Novel gamma-hydroxybutyric acid (GHB) analogs share some, but not all, of the behavioral effects of GHB and GABAB receptor agonists

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Abbreviations: GHB, γ-hydroxybutyrate; GABA, γ-aminobutyric acid; 1,4-BDL, 1,4-butanediol; GBL, γ-butyrolactone; 3-HPA, 3-hydroxyphenylacetic acid; SKF97541, 3-aminopropyl(methyl)phosphinic acid hydrochloride; CGP35348, 3-aminopropyl(diethoxymethyl)phosphinic acid; [³H]NCS-382, (5-[³H]-(2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[a][7] annulen-6-ylidene) ethanoic acid)

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Abstract

Gamma-hydroxybutyrate (GHB), a therapeutic for narcolepsy and a drug of abuse, has several mechanisms of action, which involve GHB and $GABA_B$ receptors, metabolism to GABA, and modulation of dopaminergic signaling. The aim of these studies was to examine the role of GHB and $GABA_B$ receptors in the behavioral effects of GHB. Three approaches were used to synthesize GHB analogs that bind selectively to GHB receptors and are not metabolized to GABA-active compounds. Radioligand binding assays identified UMB86, UMB72, UMB73, 2-HPA, 3-HPA, and 4-hydroxy-4-phenylbutyric acid as compounds that displace [^{3}H]NCS-382 from GHB receptors at concentrations that do not markedly affect [^{3}H]GABA binding to $GABA_B$ receptors. In rats and pigeons, GHB discriminative stimulus effects were not mimicked or attenuated by UMB86, UMB72, or 3-HPA, up to doses that decreased responding. In mice, GHB, GHB precursors (GBL and 1,4-BDL), and $GABA_B$ receptor agonists (SKF97541 and baclofen) dose-dependently produced hypolocomotion, catalepsy, ataxia, and loss of righting. The $GABA_B$ receptor antagonist CGP35348 attenuated catalepsy and ataxia that was observed after GHB and $GABA_B$ receptor agonists SKF97541 and baclofen. UMB86, UMB72, UMB73, and 3-HPA, like GHB, produced hypolocomotion, ataxia, and loss of righting; however, catalepsy was never observed with these compounds, which is consistent with the cataleptic effects of GHB being mediated by $GABA_B$ receptors. Ataxia that was observed with UMB86, UMB72, UMB73, and 3-HPA was not antagonized by CGP35348, suggesting that ataxia induced by these analogs is not mediated by $GABA_B$ receptors and might involve GHB receptors.

γ-Hydroxybutyrate (GHB) is a putative neurotransmitter and neuromodulator in brain (Maitre, 1997), a therapeutic for alcoholism (Poldrugo and Addolorato, 1999) and narcolepsy (Fuller and Hornfeldt, 2003), and a drug of abuse (Degenhardt et al., 2003; McDonough et al., 2004). GHB binds to GHB receptors (Benavides et al., 1982; Mehta et al., 2001) and γ-aminobutyric acid (GABA)$_B$ receptors (Xie and Smart, 1992; Lingenhoehl et al., 1999), and it is metabolized to GABA, which can act at GABA receptors. GHB has also been hypothesized to modulate GABA$_A$ receptor function through GABA$_B$ receptor-mediated increases in neurosteroid concentrations in rat brain (Barbaccia et al., 2002). Furthermore, several studies have reported that GHB modulates dopaminergic transmission, although the mechanism of this modulation is not well understood (Feigenbaum and Howard, 1996).

Effects of exogenously administered GHB include sedation, seizures, catalepsy, ataxia, amnesia, coma, and death (Snead and Liu, 1993; Navarro et al., 1998; Mason and Kerns, 2002). The wide range of behavioral effects that can be observed following administration of GHB is likely due to its multiple mechanisms of action. Specific GHB receptors have been hypothesized (Benavides et al., 1982; Snead and Liu, 1984, Hechler et al., 1987; Castelli et al., 2000, Mehta et. al., 2001) and recently cloned (Andriamampandry et al., 2003). However, behavioral studies suggest an important role for GABA$_B$ receptors (Carai et al., 2001; Carai et al., 2002a; Carter et al., 2003; Carter et al. 2004a; Colombo et al., 1998; Lobina et al., 1999; Ren and Mody, 2003), consistent with the finding that GHB binds to these receptors (Bernasconi et al., 1992; Xie and Smart, 1992; Mathivet et al., 1997; Lingenhoehl et al., 1999).

The relative importance of GHB receptors in mediating behavioral effects of GHB is not clear, in part because GHB has direct and indirect effects at GABA receptors. Thus, GHB receptor-selective ligands are critical tools to examine the role of GHB receptors in the various effects of GHB and related compounds. We recently reported the synthesis of the GHB receptor-selective analog 4-hydroxy-4-methylpentanoic acid (UMB68). UMB68 and GHB have similar affinities for (5-[^{3}H]-(2E)-(5-hydroxy-5,7,8,9-tetrahydro-6H-benzo[a][7] annulen-6-ylidene) ethanoic acid)-labeled ([^{3}H]NCS-382-labeled) GHB receptors; however, unlike GHB, UMB68 does not have affinity for GABA$_B$ receptors, and does not have GHB-like discriminative stimulus effects in rats (Wu et al., 2003).

In the present study, three approaches introduced aromatic substituents on the GHB molecule in order to prevent metabolism to compounds with affinity for GABA receptors. First, it has recently been shown (Macias et al., 2004) that the 4-hydroxyl group of GHB acts as a hydrogen bond acceptor when bound to GHB receptors, and not as a donor, as suggested previously (Bourguignon et al., 2000). Protecting the 4-hydroxyl of GHB as an aryl alkyl ether (UMB72, UMB73, and UMB87; Fig. 1) simultaneously converts the readily oxidizable alcohol function to the more inert ether function (Block and Beale, 2004), introduces an aromatic substituent, and allows for the retention of a hydrogen bond acceptor. Second, a phenolic hydroxyl cannot undergo metabolism to an amino group and also contains the required aromatic group. The presence of such a group in 2-HPA and 3-HPA (Fig. 1) results in a conformationally restricted analog of GHB with a similar distance between the acid and the hydroxyl group. Third, 4-hydroxy-4-phenylbutyric acid has a sterically demanding phenyl ring in the 4-position, and was

previously shown to have affinity for [³H]GHB-labeled receptors (Bourguignon et al., 2000). The same report showed that introducing 4-benzyl groups in the GHB molecule further increases the affinity for [³H]GHB-labeled receptors. UMB86 (Fig. 1) has a napthyl group that occupies the same space as the phenyl group in the 4-benzyl analogs, while its steric bulk close to the alcohol would be expected to hinder the alcohol towards enzymatic oxidation (Block and Beale, 2004).

