Discriminative Stimulus Effects of γ-Hydroxybutyrate in Pigeons: Role of Diazepam-Sensitive and -Insensitive GABA<sub>A</sub> and GABA<sub>B</sub> Receptors

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ABSTRACT

γ-Hydroxybutyrate (GHB) is an emerging drug of abuse with multiple mechanisms of action. This study is part of an effort to examine the role of GHB, GABA<sub>A</sub>, and GABA<sub>B</sub> receptors in the discriminative stimulus (DS) effects of GHB. In pigeons trained to discriminate 100 mg/kg GHB from saline, GHB and its precursors γ-butyrolactone and 1,4-butanediol produced 80 to 100% GHB-appropriate responding, whereas other compounds such as morphine, naltrexone, cocaine, and haloperidol produced no more than 34%. Compounds interacting with GABA receptors produced different maximal levels of GHB-appropriate responding. For example, the GABA<sub>A</sub> agonist muscimol produced 3%; the GABA<sub>A</sub>-positive modulators diazepam, pentobarbital, and ethanol, and the GABA<sub>B</sub> agonist baclofen produced levels ranging from 54 to 73%; and the benzodiazepine antagonist flumazenil and inverse agonist Ro 15-4513 produced no more than 34%. Compounds interacting with GABA receptors produced different maximal levels of GHB-appropriate responding. For example, the GABA<sub>A</sub> agonist muscimol produced 3%; the GABA<sub>A</sub>-positive modulators diazepam, pentobarbital, and ethanol, and the GABA<sub>B</sub> agonist baclofen produced levels ranging from 54 to 73%; and the benzodiazepine antagonist flumazenil and inverse agonist Ro 15-4513 produced 96%. The putative GHB receptor antagonist (2E)-5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[a][7]annulen-6-yldene ethanoic acid (NCS-382) produced 70% GHB-appropriate responding. The GABA<sub>A</sub> antagonist (3-aminopropyl)(diethoxymethyl)phosphinic acid (CGP 35348) completely blocked the GHB-like DS effects of NCS-382 and baclofen at a dose of 56 mg/kg. CGP 35348 also blocked the DS effects of GHB, but incompletely and only at a dose of 560 mg/kg. Together, these results are consistent with a role for diazepam-sensitive and -insensitive GABA<sub>A</sub> and GABA<sub>B</sub> receptors in the DS effects of GHB. Together with previous findings, the present results suggest that diazepam-insensitive GABA<sub>A</sub> receptors are more prominently involved in the DS effects of GHB in pigeons than in rats, whereas GABA<sub>B</sub> receptors are less prominently involved. Exploring the role of GHB receptors with NCS-382 is hampered by its GABA<sub>A</sub> receptor-mediated, GHB-like agonist activity.

γ-Hydroxybutyrate (GHB) has therapeutic use and a recent history of abuse (for review, see Nicholson and Balster, 2001). GHB was recently approved by the Food and Drug Administration to treat narcolepsy and is thought to have therapeutic potential in treating alcoholism (Gallimberti et al., 2001). GHB was recently approved by the Food and Drug Administration to treat narcolepsy and is thought to have therapeutic potential in treating alcoholism (Gallimberti et al., 2001). GHB occurs naturally in the brain, where it is believed to function as a neurotransmitter (for review, see Maitre et al., 2000). Its specific binding sites, which can be investigated with the selective radioligand [3H]NCS-382 (Mehta et al., 2001), seem to be G-protein-coupled receptors. GHB may also function as a neuromodulator, affecting, for example, the activity of central dopaminergic neurons in a naloxone-reversible manner (Feigenbaum and Howard, 1997). Its precise roles in central nervous system function, however, are not thoroughly understood.

