GABA<sub>B</sub> receptor positive modulators: enhancement of GABA<sub>B</sub> receptor agonist effects in vivo.

Wouter Koek, Charles P. France, Kejun Cheng, and Kenner C. Rice

Departments of Psychiatry and Pharmacology (W.K., C.P.F.), The University of Texas Health Science Center at San Antonio, San Antonio, TX; and Chemical Biology Research Branch (K.C., K.C.R.), National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health (NIH), Department of Health and Human Services, Bethesda, MD
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Corresponding Author: Wouter Koek, Ph.D.
Departments of Psychiatry and Pharmacology
University of Texas Health Science Center at San Antonio
7703 Floyd Curl Drive, Mail Code 7792
San Antonio, Texas 78229-3900
Tel: (210) 567 5478
Fax: (210) 567 5381
E-mail: koek@uthscsa.edu

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Abbreviations: GHB, γ-hydroxybutyrate; CGP7930, 2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethylpropyl)phenol; rac-BHFF, (R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one; CGP35348, 3-aminopropyl(diethoxymethyl)phosphinic acid; AUC, area under the curve

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Abstract

In vivo effects of GABA<sub>B</sub> receptor positive modulators suggest them to have therapeutic potential to treat CNS disorders such as anxiety, depression, and drug abuse. Although these effects are generally thought to be mediated by positive modulation of GABA<sub>B</sub> receptors, such modulation has been examined primarily in vitro. The present study was aimed at further examining the in vivo positive modulatory properties of the GABA<sub>B</sub> receptor positive modulators, 2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethylpropyl) phenol (CGP7930) and (R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one (rac-BHFF). Both compounds enhanced loss of righting induced by baclofen in mice. However, CGP7930 was less effective and rac-BHFF was less potent to enhance loss of righting induced by GHB, which, like baclofen, has GABA<sub>B</sub> receptor agonist properties. In contrast with baclofen- and GHB-induced loss of righting, the hypothermic effects of baclofen and GHB were not enhanced by rac-BHFF, and were enhanced by CGP7930 only at doses that produced hypothermia when given alone. CGP7930-induced hypothermia was not attenuated by the GABA<sub>B</sub> receptor antagonist CGP35348, at doses that blocked baclofen-induced hypothermia, and was not increased by the NOS inhibitor L-NAME, at doses that increased the hypothermic effects of baclofen and GHB. The results are evidence that CGP7930 and rac-BHFF act in vivo as positive modulators at GABA<sub>B</sub> receptors mediating loss of righting, but not at GABA<sub>B</sub> receptors mediating hypothermia. Conceivably, CGP7930, but not rac-BHFF, acts as an allosteric agonist at these latter receptors. Taken together, the results are further evidence of pharmacologically distinct GABA<sub>B</sub> receptor subtypes, possibly allowing for a more selective therapeutic interference with the GABA<sub>B</sub> system.
Introduction

Allosteric modulators alter the activity of the endogenous ligand by binding to receptor sites that are different from the orthosteric site where the endogenous ligand acts (Christopoulos, 2002; Conn et al., 2009; Pin and Prezeau, 2007; Wang et al., 2009). There is currently much interest in allosteric modulators, because by discriminating between activated and non-activated receptors, they may have a broader therapeutic window than ligands that indiscriminately alter the activity of all receptors. Allosteric modulators have been identified for various receptors, including GABA_A and GABA_B receptors. Because GABA_B receptors are implicated in various psychiatric disorders (Frankowska et al., 2007; Kerr and Ong, 1995; Pilc and Nowak, 2005), including drug dependence (Addolorato et al., 2009; Markou et al., 2004), modulation of these receptors could provide new treatments.

Several novel compounds have been characterized as positive modulators of GABA_B receptors in vitro [e.g., CGP7930 (Urwyler et al., 2001)(Adams and Lawrence, 2007), GS39783 (Urwyler et al., 2003), rac-BHFF (Malherbe et al., 2008), and BHF117 (Maccioni et al., 2009)], and in vivo results suggest them to have anxiolytic- and antidepressant-like properties (Cryan et al., 2004; Frankowska et al., 2007; Jacobson and Cryan, 2008). In addition, positive modulators of GABA_B receptors reduce self-administration of alcohol (Liang et al., 2006; Maccioni et al., 2008; Maccioni et al., 2009; Orru et al., 2005), cocaine (Filip et al., 2007), and nicotine (Mombereau et al., 2007; Paterson et al., 2008). Although all of these effects are generally thought to be mediated by positive modulation of GABA_B receptors, to date such modulation has been examined almost exclusively in vitro. Examination of positive modulating properties in vivo may
help to further understand the mechanism by which these compounds exert their potential therapeutic effects.