Of the compounds synthesized, first, 2 or 3 compounds from each of the three synthetic approaches were evaluated in the binding assays to determine if compounds from different structural classes bound selectively to GHB receptors. [³H]NCS-382 was used to examine the specific binding of GHB analogs to GHB receptors in rat cerebrocortical membranes (Mehta et al., 2001). The same analogs were also evaluated in different membrane preparations developed to study the specific binding of [³H]GABA to GABA$_A$ or GABA$_B$ receptors. Second, a representative compound from each chemical approach was examined in drug discrimination experiments. Drug discrimination data in rats have shown that GHB precursors and compounds that act at GABA$_B$ receptors occasion substantial GHB-appropriate responding, whereas pharmacologically unrelated compounds do not (Winter, 1981; Carter et al., 2003; Baker et al., 2004). Compounds were also examined in pigeons discriminating GHB because recent studies suggest that the discriminative stimulus effects of GHB in this species involve not only GABA$_B$ receptors, but also GABA$_A$ receptors (Koek et al., 2004). Third, mice were used to study the effects of the same compounds, with the addition of UMB73, on locomotion, catalepsy, ataxia, and righting, alone, and together with a GABA$_B$ receptor antagonist.

These studies were designed to compare the effects of a broad range of doses, up to and including doses that produced loss of righting or at which other limitations became apparent, of GHB and GHB precursors (GBL and 1,4-BDL), conventional GABA$_B$ receptor agonists (SKF97541 and baclofen), and selective GHB analogs (UMB86, UMB72, UMB73, and 3-HPA) on several unconditioned behaviors that are known to be affected by GHB in mice (Carai et al., 2001; Cook et al., 2002; Itzhak and Ali, 2002). GHB precursors and GABA$_B$ receptor agonists were also examined in the observational assays in mice because they serve as important controls and have been studied in the same binding and discrimination procedures (Mehta et al., 2001 and Carter et al., 2003). GBL and 1,4-BDL serve as positive controls for GHB because these compounds exert their GHB-like effects through conversion to GHB and they do not displace [^{3}H]GABA or [^{3}H]NCS-382 from rat cerebral cortex (Carter et al., 2002; Carter et al., 2003). Similarly, the prototypical GABA$_B$ receptor agonists SKF97541 and baclofen serve as positive controls. Given that GHB can produce effects through multiple mechanisms of action, it is important to compare the effects of GHB to those produced by conventional GABA$_B$ receptor agonists, as well as to related compounds that are used recreationally.

Materials and Methods

Drugs.

All chemicals used in the synthesis of the test compounds were purchased from Sigma-Aldrich (USA). All compounds showed ^{1}H NMR (300 MHz, D_2O) and mass spectra (m/z, Finnegan LCQ, negative ion mode) consistent with their assigned structures. Elemental analyses were performed by Atlantic Microlabs Inc. (USA), and were within ± 0.4% of theory. 4-Hydroxy-4-napthylbutanoic acid, sodium salt (UMB86) was prepared by adding sodium hydroxide to a methanol solution of γ-napthylbutyrolactone and stirring for 2 h. After removal of the solvent, UMB86 was re-crystallized from methanol (mp = 181-182°C). 4-(3-Phenylpropyloxy)butyric acid, sodium salt (UMB72) and 4-benzyloxybutyric acid, sodium salt (UMB73) were prepared by the method of Sheehan et al. (1971; mono-alkylation of 1,4-butanediol under basic conditions), followed by oxidation of the remaining hydroxyl group with pyridinium dichromate using standard conditions (Corey and Schmidt, 1979). The sodium salts of UMB72 and UMB73 were crystallized from methanol (mp = 228-230°C). Application of the same alkylation procedure to the phenethyl analog (UMB87) was unsuccessful, presumably due to basic elimination of phenethyl bromide to give styrene. Thus, 4-(3-phenylpropyloxy)butyric acid, sodium salt (UMB87) was prepared using the mercury assisted alkylation method of McKillop and Ford (1974), followed by pyridinium dichromate oxidation (mp = 212-214°C). 3-Aminopropyl(methyl)phosphinic acid hydrochloride (SKF97541) and 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP35348) were synthesized as described previously (Froestl et al., 1995). The radioligand [3H]NCS-382 was synthesized as described previously (Mehta et al., 2001).

[^{3}H]GABA was purchased from Perkin Elmer Life Sci. (Boston, MA). Gamma-hydroxybutyrate sodium (GHB), gamma-butyrolactone (GBL), 1,4-butanediol (1,4-BDL), (±)baclofen, 3-HPA (3-hydroxyphenylacetic acid), and 2-HPA (2 hydroxyphenylacetic acid) were purchased from Sigma-Aldrich (USA).

For *in vivo* studies, drug solutions were prepared as follows: GHB, SKF97541, CGP35348, and 3-HPA were dissolved in sterile water; baclofen was dissolved in physiological saline; GBL and 1,4-BDL were diluted in sterile water; UMB72, UMB73, and UMB86 were suspended in a solution of physiological saline and 1% TWEEN® 80 (Sigma Aldrich, St. Louis, MO). The pH of each drug solution was adjusted to 5-9 with lactic acid or sodium hydroxide (Sigma Aldrich, St. Louis, MO), as necessary.

Subjects.