GHB may exert its effects not only through specific GHB receptors, but also through other mechanisms. GHB can interact directly with GABA<sub>B</sub> receptors (Lingenhoehl et al., 1999) and indirectly with all GABA receptor subtypes because it can be metabolically converted to GABA (Vayer et al., 1985). A role for GABA<sub>B</sub> systems is suggested by the finding that the discriminative stimulus (DS) effects of GHB are mimicked by the GABA<sub>B</sub> receptor agonist baclofen and...
Drug discrimination has proven to be useful in identifying mechanisms of action because it provides sensitive and selective assays of in vivo effects of behaviorally active drugs (Colpaert and Balster, 1988). Rats can discriminate GHB from saline (Winter, 1981; Colombo et al., 1995a,b, 1998; Metcalf et al., 2001; Carter et al., 2003), and its effects seem to be selective because pharmacologically unrelated drugs (e.g., phencyclidine and ketamine) do not occasion GHB-appropriate responding (Winter, 1981; Carter et al., 2003). Also, GHB does not occasion drug-appropriate responding in discriminations that use training drugs pharmacologically unrelated to GHB (Beardsley et al., 1996; Woolverton et al., 1996). The aforementioned drug discrimination studies in rats suggest that the DS effects of GHB involve multiple mechanisms, with a prominent role for GABA<sub>B</sub> receptors, a less prominent role for GABA<sub>A</sub> receptors, and a role for GHB receptors that has not yet been clearly delineated. The present study, which is part of an effort to explore the generality of these findings across species, was aimed at examining the role of GHB, GABA<sub>B</sub>, and GABA<sub>A</sub> receptors in the DS effects of GHB in pigeons.

Although results of drug discrimination studies in rats and pigeons often seem to be similar, detailed comparisons have shown some important differences, particularly with compounds that interact with GABA receptors. For example, pentobarbital has been reported to substitute fully for the benzodiazepine midazolam in midazolam-trained rats (Gar-cha et al., 1985; Woudenberg and Slanger, 1989) but not in midazolam-trained pigeons (Evans and Johanson, 1989), squirrel monkeys (Spealman, 1985), and baboons (Ator, 2003). As suggested by others (Ator, 2003), positive modulation of GABA through the barbiturate site is generally not sufficient to produce midazolam-like DS effects in pigeons, squirrel monkeys, and baboons, although it is sufficient in rodents. Thus, it is important to examine the DS effects of drugs that interact with GABA receptors, such as GHB, in different species.

Pigeons discriminating 100 mg/kg GHB from saline were used to evaluate the following: 1) possible GHB-like DS effects of metabolic precursors of GHB [i.e., 1,4-butanediol (1,4-BDL) and γ-butyrolactone (GBL)]; 2) the role of dopamine and opioid systems in the DS effects of GHB; 3) the role of GHB, GABA<sub>B</sub>, and GABA<sub>A</sub> receptors in the DS effects of GHB, as determined by studying whether GHB, the GABA<sub>B</sub> agonist baclofen, the GABA<sub>A</sub> agonist muscimol, and the positive GABA<sub>A</sub> modulators diazepam, pentobarbital, and ethanol produce GHB-like DS effects and whether these effects can be antagonized by the GHB antagonist NCS-382, the GABA<sub>B</sub> antagonist CGP 35348, and the benzodiazepine antagonist flumazenil; and 4) the possibility that greater GHB-like DS effects can be produced by adding GABA<sub>A</sub> and GABA<sub>B</sub> receptor activation, as determined by studying whether combinations of baclofen and diazepam produce more GHB-appropriate responding when given together rather than when given alone.
mal by log-linear interpolation, and the log values of these doses were averaged to obtain a mean \( D_{50} \) value and its 95% confidence limits (CL). Potency differences were analyzed by performing Student’s \( t \) tests for related measures on individual log \( D_{50} \) values. Response-rate data were analyzed using one-way repeated measures analysis of variance for each drug that was followed, where appropriate, by comparing the results of each dose with the vehicle control rate by Dunnett’s test.

