The GHB precursor γ-butyrolactone, the benzodiazepine diazepam, the neuroactive steroid pregnanolone, and the N-methyl-α-aspartate antagonist ketamine elicited substantial GHB-appropriate responding, although none occasioned greater than 66% drug-lever responding. The barbiturate pentobarbital and the GABA<sub>A</sub> receptor agonist muscimol did not occasion greater than 17% drug-lever responding at any dose tested. The benzodiazepine antagonist flumazenil attenuated GHB-lever responding occasioned by diazepam, but not GHB. The GABA<sub>B</sub> receptor antagonist CGP 35348 antagonized GHB-lever responding occasioned by baclofen or GHB. Small doses of the purported GHB receptor antagonist (2E)-(5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[a][7]annulen-6-yldene ethanoic acid (NCS-382) attenuated partially the effects of GHB, whereas larger doses of NCS-382 alone occasioned partial GHB-lever responding. These results implicate GABA<sub>B</sub> mechanisms in the discriminative stimulus effects of GHB and further suggest that the effects of 1,4-BDL under these conditions result from its conversion to GHB. That NCS-382 shares effects with GHB could explain the lack of antagonism reported for NCS-382 in some studies.

γ-Hydroxybutyrate (GHB) is a neurotransmitter in brain and an emerging drug of abuse (Roth and Giarman, 1966; Doherty et al., 1978). Widespread abuse of GHB was first reported in 1990 and has increased in recent years as evidenced by a dramatic increase in the number of patients treated for GHB intoxication (Bernasconi et al., 1999; Nicholson and Balster, 2001; Mason and Kerns, 2002). Exogenously administered GHB can induce anesthesia, sedation, absence seizures, coma, and death (Roth and Suhr, 1970; Sneed and Liu, 1993). GHB has been used clinically as an anesthetic, to treat alcoholism, and has been recently approved by the Food and Drug Administration for the treatment of narcolepsy (Mason and Kerns, 2002).

Although GHB is widely abused, its pharmacological profile differs from other drugs of abuse. GHB does not substitute for the discriminative stimulus effects of heroin, phencyclidine (PCP), ethanol, d-amphetamine, pentobarbital, or triazolam (Beardsley et al., 1996; Woolverton et al., 1999; Metcalf et al., 2001). Conditioned place preference has been reported for GHB in rats; however, many more exposures to GHB were required to induce preference as compared with other drugs of abuse (Martellotta et al., 1997). Drug-naive mice self-administer GHB (Martellotta et al., 1998), whereas rhesus monkeys trained to self-administer either methohexitol or PCP do not (Beardsley et al., 1996; Woolverton et al., 1999).

It is likely that multiple mechanisms contribute to the effects of GHB because it binds to multiple sites in brain and because it is metabolized to and from γ-aminobutyric acid (GABA) in vivo (Roth, 1970; Doherty et al., 1975; Gold and

ABBREVIATIONS: GHB, γ-hydroxybutyrate; GABA, γ-aminobutyric acid; 1,4-BDL, 1,4-butanediol; GBL, γ-butyrolactone; CGP 35348, (3-aminopropyl) (diethoxymethyl) phosphoric acid; NCS-382, (2E)-(5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[a][7]annulen-6-yldene ethanoic acid; FR, fixed ratio; PCP, phencyclidine; PT, pretreatment.
GABA receptors with regard to their ontogeny and regional logically relevant compounds. CGP 35348, and GHB receptor antagonist NCS-382 were clofen; suggested that the low-affinity sites are GABAB receptors. High-affinity [3H]GHB binding does not correlate with GABA_A receptors with regard to their ontogeny and regional distribution (Snead, 1996).