All animals were housed individually on a 12:12 h light/dark cycle (experiments conducted during the light period) with free access to water in the home cage. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) were used for binding studies and drug discrimination experiments. Rats used in the binding studies had free access to food and weighed between 250 and 300 g. Rats discriminating 200 mg/kg GHB i.p. (n = 9; Carter et al., 2003; Wu et al., 2003) were maintained at 340 to 360 g by providing rodent chow (Rodent sterilizable diet, Harlan Teklad, Madison, WI) in the home cage after daily experimental sessions. Adult white Carneau pigeons (*Columbia Livia*; Palmetto, Sumter, SC) discriminating 100 mg/kg GHB i.m. (n = 6; Koek et al., 2004) were maintained at 80 to 90% of their free-feeding weight, ranging from 590 to 620 g, by providing mixed grain in the home cage after daily sessions. Eighty male C57BL/6J mice (The Jackson

Laboratory, Bar Harbor, ME) were used to examine directly observable effects; all mice had free access to food (Rodent sterilizable diet, Harlan Teklad, Madison, WI), weighed between 17-30 g, and were experimentally naïve before testing. On arrival, mice were allowed at least five days to habituate to the experimental room, then were handled for at least one day prior to the start of testing. All animals were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee, 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).

Binding.

Membranes were prepared as described previously (Mehta et al., 2001). Briefly, rats were decapitated and the cerebral cortex and cerebellum was dissected. Tissue was stored at -80°C until it was thawed and homogenized in ice-cold 0.32 M sucrose, pH 7.4 (20 ml/g tissue), and centrifuged at 1000 g for 10 min at 4°C. The supernatant was then centrifuged at 140,000 g for 30 min at 4°C to obtain the mitochondrial plus microsomal (P2 + P3) fraction. This fraction was dispersed in ice-cold double-distilled deionized water and homogenized with a Brinkman Polytron at a setting of 6 for two 10-sec bursts, 10 sec apart. The suspension was centrifuged at 140,000 g for 30 min at 4°C. The pellet was then resuspended in ice-cold Tris buffer (50 mM, pH 7.4) and centrifuged at 140,000 g for 30 min at 4°C. This step was repeated twice. After the final centrifugation step, the pellet was suspended in a small volume of ice-cold Tris buffer (50 mM, pH 7.4) and stored at -80°C. On the day of the assay, the tissue was thawed and washed twice with

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buffer as before (140,000 g, 30 min, 4°C) and then resuspended in the buffer for use in

the assay.

[^{3}H]NCS-382 binding was measured using a centrifugation assay as described

previously (Mehta et al., 2001). Briefly, aliquots (0.3-0.4 mg protein) of membrane-

preparation in Tris buffer (50 mM, pH 7.4) were incubated with [^{3}H]NCS-382 (16 nM) in

triplicate at 4°C for 20 min in a 1 ml total volume. Non-specific binding was determined

using NCS-382 (500 μM). The binding reaction was stopped by centrifugation (50,000 g,

10 min, 4°C). The supernatant was decanted, and the vials were rapidly rinsed twice with

4 ml ice-cold Tris buffer (50 mM, pH 7.4) without disturbing the pelleted tissue. Pellets

were solubilized with 0.3 ml of Soluene-350 (Packard, Meriden, CT) for 4-6 h.

Scintillation liquid (3 ml) was added to the solubilized material in the bio-vials.

Radioactivity was quantified by liquid scintillation spectrometry. For determination of

IC$_{50}$ values, [^{3}H]NCS-382 (16 nM) binding was carried out in the absence and presence

of concentrations of unlabeled compounds. [^{3}H]GABA (10 nM) binding to GABA$_A$

receptors in cerebral cortex was performed in a similar manner, using a 10 min

incubation period at 4°C, and GABA (100 μM) to define non-specific binding. Binding

affinity for GABA$_B$ receptors was measured in tissue from cerebellum, an area where

GHB binding to GABA$_B$ receptors has been shown previously (Mathivet et al., 1997; Wu

et al., 2003). For [^{3}H]GABA (10 nM) binding to GABA$_B$ receptors in rat cerebellum, all

of the assay tubes contained 40 μM isoguvacine HCl (ICN Biomedicals Inc., Costa Mesa,

CA) to displace [^{3}H]GABA binding to GABA$_A$ receptors. These assay tubes also

contained calcium chloride (2.5 mM), and the incubation was carried out at 25°C for 10

min. GABA (100 μM) was used to define non-specific binding. All other assay

conditions for [^{3}H]GABA binding were the same as those used for the [^{3}H]NCS-382

binding assays.

Drug Discrimination.

Discrimination experiments using rats were conducted in commercially-available

operant chambers (Model #ENV-008CT; MED Associates Inc., St. Albans, VT) located

within sound-attenuating, ventilated enclosures (Model #ENV-022M; MED Associates

Inc.), described in detail elsewhere (Carter et al., 2003). Data were collected using MED-

PC IV software and interface (MED Associates Inc.). Rats were previously trained to

discriminate 200 mg/kg GHB i.p. from saline. Discrimination training and experimental

sessions were carried out as described previously (Carter et al., 2003). Immediately prior

to each daily session, rats received 200 mg/kg GHB or saline (i.p.) and were immediately

placed into the operant chamber. A 15 min pretreatment period, during which the

chamber was dark and responses (i.e., lever presses) had no programmed consequence,

was followed by 15 min response period, during which the stimulus lights above both

levers were transilluminated and 10 consecutive responses (fixed ratio 10; FR10) on the

correct lever resulted in the delivery of a food pellet (45 mg; Research Diets; New

Brunswick, NJ). A response on the incorrect lever reset the FR requirement on the correct

lever. The response period ended after 15 min or the delivery of 100 food pellets,

whichever occurred first. Experimental sessions were conducted 5-7 days a week and the

order of training sessions was generally double alternation (e.g., saline, saline, drug,

drug). All rats had satisfied the following testing criteria before this study: at least 90% of

the total responses on the correct lever and fewer than 10 responses on the incorrect lever

before delivery of the first food pellet for five consecutive sessions, or six out of seven

sessions (Carter et al., 2003). Subsequently, rats were required to satisfy these criteria for

at least one saline and one drug training session in 2 of the 3 sessions before a test

(including the day immediately before the test). Test sessions were identical to training

sessions, except that 10 consecutive responses on either lever resulted in the delivery of

food. Test compounds (UMB72, UMB86, and 3-HPA) were studied alone (given

immediately prior to the 15 min pretreatment period in the operant chamber) and 10 min

before the training dose of GHB, up to a dose that markedly decreased responding in

more than half of the subjects (UMB72 and UMB86) or at which other limitations were

apparent (e.g., solubility; 3-HPA). The order of treatment with different doses was

unsystematic.