**Drugs.** The compounds used in this study were dissolved in sterile water or saline, unless otherwise noted, and included the following: (±)-baclofen, 1,4-BDL, GBL, haloperidol, muscimol, and pentobarbital sodium (Sigma-Aldrich, St. Louis, MO); cocaine hydrochloride, morphine sulfate, and naltrexone hydrochloride (NIDA Research Technology Branch, Rockville, MD); diazepam (Sigma/RBI, Natick, MA); flumazenil and Ro 15-4513 (gifts from F. Hoffmann-La Roche, Basel, Switzerland). NCS-382 (sodium salt) and CGP 35348 (sodium salt) were synthesized as previously described (Maître et al., 1990; Froestl et al., 1995). Diazepam was dissolved at different concentrations in the same vehicle consisting of 70% Emulphor, 20% 50% saline, 40% propylene glycol, and 10% ethanol (by volume). All compounds were injected i.m. in a volume of 0.1 to 1.0 ml except GHB, which was also administered by gavage in a volume of 0.5 to 5 ml (depending on dose) 10 min before sessions, and ethanol, which was only administered by gavage. The dose of ethanol was manipulated by varying the volume of a 20% \((v/v)\) solution of ethanol in distilled water. Doses are expressed as the form of the drug listed above.

**Results**

All six animals acquired the 100 mg/kg GHB versus saline discrimination (median sessions to criterion: 31.5, range: 19–75). The accuracy of postcriterion discrimination performance during the remainder of the study, which did not differ significantly between drug and saline training sessions, was 78.2 ± 2.8% responses (mean ± S.E.M.) on the injection-appropriate key.

Under test conditions, GHB increased responding dose dependently on the GHB-appropriate key \((D_{50} = 39 \text{ mg/kg}, 95\% \text{ CL 18–83})\) to a maximum of 99 to 100% at 100 and 178 mg/kg (Fig. 1, top panel). Only a higher dose (320 mg/kg) significantly decreased response rate (Fig. 1, bottom panel). GHB had similar effects when given by gavage, producing 100% GHB-appropriate responding \((D_{50} = 239, 95\% \text{ CL 144–398})\) and decreasing response rate (at 1000 mg/kg), but it was significantly less potent than when administered i.m. (p.o./i.m. potency ratio for GHB-appropriate responding in animals tested both p.o. and i.m. = 5.2, 95% CL 1.9–14.5). The GHB precursor GBL increased GHB-appropriate responding to a maximum of 95% with a potency \((D_{50} = 24, 95\% \text{ CL 15–38})\) similar to that of GHB and decreased response rate (at 100 mg/kg). The GHB precursor 1,4-BDL also increased GHB-appropriate responding (to a maximum of 80%), was significantly less potent \((D_{50} = 128, 95\% \text{ CL 60 - 271})\) than GHB and GBL, and did not significantly affect response rate at any of the doses tested.

GHB produced DS effects in a time-dependent manner. The percentage of GHB-appropriate responding observed with the training dose reached a maximum at 15 min and decreased to 0 at 90 min (Fig. 2). GHB produced DS-like responding with a duration of action similar to that of GHB, but because the rate-decreasing effects of 56 mg/kg were statistically significant at 10 min but not later, GBL may have a faster onset. The DS effects of 1,4-BDL had a slower onset and a longer duration than those of GHB and GBL. Because the DS effects of 1,4-BDL reached a maximum at 60
min after its administration, the dose-response data of 1,4-BDL shown in Fig. 1 were obtained using a 60-min pretreatment.

Morphine produced no more than 19.2% GHB-appropriate responding at 1 mg/kg. A higher dose suppressed responding to less than 20% of vehicle control in five of the six animals tested (Fig. 3, left panels). Naltrexone produced at most 17% GHB-appropriate responding when given alone and, except at a dose of 1 mg/kg, did not significantly affect response rate. Cocaine and haloperidol produced at most 21 and 34% GHB-appropriate responding, respectively, when tested up to and including doses that markedly decreased responding (Fig. 3, right panels).