One approach for studying drugs with atypical mechanisms of action is drug discrimination, largely because of its pharmacological selectivity (Shannon and Holtzman, 1976; Mansbach and Balster, 1991; Terry et al., 1994). Among the small number of studies on GHB with these procedures, a range of conditions (gender, strain, reinforcer, task, dose, and route) have been used to study the discriminative stimulus effects of GHB. For example, female CFN-strain rats responded for water (Winter, 1981), whereas male Long-Evans rats traversed a T-maze for food (Colombo et al., 1995, 1998); smaller doses (i.p.) were studied in the former, larger doses (i.g.) in the latter. Previous studies in rats (Winter, 1981; Colombo et al., 1995, 1998; Metcalf et al., 2001) and pigeons (C.P.F., unpublished observation) discriminating GHB have implicated GABA receptors in the discriminative effects of GHB. In rats discriminating 200 mg/kg GHB i.p. (Winter, 1981) the GABAergic compounds baclofen, muscimol, and chloridiazepoxide occasioned GHB-appropriate responding whereas the non-GABAergic compounds PCP, lysergic acid diethylamide, d-amphetamine, and apomorphine did not. In rats discriminating 300 or 700 mg/kg GHB i.g. (Colombo et al., 1998) the GABAergic compounds baclofen and diazepam occasioned GHB-appropriate responding whereas dizocilpine and WIN 55,212–2 did not. The first goal of this study was to confirm and extend results from other laboratories, with a more widely used two-lever, fixed-ratio (FR) schedule of responding for food. Evaluation of compounds under these conditions should facilitate interpretation of the results within the context of a drug discrimination literature that is largely founded on this procedure. Second, although GABA is clearly important in the behavioral actions of GHB, several important issues are unresolved, including the relative importance of GHB receptors in the GHB discriminative stimulus. Thus, a second goal of this study was to examine the role of GABA and other receptors in the GHB discriminative stimulus by examining receptor-selective agonists and antagonists in combination. This is particularly important for GHB receptors, as antagonism of GHB is not unanimously obtained with NCS-382. A third goal was to characterize two metabolic precursors of GHB, 1,4-butanediol (1,4-BDL) and γ-butyrolactone (GBL); 1,4-BDL is reported to be used by GHB abusers, although the relative interchangeability of these compounds has not been studied.

Rats discriminating 200 mg/kg GHB were used to evaluate the following: metabolic precursors 1,4-BDL and GBL; GABA_A agonist muscimol; GABA_A positive modulators diazepam, pentobarbital, and pregabalinone; GABA_A agonist baclofen; µ-opioid agonist morphine; N-methyl-D-aspartate antagonist ketamine; and the GHB antagonist NCS-382. Time course was determined for GHB, 1,4-BDL, and GBL. The benzodiazepine antagonist flumazenil, GABA_B antagonist CGP 35348, and GHB receptor antagonist NCS-382 were studied in combination with GHB and with other pharmacologically relevant compounds.
training day in 2 of the 3 days prior to a test (including the day immediately prior to the test). Test sessions were identical to training sessions with the exception that completion of the PR on either lever resulted in food pellet delivery. Two or three tests were conducted weekly in each subject, depending on performance during intervening training sessions; drugs were studied using a single-dosing procedure. On days during which time course data were obtained, the response period was shortened to 5 min to evaluate the discriminative stimulus effects of the test compound at a more focused time after administration. The PT time in the operant chamber was 15 min, except when the PT time for the test was less than 15 min, in which case the PT time in the chamber was shortened accordingly. On days during which the PT time was greater than 15 min, the animal was returned to its home cage after administration of the test compound and placed in the operant chamber 15 min prior to the scheduled response period. All antagonists were administered 10 min prior to a test compound.

Drugs. Drugs were administered i.p. (pH 6 to 8) and dissolved or diluted in sterile water or saline unless otherwise noted. Compounds studied included GHB (sodium salt), pentobarbital sodium, muscimol, (±)-baclofen, GBL, and 1,4-BDL (Sigma-Aldrich Corp., St. Louis, MO), morphine sulfate (National Institute on Drug Abuse, Research Technology Branch, Rockville, MD), ketamine hydrochloride (Ketaset; Fort Dodge Laboratories Inc., Fort Dodge, IA), diazepam (Sigma/RBI, Natick, MA), pregnanolone (Steraloids, Newport, RI), flumazenil (a gift from F. Hoffmann LaRoche 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 (EL-620, a polyoxyethylated vegetable oil; GAF Corporation, Linden, NJ), 20% sterile water, and 10% ethanol (by volume). Pregnanolone was dissolved in 45% γ-cyclodextrin (Sigma-Aldrich) in sterile water. Flumazenil was dissolved in vehicle consisting of 50% saline, 40% propylene glycol, and 10% ethanol (by volume). Doses are expressed as the weight of the free base, except where indicated otherwise.