Discrimination experiments using pigeons were conducted in sound attenuating,

ventilated operant chambers (BRS/LVE, Laurel, MD) equipped with two translucent

response keys that could be transilluminated by red lights. Data were collected using

MED-PC IV software and interface (MED Associates Inc.) to monitor and control inputs

and outputs to the operant chambers and to record the data. The procedure has been

described in detail elsewhere (Koek et al., 2004). Briefly, before each daily session,

subjects received either 100 mg/kg GHB or saline (i.m.) and were immediately placed

into the chamber. Sessions started with a pretreatment period of 15 min, during which the

lights were off and keypecks had no programmed consequence. Subsequently, the left

and the right keys were transilluminated red and 20 consecutive responses (FR20) on the

injection-appropriate key resulted in the key lights being extinguished for 4 sec, during

which time a white light illuminated the hopper where food (Purina Pigeon Checkers, St.

Louis, MO) was available. Responses on the incorrect key reset the FR requirement on the correct key. The response period ended after 30 food presentations or 15 min, whichever occurred first. Experimental sessions were conducted 5-7 days a week and the order of training sessions was generally double alternation (e.g., saline, saline, drug, drug). All pigeons had satisfied the following testing criteria before this study: at least 90% of the total responses on the correct key and fewer than 20 responses on the incorrect key before the first food presentation for at least seven of nine consecutive sessions (Koek et al., 2004). Thereafter, tests were conducted when these criteria were satisfied during two consecutive (drug and saline) training sessions. Test sessions were the same as training sessions, except that food was available after completion of 20 consecutive responses on either key. Test compounds were studied under the same temporal parameters and doses were selected by the same criteria as were used in the rat studies.

Behavioral effects in mice.

Locomotor activity was assessed using four 30x15x15 cm customized acrylic boxes (Instrumentation Services, University of Texas Health Science Center at San Antonio) that were separately enclosed in commercially-available sound-attenuating chambers (Model # ENV-022M; MED Associates Inc.). Four infrared light beams were spaced 6 cm apart and located 2 cm above the floor of each box. Occlusions of the infrared light beams were counted using commercially-available computer software (Multi-Varimex v1.00, Colombus Instruments, Colombus, OH). The floor of the boxes consisted of a parallel grid of 2.3 mm stainless steel rods mounted 6.4 mm apart or of

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perforated 16 gauge stainless steel with 6.4 mm round holes (9.5 mm staggered centers).

Floor types were counterbalanced between animals, but were always the same for

individual animals. Between tests the floor and inside of the boxes were wiped with a

damp sponge and the litter paper beneath the floor was changed. Catalepsy was measured

using a 1 cm diameter horizontal bar supported 4 cm above the floor by two 8x8 cm

square pieces of Plexiglas. Ataxia was studied by means of an inverted screen apparatus

(Instrumentation Services, University of Texas Health Science Center at San Antonio),

consisting of four 13x13 cm wire screens (No. 4 mesh) located 23 cm above the floor of

four Plexiglas containers. The four screens were connected to a rod and handle that could

be rotated 180° to simultaneously invert the four screens.

Mice were tested 1-3 times per week with at least 48 h between tests. Individual

mice were tested on average 9 times (range = 1-16). Mice were not habituated to the

locomotor activity boxes; however, the number of times that an animal had been tested

did not significantly affect the mean number of beam breaks during the second half of the

30 min session under control conditions (i.e., after saline administration; $F_{(12, 71)} = 1.10$, p

= 0.37), indicating that repeated testing of the animals did not confound the measurement

of locomotion. Doses used in this study were based on preliminary dose finding data. The

order in which the drugs and doses were tested was randomized within groups of 20 mice

(with the exception of the largest dose of each compound studied, which was studied last

due to the potential toxicity of those doses). Two groups of 20 mice were used to study

the test compounds (UMB72, UMB73, UMB86, 3-HPA) alone and two groups of 20

mice were used for the antagonism studies. On test days, one subgroup of mice (n = 4)

received 10 ml/kg saline and four subgroups of mice (n = 4 each) received a dose of a

particular drug; saline and drug conditions were randomly assigned to individual subgroups and no mouse received the same dose of drug twice. All drugs were administered i.p. in a volume of 0.1-1.0 ml.

Immediately after a test compound was administered, mice were placed in the locomotor activity chambers and beam breaks were recorded for 30 min. At the end of the 30-min session, mice were removed from the chambers and tested for loss of righting. Mice were placed in a supine position and loss of righting was defined as not placing the plantar surface of any paw on the floor within 15 sec. After testing for loss of righting, mice were tested for catalepsy by placing their front paws on a horizontal metal bar located 4 cm above the floor. The time that both paws remained on the bar was measured up to 30 sec. After these tests, animals were briefly returned to their home cage. Within 5-10 min following removal from the locomotor activity boxes, mice that had not exhibited loss of righting were tested for ataxia using the inverted screen test. Animals were placed on the screens, which were rotated 180° over a 1-sec period: failure to remain on the apparatus for 60 sec was scored as exhibiting ataxia.

For antagonism studies, a dose of 100 or 320 mg/kg CGP35348 was given 15 min prior to 1.0 mg/kg SKF97541, 17.8 mg/kg baclofen, 560 mg/kg GHB, 560 mg/kg UMB86, 1000 mg/kg UMB72, 1780 mg/kg UMB73, or 3200 mg/kg 3-HPA. These doses were chosen because they were the smallest doses that produced ataxia (an effect that was observed with all compounds) in more than 50% of the animals when tested alone. After administration of CGP35348, animals were returned to their home cage for 15 min; all other temporal parameters were the same as when test compounds were studied alone.

Locomotor activity was not studied because CGP35348 decreased locomotion when given alone (data not shown).

Data analysis.

The radioligand binding data are expressed as the mean $\pm$ 1 SEM. IC_{50} data were analyzed using DeltaGraph (DeltaPoint, USA). These data were analyzed for each individual experiment, and the mean $\pm$ 1 SEM was then calculated. For drug discrimination studies, the mean percentage of responses on the drug lever or key (drug-appropriate responding; %DR) $\pm$ 1 SEM and the mean rate of responding $\pm$ 1 SEM were plotted as a function of dose. If during a test an animal responded at a rate less than 20% of its vehicle control rate (i.e., average rate during the 5 most recent saline training sessions), discrimination data from that test were not included in the average. Mean percentages of responses on the drug lever or key were calculated only when they were based on at least half of the animals tested. One-tailed, paired t-tests (NCSS, Kaysville, UT) were used to test for differences in GHB-appropriate responding between doses of GHB analogs and saline, and between doses of GHB analogs given before the training dose of GHB and the training dose of GHB. Individual animals served as their own control and one-tailed tests were used because GHB-appropriate responding can only increase when compounds are given alone and can only decrease when compounds are given together with the training dose of GHB.