CGP7939 and rac-BHFF have been reported to increase loss of righting in mice induced by a sub-threshold dose of the GABA<sub>B</sub> receptor agonist baclofen (Carai et al., 2004; Malherbe et al., 2008). These findings, together with the observation that CGP7930 and rac-BHFF did not produce loss of righting when given alone, were taken as evidence that CGP7930 and rac-BHFF have positive modulating properties at GABA<sub>B</sub> receptors in vivo. To characterize these in vivo effects in more detail, the present study established dose-response curves for GABA<sub>B</sub> receptor agonists, and used shifts of these curves to quantify the relative potency and effectiveness of the positive modulators.

GABA<sub>B</sub> receptors can be activated by baclofen, but also by other drugs, such as GHB (Mathivet et al., 1997). However, the GABA<sub>B</sub> receptor mechanisms underlying the effects of baclofen and GHB do not appear to be identical. First, the GABA<sub>B</sub> receptor antagonist CGP35348 is often less potent to antagonize effects of GHB than effects of baclofen (Carter et al., 2006; Koek et al., 2004; Koek et al., 2007b; Koek et al., 2009). Second, NMDA antagonists enhance behavioral effects of GHB but not of baclofen (Koek et al., 2007a; Koek and France, 2008). Preferential activity of GHB at GABA<sub>B</sub> heteroreceptors on glutamatergic neurons and of baclofen at GABA<sub>B</sub> autoreceptors on GABAergic neurons could conceivably account for some of these differences (Carter et al., 2009). Recent in vitro evidence suggests that CGP7930 and its analogue BSPP selectively potentiate activity at GABA<sub>B</sub> autoreceptors, but not at heteroreceptors (Chen et al., 2006; Parker et al., 2008). This suggests the possibility, examined here, that
CGP7930, and perhaps rac-BHFF, preferentially enhance in vivo effects of baclofen compared with those of GHB.

GABA_B receptor activation not only produces loss of righting, but also other in vivo effects, such as hypothermia (Kaupmann et al., 2003). Hypothermia, which occurs at lower doses than loss of righting, is likely mediated by a population of GABA_B receptors in a particular brain region (i.e., hypothalamus) that differs from the population of GABA_B receptors involved in loss of righting. To examine if these GABA_B receptor populations differ in their susceptibility to enhancement by positive modulators, the present study characterized the effects of CGP7930 and rac-BHFF on baclofen- and GHB-induced hypothermia, which have not been studied before, and compared these effects with those on loss of righting, which to date have been studied at a single agonist dose.

Surprisingly, the present study found CGP7930 to produce hypothermia when given alone. To study the involvement of GABA_B receptors in these effects, their antagonism by CGP35348 was examined, in comparison with antagonism of baclofen- and GHB-induced hypothermia. In rats, the NOS inhibitor L-NAME enhances baclofen-induced hypothermia (Rawls et al., 2004; Rawls et al., 2006). Preliminary observations in our laboratory (unpublished) showed this enhancement to occur also in mice. Thus, the present study further examined the mechanisms underlying CGP7930-, baclofen-, and GHB-induced hypothermia by testing if L-NAME enhanced the hypothermic effects of CGP7930 in a manner similar to that observed with baclofen and GHB.
Methods

Animals

One hundred and sixty adult male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), weighing 26-37 g at the beginning of the experiments, were housed in groups of four in an environmentally controlled room (temperature, 24°C; relative humidity, 45%) under a 14/10-h light/dark cycle (light on at 0700 h) with food (rodent sterilizable diet; Harlan Teklad) and water continuously available. The animals were maintained and the 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 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996).

Apparatus

Body temperature was measured using a digital thermometer (model BAT7001H) and a thermistor probe (model RET-3), both manufactured by Physitemp Instruments (Clifton, NJ, USA).