Data Analysis. The percentage of responses on the GHB lever during the response period is plotted as drug-appropriate responding (%DR). Rate of lever pressing on both levers is plotted in responses per second. Data are reported as the average ± S.E.M. for animals whose response rate was greater than 20% of their control vehicle rate. When a rat responded at a rate that was less than 20% of its vehicle control rate, discrimination data from that test were not included in the average. The control rate of responding was the average of the response rates from the 5 previous saline training days. Drugs (except for the antagonists flumazenil, CGP 35348, and NCS-382) were studied up to doses that markedly decreased rates of responding (Fig. 2). Baclofen occasioned 84.1 ± 12.5% drug-appropriate responding, but only at a dose that decreased the rate of responding to 14.4% of the vehicle control rate (Fig. 1).

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Results

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![Fig. 1. Effects of 1,4-BDL (□) and GBL (○) in rats trained to discriminate 200 mg/kg GHB (●) from saline. The percentage of responses on the drug-appropriate lever (%DR; top panel) and the rate of responding (responses/sec; bottom panel) are plotted as a function of dose. Data points and error bars represent the mean ± S.E.M. for 7 or 8 animals. The points above V represent data obtained after administration of the appropriate vehicle.](image1)

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13.1% drug-appropriate responding at a dose of 3.2 mg/kg. A dose of 5.6 mg/kg baclofen decreased rate of responding to less than 10% of the vehicle control rate (Fig. 2).

GABA<sub>A</sub> positive modulators diazepam and pregnanolone dose-dependently increased GHB-lever responding up to doses that markedly decreased rates of responding (Fig. 2). Diazepam occasioned 65.9 ± 17.3% drug-appropriate responding at a dose of 3.2 mg/kg. Pregnanolone elicited 28.4 ± 23.9% drug lever responding at 5.6 mg/kg. Diazepam and pregnanolone decreased rate of responding to 20% (or less) of the vehicle control rate at a dose of 10 mg/kg (Fig. 2). The GABA<sub>A</sub> direct-acting agonist muscimol and barbiturate-site positive modulator pentobarbital did not occasion greater than 17% GHB-appropriate responding at any dose tested, up to doses that markedly decreased rate of responding (Table 1).

Morphine and ketamine occasioned less than 40% drug-appropriate responding up to doses that decreased rate of responding (Table 1). Doses of 1.0 and 3.2 mg/kg morphine occasioned 37.1 ± 18.1% and 36.9 ± 20.7% drug-appropriate responding. Ketamine elicited 35.4 ± 17.3% and 32.2 ± 16.3% drug-appropriate responding at 1.0 and 3.2 mg/kg. Both morphine and ketamine decreased rate of responding to less than 16% of the vehicle control rate at 10 mg/kg (Table 1).

**Time Course Studies.** Discriminative stimulus effects of GHB were evident 15 min after administration (Fig. 3). At 30 min postinjection, the discriminative stimulus effects of 200 mg/kg GHB were still present as evidenced by substantial drug-appropriate responding. However, 60 min after administration of 200 mg/kg GHB, drug lever responding was similar to that observed with vehicle (Fig. 3). 1,4-BDL exhibited a longer duration of action than GHB, as evidenced by substantial drug-appropriate responding 60 and 90 min after administration (99.3 ± 0.3% and 40.7 ± 17.8%, respectively; Fig. 3). The largest dose of GBL that did not decrease responding (32 mg/kg) did not occasion greater than 40% GHB-appropriate responding when longer pretreatment intervals were used (upper panel, Fig. 3).