In the locomotor activity assay, beam breaks were counted in 5-min periods and the number of beam breaks occurring in the last 3 periods (between 15 and 30 min after the i.p. injection) were analyzed because preliminary results showed all drugs to be

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maximally active within this interval. The number of beam breaks from the last 3 (5-min)

periods were averaged and transformed to a percent of control where the control value

was the average of the mean number of beam breaks from the last 3 (5-min) periods after

all saline tests (n = 84). For graphical presentation, transformed data from individual

animals were used to calculate the mean $\pm$ 1 SEM at different doses of drug. The

dependent variable in the catalepsy assay was the time that both paws remained on the

horizontal bar, up to 30 sec, which was averaged across animals. Data for the inverted

screen and loss of righting assays were quantal (i.e., present or absent) and were analyzed

as the percentage of animals that exhibited ataxia or loss of righting.

The ED_{50} values and 95% CL for the observational procedures in mice were

calculated with the Litchfield-Wilcoxin procedure (Pharm Tools Pro v1.1.27, Elkins

Park, PA); when less than two intermediate percentages were observed, 0% and 100%

effects were transformed by Berkson's adjustment (Hubert, 1984) to enable the use of the

Litchfield-Wilcoxon procedure. Correlation analyses of ED_{50} values between assays were

performed in a pair-wise fashion; compounds for which ED_{50} values could not be

calculated were excluded from individual analyses (NCSS, Kaysville, UT).

Results

Binding.

GHB displaced [^{3}H]NCS-382 with an IC_{50} of 25 µM (Table 1). Three ether

analogs (UMB72, UMB73, and UMB87) had lower affinity at GHB receptors, with

IC_{50}'s ranging from 195-352 µM. 2-HPA, the 2-phenol corresponding to GHB, had

similar affinity as the ether analogs at GHB receptors with an IC_{50} of 146 µM, whereas

the corresponding 3-phenol (3-HPA) had higher affinity, similar to that of GHB (IC_{50} =

12 µM). The 4-phenyl analog (4-hydroxy-4-phenylbutyric acid) and the 4-napthyl analog

of GHB (UMB86) had IC_{50} values (48 and 13 µM, respectively) similar to GHB, in the

low micromolar range.

At 1000 µM, GHB displaced 36% and 41% of [^{3}H]GABA binding from $GABA_A$

and $GABA_B$ receptors, respectively. With the exception of UMB87 (93% inhibition),

GHB analogs did not alter [^{3}H]GABA binding to $GABA_B$ receptors more than 14% at

1000 µM. At the same concentration, the ether analogs UMB72 and UMB73 did not

affect $GABA_A$ binding more than 18%, whereas UMB87 displaced 50% of [^{3}H]GABA

binding at $GABA_A$ receptors. The phenolic hydroxyl analogs 2-HPA and 3-HPA

displaced 17% and 44% of $GABA_A$ binding, respectively. The napthyl-substituted

analog, UMB86 displaced 30% of binding at $GABA_A$ receptors at 1000 µM.

Drug discrimination.

Under test conditions, saline occasioned less than 5% drug-appropriate

responding in rats and pigeons ($0.4 \pm 0.2\%$ and $2.6 \pm 0.2\%$, respectively; Fig. 2, leftmost

column, "SAL"). GHB dose-dependently occasioned GHB-appropriate responding in

both groups of animals under test conditions; the training dose of 200 mg/kg or 100

mg/kg occasioned 84.8 ± 10.8 and 98.5 ± 1.1 percent GHB-appropriate responding in rats

and pigeons, respectively (Fig. 2). When given alone, UMB86, UMB72, and 3-HPA

occasioned, at most, 12, 17, and 34% GHB-appropriate responding in rats, respectively

(Fig. 2, panel A, closed circles). The greatest amount of GHB-appropriate responding

observed at 1780 mg/kg 3-HPA was not significantly different from saline. UMB86 was

the only analog that occasioned any GHB-appropriate responding in pigeons (Fig. 2,

panel B, closed circles) but this effect was not statistically significant. UMB86 and

UMB72 were studied up to doses that markedly decreased responding; the study of larger

doses of 3-HPA was limited by its solubility. The order of potency to decrease

responding was UMB86 > UMB72 > 3-HPA.

In rats, doses of 100 mg/kg UMB86, 320 mg/kg UMB72, and 1780 mg/kg 3-HPA

decreased GHB-appropriate responding (elicited by the training dose) to 71, 59, and 52%,

respectively (Fig. 2, panel A, open circles); however, none of these decreases reached

statistical significance. In pigeons, doses of 100 mg/kg UMB86, 178 mg/kg UMB72, and

1780 mg/kg 3-HPA decreased GHB-appropriate responding (elicited by the training

dose) to 72, 74, and 90%, respectively (Fig. 2, panel B, open circles); as in rats, these

decreases did not reach statistical significance.

Behavioral effects in mice.

In the locomotion studies, the mean (± 1 SEM) number of beam breaks per 5-min

period in the 15-30 min after saline administration for all saline tests was 99.7 ± 3.8 (n =

84). This value ranged from 93.2 ± 3.9 (25-30 min) to 105.5 ± 4.2 (15-20 min) and did

not significantly differ between the three 5-min periods ($F_{(2, 248)} = 2.28$, $p > 0.05$). All compounds dose-dependently and completely inhibited locomotion; the order of potency to decrease locomotion was SKF97541 > baclofen > GBL > 1,4-BDL > GHB > UMB86 > UMB72 > UMB73 > 3-HPA (Table 2; Fig. 3). At larger doses, catalepsy was observed with SKF97541, baclofen, GBL, 1,4-BDL, and GHB, and not with UMB86, UMB72, UMB73, or 3-HPA (Fig. 3, middle column). Catalepsy was never observed under control conditions (data not shown). The cataleptic effects of SKF97541, baclofen, GBL, 1,4-BDL, and GHB were biphasic; catalepsy increased with smaller doses and subsequently decreased with larger doses. The order of potency of these compounds to produce catalepsy was SKF97541 > baclofen > GBL > 1,4-BDL > GHB, and was highly correlated with their potency to decrease locomotion ($r = 0.998$).