The GABA<sub>B</sub> agonist baclofen produced a maximum of 66% GHB-appropriate responding at a dose (17.8 mg/kg) that did not significantly affect the rate of responding (Fig. 4, left panels). At a higher dose (32 mg/kg), baclofen markedly decreased responding in more than half the animals tested. The putative GHB receptor antagonist NCS-382 produced GHB-appropriate responding when given alone and produced 70% GHB-appropriate responding at a dose (320 mg/kg) that also significantly decreased response rate. Compounds interacting with GABA<sub>A</sub> receptors (Fig. 4, right panel) increased the percentage of GHB-appropriate responding to different extents, with maximal effects ranging from 3% (obtained with the GABA<sub>A</sub> receptor agonist muscimol) to intermediate levels (54, 68, and 73%, as observed with the positive GABA<sub>A</sub> modulators diazepam, ethanol, and pentobarbital, respectively) to levels similar to that obtained with the training dose of GHB (96%, as observed with the benzodiazepine antagonist flumazenil and the negative GABA<sub>A</sub> modulator Ro 15-4513). The potency of Ro 15-4513 to produce GHB-appropriate responding (D<sub>50</sub> = 0.069 mg/kg, 95% CL 0.029–0.17) was similar to that of flumazenil (D<sub>50</sub> = 0.047, 95% CL 0.017–0.13). Ro 15-4513, however, had no significant effects on response rate.

When tested alone, the training dose of GHB produced 99%

**Discussion**

The number of sessions needed to acquire the GHB discrimination in pigeons was similar to those reported with a training dose of 200 mg/kg GHB in rats (Winter, 1981; Carter et al., 2003), suggesting that the discriminability of 100 mg/kg GHB administered i.m. to pigeons is not markedly different than that of 200 mg/kg GHB administered i.p. to rats. The lowest dose that suppressed responding in pigeons was 320 mg/kg, as was the case in rats (Carter et al., 2003). Thus, pigeons can reliably discriminate GHB from vehicle, and their sensitivity to the DS and rate-decreasing effects of GHB does not seem to differ markedly from that of rats.

GBL and 1,4-BDL produced GHB-like DS effects, consistent with the hypothesis that they exert their effects because of metabolism to GHB (Roth et al., 1966; Roth and Giorman, 1968). The faster onset of GBL versus GHB is compatible with the hypothesis that the bioavailability of GHB as a metabolite of GBL is greater than after administration of GHB itself because GBL is more rapidly absorbed than GHB (Lettieri and Fung, 1978). GBL and 1,4-BDL produced more than 80% GHB-appropriate responding in pigeons, whereas only 1,4-BDL did so in rats (Carter et al., 2003). GBL produced at most 50% GHB-appropriate responding in rats (Winter, 1981; Carter et al., 2003). GBL and 1,4-BDL are metabolized to GHB by different enzymes, which has been suggested to account for the low level of GHB-appropriate responding obtained with GBL in rats (Carter et al., 2003). That 1,4-BDL has GHB-like DS effects in pigeons and rats is consistent with reports of recreational 1,4-BDL use by human GHB abusers (Mason and Kerns, 2002). Its marked GHB-like effects observed in pigeons here are consistent with GBL being similarly abused (Graeme, 2000).

Although GHB modulates dopamine function (Feigenbaum and Howard, 1997), neither cocaine nor haloperidol produced more than 35% GHB-appropriate responding, and haloperidol did not attenuate the DS effects of GHB. Also, neither morphine nor naltrexone produced more than 20% GHB-appropriate responding, and naltrexone did not attenuate the DS effects of GHB. These results, which confirm and extend those obtained in GHB-discriminating rats (Winter,

**Fig. 3.** Effects of morphine and naltrexone (left panels) and of cocaine and haloperidol (right panels) in pigeons (n = 6) trained to discriminate 100 mg/kg GHB from saline. See Fig. 1 for other details.
1981; Carter et al., 2003), suggest that the DS effects of GHB do not primarily involve modulation of dopamine or opioid systems.