**Antagonism Studies.** The purported GHB antagonist NCS-382 in combination with 200 mg/kg GHB decreased drug-appropriate responding from 83.1 ± 12.5% (control) to 38.0 ± 16.9% at a dose of 32 mg/kg NCS-382 (Fig. 4). Doses of 10 and 32 mg/kg NCS-382 also attenuated the modest rate decreasing effects of 200 mg/kg GHB. At 56 and 100 mg/kg NCS-382 (in combination with GHB), drug-appropriate responding and rate of responding were not markedly different from 200 mg/kg GHB alone (Fig. 4). When administered alone, NCS-382 occasioned substantial responding on the GHB lever (56 and 100 mg/kg) and dose-dependently decreased rate of responding (Fig. 4, Table 1). A maximum of 49.5 ± 19.1% drug-appropriate responding was obtained with 100 mg/kg NCS-382.

GHB-lever responding occasioned by diazepam was attenuated dose-dependently by the benzodiazepine-site antagonist flumazenil (Fig. 5). In contrast, the same doses of flumazenil failed to antagonize the discriminative stimulus effects of 200 mg/kg GHB (closed symbols, upper panel, Fig. 5). Pretreatment with 5.6 mg/kg flumazenil decreased drug-appropriate responding occasioned by diazepam to less than 20% of control; the same dose of flumazenil had no clear effect on the discriminative stimulus effects of GHB. Rate-decreasing effects of GHB and diazepam were less when these drugs were studied in combination with flumazenil.

Baclofen elicited GHB-lever responding that was antagonized by the GABA<sub>B</sub> receptor antagonist CGP 35348 (Fig. 6). The same doses of CGP 35348 that attenuated GHB-appropriate responding occasioned by baclofen also dose-dependently antagonized the discriminative stimulus effects of the training dose of GHB (Fig. 6). Rate of responding with GHB or baclofen in combination with CGP 35348 was slightly higher than with either GHB or baclofen alone.

**Discussion.**

With reports of increased recreational use of GHB as well as the recent marketing of GHB for the treatment of narco-
lepsy there has been a renewed interest in understanding the mechanism(s) by which this molecule exerts abuse-related and therapeutic effects. Since its isolation, much has been learned about GHB, although the mechanisms that mediate the behavioral actions of GHB are not fully described. Among the many procedures that have been used to study GHB is drug discrimination, a procedure that has a high degree of pharmacologic specificity. Previous discrimination studies with GHB in rats (Winter, 1981; Colombo et al., 1995, 1998; Metcalf et al., 2001) suggested a role for GABAergic mechanisms; however, the extent to which GABA or other receptors mediate these effects of GHB and the extent to which other metabolically related compounds share effects with GHB were not fully explored. The current study established stimulus control with GHB in rats responding under conditions that have been used widely in drug discrimination research (two-lever, FR, food-maintained responding); for compounds that were studied in both the current procedure and with

Fig. 3. Time course for GHB (○), 1,4-BDL (○), and GBL (△). The percentage of responses on the drug-appropriate lever (%DR; top panel) and the absolute rate of responding (responses/sec; bottom panel) are plotted as a function of pretreatment time. Data points and error bars represent the mean ± S.E.M. for 7 animals.

Fig. 4. Effects of NCS-382 alone (○) and in combination (●) with 200 mg/kg GHB in rats trained to discriminate 200 mg/kg GHB from saline. Discrimination data for 32 mg/kg NCS-382; n = 6 (of 7 animals tested). 100 mg/kg NCS-382; n = 6 (of 8 animals tested). 178 mg/kg NCS-382; n = 4 (of 7 animals tested). 56 mg/kg NCS-382 + 200 mg/kg GHB; n = 5 (of 8 animals tested). See Fig. 1 for other details.

Fig. 5. Effects of flumazenil in combination with diazepam (△) and GHB (●) in rats trained to discriminate 200 mg/kg GHB from saline. Discrimination data for 1.0 mg/kg flumazenil in combination with diazepam; n = 4 (of 8 animals tested). 3.2 flumazenil in combination with diazepam; n = 5 (of 7 animals tested). See Fig. 1 for other details.