At larger doses, ataxia and loss of righting were observed, with average ED_{50} values that were 1.6 ± 0.2 and 4.5 ± 0.6 times greater than those for decreasing locomotion, respectively (Table 2). The potency to produce ataxia and loss of righting was highly correlated with the potency to decrease locomotion ($r = 0.993$ and $r = 0.996$, respectively). UMB73 and 3-HPA failed to produce loss of righting in more than 50% of the animals. Larger doses were not tested because 75% and 87% lethality was observed following 1780 mg/kg UMB73 and 3200 mg/kg 3-HPA, respectively (data not shown). Lethality was generally observed at least 24 hours after administration, and also occurred after doses of 1,4-BDL (25% at 1780 mg/kg), UMB72 (12.5% at 1000 mg/kg and 62.5% at 1780 mg/kg), and UMB86 (50% at 1000 mg/kg and 100% at 1780 mg/kg), but was never observed following administration of SKF97541, baclofen, GBL, or GHB.

The GABA$_B$ receptor antagonist CGP35348 decreased the number of animals that exhibited ataxia after receiving 1 mg/kg SKF97541, 17.8 mg/kg baclofen, or 560 mg/kg GHB (Fig. 4). CGP35348 failed to attenuate the ataxia that was observed following administration of 560 mg/kg UMB86, 1000 mg/kg UMB72, 1780 mg/kg UMB73, or 3200 mg/kg 3-HPA. A dose of 320 mg/kg CGP35348 completely blocked catalepsy in mice treated with SKF97541 or baclofen (data not shown). Catalepsy increased from 15.6 ± 4.9 sec to the maximum of 30.0 sec when 100 mg/kg CGP35348 was given prior to 560 mg/kg GHB. A larger dose of CGP35348 (320 mg/kg) together with GHB decreased catalepsy to 8.3 ± 3.3 sec (data not shown).

Discussion

The main finding of the present study is that selective GHB analogs shared some, but not all, of the effects of GHB. Three chemical approaches were taken to synthesize GHB analogs that could not be metabolized to GABAergic ligands. Radioligand binding studies identified several GHB analogs that were selective for GHB receptors and a selective compound from each chemical class was studied in several behavioral procedures. Selective GHB analogs (UMB86, UMB72, and 3-HPA) did not occasion GHB-like discriminative stimulus effects in rats and pigeons, or catalepsy in mice. However, UMB86, UMB72, 3-HPA, and UMB73, like GHB, decreased locomotion and, at larger doses, produced ataxia. The ataxic effects of these compounds were not attenuated by CGP35348, suggesting that GHB receptors might mediate the effects of these compounds and contribute to the effects of GHB and GHB precursors.

Increasing evidence (e.g., cross-substitution between GHB and baclofen) in rats and pigeons suggests that GABAergic activity is a major component of the discriminative stimulus effects of GHB (Colombo et al., 1998; Lobina et al., 1999; Carter et al., 2003; Carter et al., 2004a; Koek et al., 2004; Baker et al., 2004). Thus, compounds that do not bind to GABA receptors might not be expected to produce or attenuate GHB-like discriminative stimulus effects, which is consistent with the relatively small amount of GHB-appropriate responding observed after administration of selective GHB receptor ligands (Wu et al., 2003; current study) and the failure of UMB86, UMB72, or 3-HPA to fully antagonize the discriminative stimulus effects of the training dose in either rats or pigeons. However, the apparent partial effects of 1780 mg/kg 3-HPA alone in rats, or 178 mg/kg of UMB72 together with the training dose of GHB in rats and pigeons, might

suggest a small contribution of GHB receptor activity in the discriminative stimulus effects of GHB.

In mice, doses of GHB larger than those required to decrease operant responding (200 mg/kg i.g.; Cook et al., 2002) have been shown to produce catalepsy (300 mg/kg i.p.; Itzhak and Ali, 2002), ataxia (300-1000 mg/kg i.g.; Cook et al., 2002) and loss of righting (560-1000 mg/kg, i.p.; Carai et al., 2001). The mechanism of action of large doses of GHB in mice might be relevant to large doses of GHB (that result in overdose and lethality) in humans. As the dose of GHB is increased, additional mechanisms of action are likely to contribute to its behavioral profile. Thus, GHB or dopamine receptor-mediated mechanisms that do not appear to play a prominent role in the discriminative stimulus effects of GHB in rats (Winter et al., 1981; Wu et al., 2003; Carter et al., 2004a), could play a more predominant role in mediating the effects of GHB that are observed at larger doses.

In observational studies in mice, SKF97541, baclofen, GBL, 1,4-BDL, GHB, UMB86, UMB72, UMB73, and 3-HPA dose-dependently decreased locomotor activity, produced ataxia, and produced loss of righting. These results are consistent with the sedative and muscle relaxant properties of GHB, GHB precursors, and $GABA_B$ receptor agonists (Carai et al., 2001; Carai et al., 2002a; Carai et al., 2002b; Cook et al., 2002; Quang et al., 2002), and extend these findings to structurally-related analogs of GHB. These GHB receptor-specific analogs, which unlike GHB and $GABA_B$ receptor agonists do not readily bind to $GABA_B$ receptors, did not produce catalepsy when studied up to lethal doses. This finding is similar to other studies showing that transgenic mice that do not express functional $GABA_B$ receptors do not exhibit hypolocomotion or hypothermia

after administration of baclofen, GHB, or GBL (Kaupmann et al., 2003; Queva et al.,

2003). The order of potency of SKF97541, baclofen, GBL, 1,4-BDL, GHB, UMB86,

UMB72, UMB73, and 3-HPA was the same across the assays employed in this study,

including the potency to produce catalepsy, when it was observed. In general, doses

required to produce ataxia and loss of righting were larger than those needed to decrease

locomotor activity or produce catalepsy.