Procedure

Righting was assessed repeatedly in the same animals before and at different intervals (15 – 120 min) after drug administration. Loss of the righting reflex was scored as 1, otherwise as 0. Righting was considered absent when a mouse, after having been
placed on its back, did not right itself within 15 s (i.e., the plantar surface of none of the feet made full contact with the floor).

Immediately before drug administration, baseline temperature was measured by inserting the lubricated probe 2 cm into the rectum. Thereafter, body temperature was recorded repeatedly in the same animals, at different intervals (ranging from 15 – 120 min) after drug administration.

Drug tests were conducted in groups of mice (body temperature: n=4-6; righting: n=8) selected non-systematically from the population of mice (body temperature: 80; righting: 80) available for the present studies. Individual mice were tested with drugs on average 10 times (range=5-15), and no mouse received the same drug test twice. In an effort to control for effects of repeated testing, drug doses were tested in a nonsystematic order with at least 1 week between tests.

Data Analysis

Effects of drugs given alone on loss of righting were examined by calculating the percentage of animals showing loss of righting, for each dose and each post-administration interval. To further examine drugs given alone, and to examine drugs given together, for each dose (or dose combination) the loss of righting scores obtained from 0 to 120 min post-administration were summed for each animal (maximum total score = 6). Total scores were averaged across animals, and mean values ± S.E.M. were plotted as a function of dose. Dose-response data were analyzed by log-linear regression (Tallarida, 2000) of individual values by use of GraphPad Prism, with the following equation: effect = slope x log(dose) + intercept. Deviations from linearity were examined
by the replicates test. F ratio tests in GraphPad Prism were used to compare dose-
response curves with respect to their slopes and intercepts. For example, a non-
significant F ratio for slopes and a significant F ratio for intercepts show that dose-
response curves are parallel but occupy different positions on the dose axis. Effects of
320 mg/kg CGP35348 on loss of righting produced by CGP7930 or rac-BHFF together
with baclofen or GHB were analyzed by Student’s t test.

Effects of drugs on body temperature when given alone were analyzed by two-
way analysis of variance (ANOVA) with dose as between-subjects factor and time as
within-subjects factor, followed by Dunnett’s test (NCSS 2007; Kaysville, UT, USA). To
examine effects of drugs when given together, for each dose combination the area under
the body temperature time curve (AUC) from 0 to 120 min post-administration was
calculated for each animal by means of GraphPad Prism version 5.02 for Windows
(GraphPad Software, San Diego, CA, USA), which used the trapezoid rule to calculate
the area below the baseline value obtained immediately before drug administration.
AUC values were averaged across animals, and the mean values ± S.E.M. were plotted as
a function of dose. Dose-response data were analyzed by log-linear regression as
described for loss of righting. In addition, pretreatment interval effects were analyzed by
two-way ANOVA with interval and dose as between-subjects factors. Antagonist effects
were analyzed by calculating, for each dose of the antagonist, the agonist dose needed to
produce 50% of the maximal response (ED50) and the ratio of this ED50 with the agonist
ED50 after vehicle. Dose ratios were plotted as a function of antagonist dose, and the
resulting Schild plot (Arunlakshana and Schild, 1959) was analyzed with linear
regression.
Drugs

Baclofen and L-NAME (Nω-nitro-L-arginine methyl ester) HCl were purchased from Sigma-Aldrich (St. Louis, MO). GHB (γ-hydroxybutyrate) was provided by the National Institute on Drug Abuse (Bethesda, MD). CGP7930 (2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethylpropyl)phenol) and rac-BHFF [(R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one] were synthesized by K. Cheng at the National Institute on Drug Abuse (Bethesda, MD), and CGP35348 (3-aminopropyl(diethoxymethyl)phosphinic acid) was synthesized by J. Agyin at the University of Texas Health Science Center (San Antonio, TX). All compounds were dissolved in sterile water, except CGP7930, which was suspended in sterile water with 0.6% methylcellulose, and rac-BHFF, which was suspended in a 4:1:15 mixture containing Cremophor EL, 1,2-propanediol and distilled water (Malherbe et al., 2008). All compounds were injected intraperitoneally, except CGP7930, which was also administered orally, in a volume of 5 – 20 ml/kg. When more than one drug was administered, they were administered at the same time (except where noted). Doses are expressed as the form of the compound listed above.
Results