Fig. 6. Effects of CGP 35348 in combination with baclofen (△) and GHB (●) in rats trained to discriminate 200 mg/kg GHB from saline. Discrimination data for 10 mg/kg CGP 35348 in combination with baclofen; n = 6 (of 7 animals tested). See Fig. 1 for other details.
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other procedures, substitution data appear relatively consistent across different subjects, doses, routes of administration, gender, strain, and reinforcer. Moreover, the time required to establish stimulus control was similar to what has been reported for drug discrimination in rats with GHB or other drugs (Winter, 1981; Engel et al., 2001).

Abuse of other GHB-related compounds has reportedly increased, perhaps, because GHB was designated Schedule I (Mason and Kerns, 2002). This study provides clear evidence that one of these metabolic precursors of GHB, 1,4-BDL, shares discriminative stimulus effects with GHB. 1,4-BDL does not bind to high-affinity GHB sites, GABA_A or GABA_B receptors and is believed to exert its effects by conversion to GHB (Roth and Giarman, 1968; Benavides et al., 1982; Snead and Liu, 1984; Carter et al., 2002), an hypothesis that is consistent with the similar discriminative stimulus effects of these two compounds in rats as well as reports of 1,4-BDL use by human GHB abusers (Mason and Kerns, 2002). Discriminative stimulus effects of GHB were evident 15 min after administration, whereas an equi-effective dose of 1,4-BDL produced comparable effects 30 to 90 min after administration. The comparatively longer time course of 1,4-BDL is also consistent with 1,4-BDL being converted to GHB (Roth and Giarman, 1968; Maxwell and Roth, 1972; Snead et al., 1989). The longer duration of action of 1,4-BDL could be preferable in future studies with chronic dosing.

1,4-BDL and GBL are metabolized to GHB by different enzymes (alcohol dehydrogenase and peripheral lactonases, respectively); thus, differences in pharmacokinetics and distribution of enzymes could account for the comparatively smaller effect obtained with GBL (Roth et al., 1966; Maxwell and Roth, 1972; Gold and Roth, 1977; Snead et al., 1989). The limited effects that were obtained in this study with GBL, as well as with morphine and ketamine, are consistent with effects of the same or similar (PCP) drugs in female rats discriminating the same dose of GHB while under other conditions (Winter, 1981).

NCS-382 antagonizes some effects of GHB including increases in cGMP levels and inositol phosphate turnover in hippocampus, release of dopamine in striatum, and seizures in rats and mice (Maitre et al., 1990). However, under other conditions NCS-382 not only fails to antagonize GHB it can enhance some actions of GHB (Schmidt-Mutter et al., 1998; Carai et al., 2001; Cook et al., 2002); the mechanism of this enhancement is not established. In the current study smaller doses of NCS-382 partially attenuated GHB-lever responding; however, larger doses of NCS-382 failed to antagonize the discriminative stimulus effects of GHB and when these doses were studied alone they elicited considerable responding on the GHB-associated lever. Similarly under other conditions smaller doses of NCS-382 (25, 50 mg/kg) antagonized the discriminative stimulus effects of GHB, whereas larger doses (300, 500 mg/kg) enhanced the sedative/hypnotic effects of GHB (Colombo et al., 1995; Schmidt-Mutter et al., 1998; Carai et al., 2001). These biphasic actions of NCS-382 further support the notion that the GHB discriminative stimulus comprises multiple mechanisms. The likelihood of obtaining antagonism of GHB with NCS-382 appears to depend on dose as well as behavioral endpoint, as NCS-382 might be an antagonist at some receptors (e.g., GHB) and an agonist at others (e.g., GABA).