It is known that peripheral (i.p; Mehta and Ticku, 1987) and central (ventromedial

thalamic nucleus; Wüllner et al., 1987) administration of baclofen produces catalepsy in

rats. Baclofen-induced catalepsy is blocked by the $GABA_B$ receptor antagonist δ-

aminovaleric acid, and not by bicuculline, bromocriptine, or scopolamine, indicating that

the effect is mediated by $GABA_B$ receptors (Mehta and Ticku, 1987). The presence of

catalepsy following a dose of 320 mg/kg GHB (i.p.) in this study is consistent with GHB

having agonist-like activity at $GABA_B$ receptors (Lingenhoehl et al., 1999) and with

previous reports of catalepsy following a dose of 300 mg/kg GHB (i.p.) in Swiss Webster

mice (Itzhak and Ali, 2002) and 560 mg/kg GHB (i.p.) in Sprague-Dawley rats (Sevak et

al., 2004). That the effects of GHB, GHB precursors, and $GABA_B$ receptor agonists on

catalepsy were biphasic is likely due to other effects of these compounds that interfere

with catalepsy at larger doses, such as ataxia and muscle atonia. The absence of catalepsy

following the administration of selective GHB analogs UMB86, UMB72, UMB73, and 3-

HPA and the attenuation of catalepsy produced by SKF97541, baclofen, and GHB by

CGP35348 provides strong evidence that activity at $GABA_B$ receptors is necessary for

catalepsy produced by GHB and its precursors in mice.

It is well-established that $GABA_B$ receptor activation inhibits neurotransmitter release in brain and spinal cord (for review, see Bowery et al., 1980; Bowery et al., 2002). Administration of GHB (Feigenbaum and Howard, 1996) or baclofen (Bowery et al., 1980; Balsara et al., 1981) inhibits presynaptic dopamine release in unanaesthetized animals, which is likely to account for their neuroleptic-like behavioral effects (e.g., catalepsy; Balsara et al., 1981; Hechler et al., 1993; Itzhak and Ali, 2002). Catalepsy produced by neuroleptics is enhanced by GHB (Navarro et al., 1998) or baclofen (Davies and Williams, 1978; Richardson and Richardson, 1982), although the cataleptic effects of neuroleptics and $GABA_B$ receptor agonists involve different mechanisms (Sevak, et al., 2004). Haloperidol-induced catalepsy is attenuated by the N-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine, whereas dizocilpine enhances GHB-induced catalepsy (Sevak et al., 2004). Together, these findings suggest that GHB- and baclofen-induced catalepsy is not due to antagonism at dopamine receptors, but rather, to $GABA_B$ receptor-mediated inhibition of neurotransmitter release, a mechanism of action that is not shared with the selective GHB analogs used in this study.

The novel GHB receptor-selective analogs in this study have begun to serve as pharmacological tools to study the structure-activity relationship of GHB receptors and the contribution of GHB receptors to the behavioral profile of GHB and related compounds. The present data support previous findings that the discriminative stimulus effects of GHB involve actions at $GABA_B$ receptors and further provide evidence that the cataleptic and ataxic effects of GHB are dependent on activity at $GABA_B$ receptors. UMB86, UMB72, UMB73, and 3-HPA lack affinity for GABA receptors; thus, activity at non-GABAergic (e.g., GHB) receptors might be responsible for the effects that these

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compounds share with GHB (e.g., ataxia) that are not attenuated by the $GABA_B$ receptor antagonist CGP35348. Because the affinity of some of these compounds for GHB receptors *in vitro* differs from their potency *in vivo* (e.g. 3-HPA), other factors such as bioavailability and efficacy might contribute to the differences observed between these compounds and GHB.

To the extent that different behavioral effects of GHB are mediated by different mechanisms (e.g. GHB, $GABA_B$, or other receptors), compounds that are selective for GHB receptors might retain some of the therapeutic effects of GHB, while having fewer undesired effects. The present studies have identified UMB86, UMB72, UMB73, and 3-HPA as selective GHB analogs that exhibit some, but not all, of the behavioral effects of GHB and its precursors GBL and 1,4-BDL; these compounds will be helpful tools to investigate the role of GHB receptors in the behavioral profile of GHB.

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Acknowledgements

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Legends for figures:

Figure 1. Structures of gamma-hydroxybutyric acid, sodium (GHB), 4-hydroxy-4-methylpentanoic acid, sodium (UMB68), 4-hydroxy-4-phenylbutyric acid, sodium, 4-hydroxy-4-napthylbutanoic acid, sodium (UMB86), 4-benzyloxybutyric acid, sodium (UMB73), 4-(2-phenethyloxy)butyric acid, sodium (UMB87), 4-(3-phenylpropyloxy)butyric acid (UMB72), 2-hydroxyphenylacetic acid (2-HPA), and 3-hydroxyphenylacetic acid (3-HPA).

Figure 2. Effects of UMB86, UMB72, and 3-HPA alone (closed circles) and together with the training dose of GHB (open circles) in rats trained to discriminate 200 mg/kg GHB i.p. (panel A) and in pigeons trained to discriminate 100 mg/kg GHB i.m. (panel B) from saline. The percentage of responses on the drug-appropriate lever (%DR) and the rate of responding (responses/sec) are plotted as a function of dose. Data points and error bars in panel A represent the mean ± 1 SEM for at least 8 of the 9 rats, with the exception of the following doses: 560 mg/kg UMB72 + 200 mg/kg GHB (n = 5); 560 mg/kg 3-HPA + 200 mg/kg GHB (%DR, n = 6; rate, n = 7); 1780 mg/kg 3-HPA (n = 4). Data points and error bars in panel B represent the mean ± 1 SEM for at least 5 of the 6 pigeons, with the exception of 178 mg/kg UMB86 (n = 4). Data above, "SAL" or "GHB" show the effects of saline or the training dose of GHB, respectively.

Figure 3. Locomotor activity, catalepsy, ataxia, and loss of righting in mice treated with GABA$_B$ agonists (SKF97541 and baclofen), GHB and GHB precursors (GBL and 1,4-BDL), and GHB analogs (UMB86, UMB72, UMB73, and 3-HPA). Compounds are arranged from most potent (SKF97541) to least potent (3-HPA) to decrease locomotor activity. Locomotor activity is plotted as the average ± 1 SEM percent control of the number of beam breaks that were observed under saline conditions for individual animals. The average (± 1 SEM) number of beam breaks per 5-min period in the 15-30 min after saline administration for all saline tests was 99.7 ± 3.8 (n = 84). Catalepsy is plotted as the average time ± 1 SEM that animals exhibited cataleptic behavior up to 30 sec. Ataxia and loss of righting (LOR) are plotted as the percent of animals that exhibited these behaviors. All behavioral effects are shown as a function of dose of drug (mg/kg) using the same abscissa. N = 8 in all cases except, 56 mg/kg baclofen (n = 4). GHB data are from Carter et al., in press.