Baclofen and GHB produced loss of righting in a dose- and time-dependent manner (Fig. 1, left panels). Using the total of the loss of righting scores at each of the time points as a measure of drug effect, dose-response data were collected for baclofen and GHB, given alone and given together with 320 mg/kg CGP35348 (Fig. 1, upper right panel, filled and unfilled symbols, respectively). None of the dose-response data obtained in the present study deviated significantly from linearity, unless stated otherwise. The dose-response curves of GHB and baclofen had a common slope (F[1,52]=2.89, P>0.05) and significantly different ED₅₀ values (baclofen: 34 (95% CL: 28 – 41) mg/kg; GHB: 870 (750 - 2890) mg/kg; F[1,53]=67.53, P<0.0001). Thus, baclofen was almost 30-fold more potent than GHB to produce loss of righting.

CGP35348 significantly increased the ED₅₀ values for baclofen (F[1,37]=37.63, P<0.0001) and GHB (F[1,45]=37.37, P<0.0001) in a similar manner (3- and 3.3-fold, respectively). Unlike baclofen and GHB, the positive GABAₐ receptor modulators CGP7930 and rac-BHFF did not produce any loss of righting (Fig. 1, lower right panel).

CGP7930 dose-dependently enhanced both baclofen- and GHB-induced loss of righting, but did so in a different manner (Fig. 2, upper panels). CGP7930 shifted the dose-response curve of baclofen to the left in a parallel manner. The dose-response curves of baclofen in the presence of CGP7930 had a common slope (F[3,80]=1.16, P>0.20) and significantly different ED₅₀ values (F[3,83]=14.82, P<0.0001). At the highest dose of 320 mg/kg, CGP7930 decreased the ED₅₀ of baclofen 2.8-fold, from 34(28-41) to 12(10-15) mg/kg. In contrast, CGP7930 shifted the dose-response curve of GHB in a non-parallel manner, as evidenced by significant different slope values for the
dose-response curves of GHB in the presence of CGP7930 (F[2,81]=3.54, P<0.05). As a result, the extent to which CGP7930 increased the potency of GHB depended on the effect level, with an almost 2-fold shift at maximal effect levels, but no apparent shift at intermediate and minimal effect levels. CGP7930 enhanced the effects of baclofen in a different manner than the effects of GHB; however, in both cases CGP35348 attenuated the enhanced effects. A dose of 320 mg/kg CGP35348 significantly attenuated the effects of 320 mg/kg CGP7930 combined with 17.8 mg/kg baclofen (t=2.90, df=14, P<0.05) and combined with 1000 mg/kg GHB (t=2.51, df=13, P<0.05) (Fig. 2, upper panels: filled and unfilled downward triangles).

rac-BHFF dose-dependently enhanced baclofen- and GHB-induced loss of righting (Fig. 2, middle panels), as evidenced by parallel (F[3,104]=2.25, P>0.05), leftward shifts (F[3,107]=7.49, P<0.0001) of the dose-response curves. At the highest dose of 100 mg/kg, rac-BHFF decreased the ED50 of baclofen 1.9-fold, from 31(27-35) to 16(10-15) mg/kg, and decreased the ED50 of GHB 1.6-fold, from 740(640-850) to 460(400-540) mg/kg. A dose of 320 mg/kg CGP35348 attenuated the enhanced effects of 100 mg/kg rac-BHFF combined with 17.8 mg/kg baclofen (t=2.90, df=14, P<0.05) and combined with 320 mg/kg GHB (t=2.51, df=13, P<0.05) (Fig. 2, middle panels: filled and unfilled upward triangles).

To characterize the enhancing properties of CGP7930 and rac-BHFF, ED50 values for baclofen and GHB in the presence of different doses of the modulators were used to calculate dose ratios for each modulator/agonist combination, except for the combination of CGP7930 and GHB, which did not yield parallel shifts. These ratios, shown in a Schild-like plot (Fig. 2, lower panel), could be fitted with straight lines with a common
slope (F[2,3]=0.60, P>0.20) not significantly different from 1 (i.e., 1.2[0.92-1.5]) and significantly different intercepts (F[2,5]=6.53, P<0.05). These lines were used to estimate the dose of the modulator needed to shift the agonist dose-response curve 2-fold to the left, which was 220 mg/kg for CGP7930 combined with baclofen, 89 mg/kg for rac-BHFF combined with baclofen, and 120 mg/kg for rac-BHFF combined with GHB. Thus, rac-BHFF was 2.5-fold more potent than CGP7930 to enhance effects of baclofen, and was 1.3-fold more potent to enhance baclofen than to enhance GHB.