GABA_B receptors appear to play an important role in the discriminative stimulus effects of GHB. Baclofen occasioned greater than 84% GHB-lever responding in 7 of 8 animals, whereas the positive GABA_A modulators diazepam and pregnanolone occasioned this level of responding in fewer animals (4 of 8 and 4 of 7, respectively) and only at doses that markedly decreased responding. The inability of flumazenil to antagonize the discriminative stimulus effects of GHB is consistent with GHB not binding to benzodiazepine receptors (Serra et al., 1991). On the other hand, the selective GABA_B receptor agonist CGP 35348 attenuated GHB-lever responding occasioned by either baclofen or GHB, confirming a significant GABA_B component in these effects of GHB.

It is not clear whether the GABA_B-like activity of GHB results from it binding directly to GABA_B receptors or through the conversion of GHB to GABA. GHB and its antagonist NCS-382 do not alter [3H]GABA binding to GABA_B receptors and GABA_A receptor ligands do not affect [3H]GHB binding (Snead, 1996). GHB is less effective at displacing [3H]GABA from GABA_B receptors in the presence of the GHB dehydrogenase inhibitors valproate and ethosuximide (Hechler et al., 1997), further indicating that GHB is metabolized to GABA to act at GABA_B receptors. More recently (±)baclofen and GABA were shown to displace 20–30% of [3H]-NCS-382 binding in rat cerebral cortex (Mehta et al., 2001). Further evidence that GHB can act directly at GABA_B receptors was provided by Lingenhoehl et al. (1999), who showed that GHB activates recombinant GABA_B receptors in Xenopus oocytes. Thus, in vivo GHB might act both directly at GABA_B receptors and indirectly by its conversion to GABA.

GHB does not appear to interact directly with GABA_A receptors. It does not displace [3H]muscimol, [3H]flunitrazepam, or [3H]TBPS (3-tert-utylbicyclo-phosphorothionate) from the GABA_A receptor complex (Serra et al., 1991). Conversely, [3H]NCS-382 is not displaced by GABA_A receptor ligands such as muscimol and (+)bicuculline (Mehta et al., 2001). However, behavioral studies indicate that GHB exerts some effects through the GABA_A receptor complex. In previous studies diazepam, muscimol, and chlor diazepoxide occasioned substantial drug-appropriate responding (Winter, 1981; Colombo et al., 1998), although the doses required for these effects markedly decreased behavioral output (e.g., rate). Similarly, in the current study GHB-like responding occurred only with doses of diazepam that markedly decreased responding. Flumazenil reversed the rate decreasing effects of diazepam and GHB; however, it is unclear whether these effects were attributable to functional antagonism at benzodiazepine receptors or a general increase in rate occasioned by flumazenil. One hypothesized mechanism by which GHB could indirectly modulate the GABA_A receptor complex is by increasing brain concentrations of neurosteroids (Barraccia et al., 2002). If discriminative stimulus effects of GHB are mediated by neuroactive steroids, then neuroactive steroids that positively modulate the GABA_A receptor complex should occasion GHB-appropriate responding as should other positive modulators (e.g., barbiturates). Pregnanolone (and pentobarbital) elicited little GHB-appropriate responding, further suggesting only a minor role for GABA_A systems in these effects of GHB.

This study had several goals. First, stimulus control was established with GHB under conditions that are used widely in drug discrimination research and substitution data gen-
erated with this procedure support results from previous studies that used different parameters and subjects. The generality of the GHB stimulus, therefore, appears to extend across a range of experimental conditions. Second, the role of GABAergic, especially GABAA receptors, systems was firmly established both by substitution of baclofen for GHB and, more importantly, by unequivocal antagonism of both baclofen and GHB by CGP 35448. Although GABAA receptors might also contribute to these effects of GHB, their contribution is comparatively small. Third, results from this study strongly suggest that the inconsistent literature on NCS-382 with GHB is due to GHB, and possibly NCS-382, exerting effects through more than one mechanism. Finally, greater difficulty in obtaining GHB is thought to have stimulated increased recreational use of pharmaco logically related compounds such as 1,4-BDL. This study demonstrates a qualitative similarity between GHB and its precursor 1,4-BDL. To the extent that the pharmacodynamics of 1,4-BDL and GHB are qualitatively the same, 1,4-BDL might be expected to have multiple mechanisms of action and to have abuse liability similar to that of GHB.

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