Studies in rats (Winter, 1981; Colombo et al., 1995a, 1998; Metcalf et al., 2001; Carter et al., 2003) implicate GABA receptors in the DS effects of GHB. Consistent with this view, the GABA<sub>B</sub> agonist baclofen produced intermediate GHB-like responding (66%) in pigeons. In rats, baclofen produced intermediate responding in one study (Winter, 1981) but fully substituted for GHB in two other studies (Colombo et al., 1998; Carter et al., 2003). Baclofen was at least 80-fold more potent than GHB in rats (Carter et al., 2003) but was only 4-fold more potent than GHB in pigeons. In the present study, CGP 35348 completely blocked the GHB-like effects of baclofen at a dose of 56 mg/kg while incompletely attenuating the DS effects of GHB even at a 10-fold higher dose. In contrast, CGP 35348 blocked the DS effects of GHB in rats at the same dose (56 mg/kg) that blocked the GHB-like DS effects of baclofen (Carter et al., 2003). That baclofen was less potent and less efficacious in producing GHB-like DS effects in pigeons than in rats, and that CGP 35348 was less potent and less able to antagonize the DS effects of GHB in pigeons than in rats, suggests a possible species difference in the extent to which GABA<sub>B</sub> receptors are involved in these effects. The relative importance of GABA<sub>B</sub> mechanisms, however, may also be influenced by the training dose of GHB (Colombo et al., 1998). Varying the training dose and temporal conditions in both species could help to delineate conditions that determine to what extent GABA<sub>B</sub> receptors are involved in the DS effects of GHB.

GABA<sub>A</sub> receptors may also play a role in the DS effects of GHB. Ethanol, which positively modulates GABA<sub>A</sub> receptors, produced 68% GHB-appropriate responding, which is in the middle of the range of values previously reported in rats, i.e., 31% (Winter, 1981), 71% (Metcalf et al., 2001), and 91% (Colombo et al., 1995b). The maximal effect of pentobarbital, which positively modulates GABA<sub>A</sub> through the barbiturate site, was 73%. In contrast, in a previous study in rats (Carter et al., 2003), pentobarbital did not produce any GHB-appropi-
appropriate responding. However, the doses of pentobarbital that could be evaluated for possible GHB-like DS effects in the present study in pigeons and in the previous study in rats differed markedly, possibly due to a differential sensitivity to its response rate-decreasing effects. In rats, the highest dose tested for GHB-like DS effects was 3.2 mg/kg pentobarbital because 10 mg/kg eliminated responding in all animals. In pigeons, however, pentobarbital produced GHB-appropriate responding only at 10 and 17.8 mg/kg without affecting response rate and eliminated responding at 32 mg/kg. Diazepam produced no more than 54% GHB-appropriate responding. In contrast, the GHB precursors GBL and 1,4-BDL did not produce midazolam- or pentobarbital-appropriate responding (McMahon et al., 2003). This apparent asymmetrical generalization resembles that between ethanol and benzodiazepines; i.e., whereas benzodiazepines produce ethanol-appropriate responding in ethanol-trained animals, ethanol does not produce benzodiazepine-appropriate responding in benzodiazepine-trained animals (De Vry and Slangen, 1986). Together, the maximum percentage of GHB-appropriate responding obtained with the GABA_A receptor modulators ethanol (68%), pentobarbital (73%), and diazepam (54%), and with the GABA_A receptor agonist muscimol (3%), seem to be consistent with a limited role for GABA_A receptors in the DS effects of GHB under these conditions. The finding that diazepam and baclofen did not produce more GHB-appropriate responding when given together than when given alone suggests that full GHB-like DS effects cannot be produced by adding GABA_A and GABA_B receptor activation. Thus, the DS effects of GHB in pigeons involve other (possibly GHB) receptors in addition to GABA_A and GABA_B receptors.