When given alone, baclofen, GHB, and CGP7930, but not rac-BHFF, decreased body temperature in a dose- and time-dependent manner (Fig. 3, left and middle panels) (baclofen and GHB, dose: F[4,23]=9.48, P<0.001; time: F[6,138]=19.42, P<0.001; dose x time: F[24,138]=8.28, P<0.001; CGP7930, dose: F[5,21]=25.15, P<0.001; time: F[6,126]=30.43, P<0.001; dose x time: F[30,126]=7.27, P<0.001; rac-BHFF, dose: F[5,18]=1.15, P>0.20; time: F[6,108]=21.46, P<0.001; dose x time: F[30,108]=1.91, P<0.01). The lowest dose that produced statistically significant hypothermia was 3.2 mg/kg for baclofen, 178 mg/kg for GHB, and 100 mg/kg for CGP7930. Baclofen and GHB produced maximal hypothermia at 30 – 60 min after injection, and maximal effects of CGP7930 were apparent around 90 min after injection. The lowest body temperature observed with CGP7930 was 34 (0.3) °C, which was not significantly different (t[8]≤1.96, P>0.05) from that obtained with baclofen (32.6 [0.5]) or GHB (32.3 [1.2]). None of the values obtained with rac-BHFF differed significantly from vehicle control. Using area under the body temperature time curve (AUC) as a measure of drug effect, dose-response data were collected for baclofen and GHB, given alone and given together with 320 mg/kg CGP35348 (Fig. 3, upper right panel, filled and unfilled symbols,
respectively). The dose-response curves of GHB and baclofen had a common slope (F[1,44]=0.41, P>0.20) and significantly different ED50 values (baclofen: 4.7 (3.8-5.8) mg/kg; GHB: 250 (210-280) mg/kg; F[1,45]=72.41, P<0.0001). Thus, baclofen was 53-fold more potent to produce hypothermia than GHB. CGP35348 significantly increased the ED50 value for baclofen 3.4-fold (F[1,29]=45.02, P<0.0001), but did not significantly alter the ED50 for GHB (F[1,29]=0.01, P>0.20). CGP7930 produced hypothermia (ED50 = 100 [73-130]), like baclofen and GHB, but its dose-response curve (Fig. 3, lower right panel) was significantly shallower (F[1,39]=19.78, P<0.0001). CGP7930 also produced hypothermia when administered orally (data not shown), with an ED50 (i.e., 140 (104-201) mg/kg) that did not differ significantly (F[1,35]=2.64, P>0.10) from its ED50 after i.p. administration (i.e., 100 (73-130) mg/kg), and with a common slope (F[1,34]=2.52, P>0.10). In contrast with CGP7930, rac-BHFF did not produce hypothermia, as evidenced by the slope of the regression line not being significantly different from zero (F[1,18]=2.29, P>0.10).

CGP7930 enhanced the hypothermic effects of baclofen and GHB (Fig. 4, upper panels). At 320 mg/kg, CGP7930 significantly shifted the dose-response curves of baclofen (F1,36]=18.84, P<0.001) and GHB (F[1,37]=14.39, P<0.001) to the left, in a parallel manner for GHB (F[1,36]=2.08, P>0.10) but not for baclofen (F[1,36]=6.50, P<0.05). At lower doses of CGP7930 (i.e., 32 and 100 mg/kg), the dose-response curves of baclofen and GHB were similar to control, and had a common slope (baclofen: F[2,54]=1.17, P>0.20; GHB: F[2,50]=0.90, P>0.20) and a common ED50 (baclofen: F[2,56]=2.26, P>0.10; GHB: F[2,52]=0.07, P>0.20). None of the dose-response curves deviated from linearity, except the dose-response curve of baclofen in the presence of 32
mg/kg CGP7930 (P<0.001). When 100 mg/kg CGP7930 was given 60 min before baclofen (3.2, 5.6 mg/kg) or GHB (178, 320 mg/kg) (data not shown), the results were not significantly different (F[1,12]<=1.32, P>0.20) from those obtained when CGP7930 was co-administered with baclofen or GHB (Fig. 4, top panels). In contrast with CGP7930, rac-BHFF did not alter the hypothermic effects of baclofen and GHB (Fig. 4, lower panels). The dose-response curves of baclofen and GHB obtained in the presence of different doses of rac-BHFF could be fitted with common slopes (baclofen: F[2,40]=0.07, P>0.20; GHB: F[2,36]=0.92, P>0.20, respectively) and common ED50 values (baclofen: F[2,42]=0.13, P>0.20; GHB: F[2,38]=0.87, P>0.20).