Figure 4. Ataxia in mice pretreated with different doses of the GABA$_B$ antagonist CGP35348 prior to treatment with SKF97541, baclofen, GHB, or GHB analogs UMB86, UMB72, UMB73, or 3-HPA. Animals were given saline, 100 mg/kg CGP35348, or 320 mg/kg CGP35348 15 min prior to administration of 1.0 mg/kg SKF97541, 17.8 mg/kg baclofen, 560 mg/kg GHB, 560 mg/kg UMB86, 1000 mg/kg UMB72, 1780 mg/kg UMB73, or 3200 mg/kg 3-HPA. The percent of animals that exhibited ataxia is plotted as a function of treatment; N = 8 in all cases.

Table 1. IC_{50} values of GHB and structural analogs using [^{3}H]NCS-382 (16 nM) as a radioligand in rat cerebrocortical membranes, and effects of GHB and analogs (1 mM) on [^{3}H]GABA (10 nM) binding to $GABA_A$ receptors in rat cerebrocortical membranes and to $GABA_B$ receptors in rat cerebellar membranes. Each value is the mean ± 1 SEM of at least three experiments, and each experiment was performed in triplicate.

Compound	[^{3}H]NCS-382	[^{3}H]GABA	
	IC_{50} ± SEM (µM) GHB receptor	% Change in binding ± SEM $GABA_A$ receptor	$GABA_B$ receptor
3-HPA	12 ± 6	-44 ± 2	-14 ± 4
UMB86	13 ± 6	-30 ± 2	-10 ± 6
GHB	25 ± 2[a]	-36 ± 4[b]	-41 ± 3[b]
4-hydroxy-4-phenylbutyric acid	48 ± 1	-40 ± 2	-13 ± 8
2-HPA	146 ± 15	-17 ± 1	-6 ± 4
UMB87	195 ± 28	-50 ± 4	-93 ± 2
UMB73	294 ± 39	18 ± 11	-10 ± 7
UMB72	352 ± 37	0 ± 1	-2 ± 4

[a]Mehta et al. 2001; [b]Wu et al. 2003.

Table 2. ED$_{50}$ values (mg/kg) and 95% confidence limits for compounds across the four assays in mice.

	Locomotor activity			Catalepsy			Ataxia			Loss of righting		
	ED$_{50}$	95% CL		ED$_{50}$	95% CL		ED$_{50}$	95% CL		ED$_{50}$	95% CL	
SKF97541	0.32	0.07	0.58	0.42	0.29	0.62	0.60	0.35	0.89	1.55	0.97	2.21
baclofen	8.7	5.5	13.2	14.7	8.1	31.9	16.6	10.8	24.3	34.2	21.0	264.7
GBL	86.7	54.5	123.6	99.9	66.8	149.3	133.6	86.8	205.8	392.2	279.9	647.6
1,4-BDL	177.6	ND	ND	209.3	ND	ND	197.7	73.3	356.6	839.4	501.1	1718.6
GHB[a]	220.8	82.9	383.9	221.0	157.7	364.3	584.1	276.3	1043.8	1702.7	1093.3	3329.5
UMB86	304.1	133.9	471.7	ND			318.6	119.4	525.7	999.1	668.1	1493.1
UMB72	422.8	288.3	619.3	ND			629.5	435.0	988.4	1072.6	735.8	1650.8
UMB73	750.7	514.5	1098.4	ND			1235.6	881.8	2037.2	ND		
3-HPA	1441.3	870.3	2025.5	ND			1677.3	1125.8	2869.3	ND		

[a]from Carter et al., in press

ND – value could not be determined

Fig 1.

GHB

UMB68

4-Hydroxy-4-phenylbutyric
acid, sodium salt

UMB86

UMB73

UMB87

UMB72

2-HPA

3-HPA

Figure 2

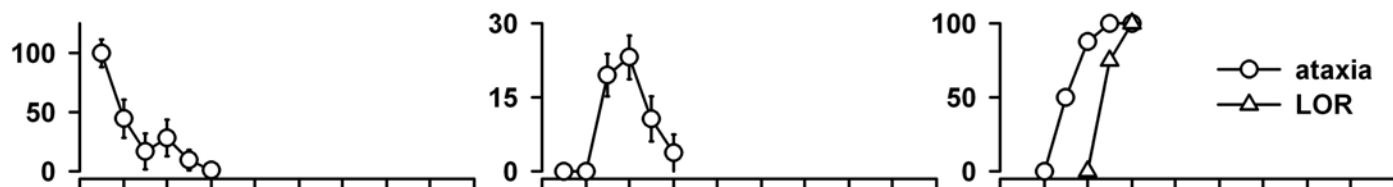**Fig 3**

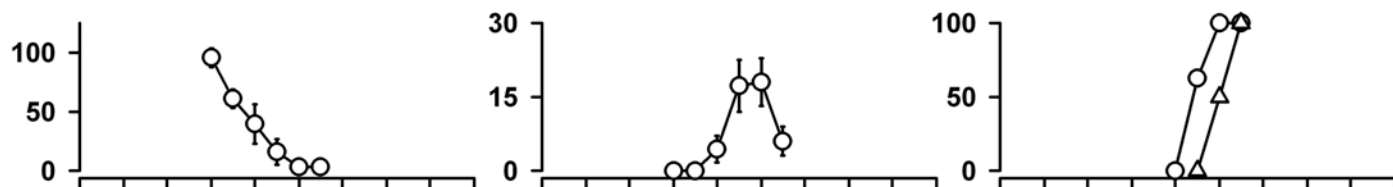**Beam breaks (% control)** **Time cataleptic (sec.)** **% Animals**

Dose (mg/kg)

Rows (top to bottom): SKF97541, baclofen, GBL, 1,4-BDL, GHB, UMB86, UMB72, UMB73, 3-HPA

Legend: ○ ataxia, △ LOR

Fig 4