GHB may exert its effects not only through GABA receptors, but also through specific GHB receptors that can be investigated with [3H]NCS-382 (Mehta et al., 2001). NCS-382 was initially reported to antagonize several effects of GHB (e.g., increased inositol phosphate turnover in hippocampus and increased dopamine release in striatum) and GHB-induced seizures (Maitre et al., 1990). Since that time, NCS-382 has been reported to antagonize some behavioral effects of GHB, such as self-administration in mice (Martel-lotta et al., 1998) and DS effects in rats (Colombo et al., 1995a). More recent studies, however, suggest that the antagonism by NCS-382 of the behavioral depressant effects of GHB is very limited (Carai et al., 2001; Cook et al., 2002; Lamb et al., 2003) and that NCS-382 can enhance certain effects of GHB, such as loss of righting (Carai et al., 2001). In a recent study of rats, NCS-382 partially attenuated the DS effects of GHB, but its antagonism was limited by partial GHB-like effects when given alone (Carter et al., 2003). In this study, NCS-382 failed to attenuate the DS effects of GHB and when given alone produced substantial (70%) GHB-appropriate responding. NCS-382-induced GHB-appropriate responding was antagonized by CGP 35348, suggesting that NCS-382 has agonist actions at GABA_B receptors. GABA_B receptors have been reported to mediate the GHB-like effects of NCS-382 on intestinal motility in mice (Carai et al., 2002). The present results extend this finding to NCS-382-induced GHB-like DS effects and suggest that GABA_B receptors may be involved in the enhancement of some actions of GHB by NCS-382. Selective GHB receptor antagonists that lack GHB-like effects would greatly facilitate the study of drug actions at specific GHB receptors. Because of its sensitivity in detecting GHB-like effects of NCS-382, a
GHB discrimination in pigeons may be useful in the search for such antagonists.

At 5.6 mg/kg, flumazenil blocked the GHB-like effects of diazepam in rats (Carter et al., 2003). In this study, flumazenil did not attenuate the GHB-like effects of diazepam; instead, it substituted completely for GHB when given alone at 0.1 mg/kg. Flumazenil can function as a DS in monkeys (Gerak and France, 1999), rats (De Vry and Slanger, 1985; Woudenberg and Slanger, 1990), and pigeons (Wong et al., 1993). In monkeys treated daily with diazepam, flumazenil (0.32 mg/kg) has DS effects that are related to its antagonist actions at diazepam-sensitive GABA_A receptors (Gerak and France, 1999) and that are not mimicked by GBL and 1,4-BDL (McMahon et al., 2003), suggesting that GHB and its precursors lack antagonistic actions at diazepam-sensitive GABA_A receptors. In contrast, in monkeys not treated daily with diazepam, the DS effects of flumazenil (1 mg/kg) seem to involve other mechanisms (Gerak and France, 1999). Whereas a flumazenil discrimination in rats has been demonstrated only at doses of 10 mg/kg and higher, pigeons discriminate a 100-fold lower dose of flumazenil. In 0.1 mg/kg flumazenil-trained pigeons, only compounds with high affinity for diazepam-insensitive GABA_A receptors produce flumazenil-appropriate responding (e.g., Ro 15-4513), with potencies that correlate positively with their affinities at diazepam-insensitive GABA_A receptors, suggesting that the DS effects of flumazenil in this species are mediated by diazepam-insensitive GABA_A receptors (Wong et al., 1993; Acri et al., 1995, 1997). Despite the opposite functioning of flumazenil and Ro 15-4513 at diazepam-sensitive GABA_A receptors, both compounds produced GHB-appropriate responding, with similar potency. This agrees with the finding that Ro 15-4513 and flumazenil have similar potency to produce flumazenil-like DS effects in pigeons (Wong et al., 1993), and is consistent with a role for diazepam-insensitive GABA_A receptors, for which both have high affinity (Wong et al., 1993), in their GHB-like effects. Thus, a GHB discrimination in pigeons may be especially useful in investigating the role of diazepam-insensitive GABA_A receptors in the effects of GHB.

In summary, the DS effects of GHB in pigeons involve multiple mechanisms and seem to be similar, but not identical, to those in rats. In pigeons, diazepam-insensitive GABA_A receptors may be more prominently involved in the DS effects of GHB than in rats, whereas GABA_B receptors may be less prominently involved. Varying the training dose of GHB in both species will help to examine the extent to which the DS effects of GHB are species-dependent and could help to delineate the conditions that determine the relative importance of diazepam-sensitive and -insensitive GABA_A and GABA_B receptors in these effects.

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