CGP7930-induced hypothermia was not significantly attenuated by the GABA<sub>B</sub> receptor antagonist CGP35348; the dose-response curves shown in the upper left panel of Fig. 5 had a common slope (F[2,37]=0.24, P>0.20) and a common ED50 (F[2,39]=1.62, P>0.20). In contrast, CGP35348 shifted the dose-response curve for baclofen-induced hypothermia to the right (F[3,59]=29.33, P<0.001), in a parallel manner (F[3,56]=0.62, P>0.20) (Fig. 5, upper middle panel). These antagonist effects of CGP35348 were quantified by means of a Schild regression plot (not shown). The plot, with a slope [i.e., -0.54 (-0.77, -0.31)] significantly different from -1 (P<0.05), yielded an apparent pA<sub>2</sub> value, as an empirical potency estimate, of 3.55 (3.37 – 3.80), and did not differ significantly (F[2,2]=1.05, P>0.20) from the plot based on data obtained only at 60 min after the injection of the antagonist (data not shown). Doses of CGP35348 that antagonized the effects of baclofen failed to antagonize the effects of GHB (Fig. 5, upper right panel). At the highest dose tested (i.e., 1000 mg/kg), CGP35348 significantly (P<0.05) shifted the dose-response curve of GHB to the left, in a non-parallel manner.
This dose of CGP35348 significantly decreased body temperature when given alone (dose: F[4, 25] = 2.51, P = 0.067; time: F[6, 150] = 7.93, P < 0.001; dose x time: F[24, 150] = 2.72, P < 0.001) to a minimum value of 36.7 (0.35) °C, 45 min after injection (data not shown).

CGP7930-induced hypothermia was not significantly affected by L-NAME. The dose-response curves shown in the lower left panel of Fig. 5 had a common slope (F[2, 105] = 1.87, P > 0.10) and a common ED$_{50}$ (F[2, 107] = 1.75, P > 0.10). In contrast, L-NAME dose-dependently enhanced baclofen- and GHB-induced hypothermia (Fig. 5, lower middle and right panels) at doses that did not lower body temperature when given alone (data not shown; dose: F[4, 25] = 0.09, P > 0.20; time: F[6, 150] = 11.42, P < 0.001; dose x time: F[24, 150] = 1.67, P = 0.034). At 10 – 100 mg/kg, L-NAME significantly shifted the dose-response curves of baclofen (F[3, 79] = 11.10, P < 0.001) and GHB (F[3, 71] = 7.58, P < 0.001) to the left, in a parallel manner (baclofen: F[3, 76] = 0.44, P > 0.20; GHB: F[3, 68] = 0.85, P > 0.20). L-NAME maximally shifted the baclofen dose-response curve 2.4-fold to the left at 32 and 100 mg/kg, and maximally shifted the GHB dose-response curve 2.5-fold to the left at 100 mg/kg. Thus, L-NAME appeared to be more potent to enhance the hypothermic effects of baclofen than those of GHB.
Discussion

The main finding of the present study is that the positive GABA<sub>B</sub> receptor modulators CGP7930 and rac-BHFF enhanced baclofen- and GHB-induced loss of righting, but not hypothermia. These results suggest that CGP7930 and rac-BHFF act in vivo as positive modulators at GABA<sub>B</sub> receptors involved in loss of righting, but not at GABA<sub>B</sub> receptors mediating hypothermic effects of GABA<sub>B</sub> receptor agonists. Thus, different GABA<sub>B</sub> receptor populations may differ in their susceptibility to positive modulatory effects, possibly allowing for a more selective therapeutic interference with the GABA<sub>B</sub> system.

Baclofen and GHB induced loss of righting in mice, consistent with previous observations (Carter et al., 2005), and antagonism by CGP35348 confirmed a role for GABA<sub>B</sub> receptors in these effects. CGP7930 and rac-BHFF enhanced baclofen- and GHB-induced loss of righting without producing loss of righting when given alone, in agreement with previous findings (Carai et al., 2004; Malherbe et al., 2008). Antagonism of the enhancement by CGP35348 indicated the involvement of GABA<sub>B</sub> receptors. In contrast with previous studies using a sub-threshold dose of baclofen (Carai et al., 2004; Malherbe et al., 2008) and GHB (Carai et al., 2004), dose-response curves for baclofen and GHB were established. CGP7930 shifted the baclofen dose response curve to the left in a parallel manner, but increased the slope of GHB dose response curve. Conceivably, this may be related to different GABA<sub>B</sub> receptors mediating the effect of baclofen and GHB (i.e., GABA<sub>B</sub> autoreceptors and heteroreceptors, respectively; e.g., (Carter et al., 2009), and to CGP7930 selectively potentiating autoreceptor activity (Chen et al., 2006). BHFF was 2.5–fold more potent than CGP7930 to enhance baclofen, and was less potent
to enhance GHB than to enhance baclofen. Taken together, these results suggest the possibility that GABA<sub>B</sub> receptor positive modulators preferentially enhance in vivo effects of baclofen compared with those of GHB. If confirmed, this would be further evidence that the GABA<sub>B</sub> receptor mechanisms involved in the effect of baclofen and GHB are not identical.

Baclofen decreased body temperature, in agreement with previous observations (Gray et al., 1987; Jacobson and Cryan, 2005), and did so by activating GABA<sub>B</sub> receptors, evidenced by antagonism by CGP35348. Schild analysis yielded a regression plot with a slope different from -1, suggesting possible non-equilibrium (e.g., Kenakin, 1997). However, the regression plot was not different when, instead of the 2 h AUC data, only data were used that were obtained 60 min after administration of CGP35348, when agonist effects of baclofen and antagonist effects of CGP35348 are likely maximal (Koek et al., 2007b). This suggests that other factors, such as a heterogeneous receptor population, might be involved (Kenakin, 1997). An apparent pA<sub>2</sub> value of 3.55 was calculated as an empirical estimate of the potency with which CGP35348 antagonized the hypothermic effects of baclofen. This potency estimate, which is equivalent to 70 mg/kg CGP35348, agrees with the previous finding that 100 mg/kg CGP35348 shifted the dose-response curve for the cataleptic effects of baclofen 2-fold to the right (Koek et al., 2007b). These results obtained with CGP35348, together with the finding that baclofen does not produce hypothermia in GABA<sub>B</sub> receptor knockout mice (Queva et al., 2003), indicate that baclofen-induced hypothermia is mediated by GABA<sub>B</sub> receptors and, therefore, useful as an in vivo assay of GABA<sub>B</sub> receptor activation.
Like baclofen, GHB produces hypothermia by activating GABA$_B$ receptors, evidenced by its lack of hypothermic effects in GABA$_B$ receptor knockout mice (Kaupmann et al., 2003). Consistent with the involvement of GABA$_B$ receptors, CGP35348 antagonizes many of the effects of GHB, but is often less potent to antagonize effects of GHB than of baclofen (Carter et al., 2006; Koek et al., 2004; Koek et al., 2007b; Koek et al., 2009). This has been taken to indicate that the GABA$_B$ receptor mechanisms underlying the effects of GHB are not identical to those of prototypical GABA$_B$ agonists such as baclofen (Koek et al., 2009). Consistent with this, in the present study CGP35348 failed to antagonize GHB-induced hypothermia at doses that antagonized baclofen-induced hypothermia. At the highest dose, CGP35348 shifted the GHB dose-response curve to the left, and produced hypothermia when given alone. Thus, its hypothermic effects appeared to limit its antagonism of GHB-induced hypothermia. The finding that high doses of CGP35348 produced hypothermia, like GABA$_B$ agonists, may be related to its partial agonist properties at GABA$_B$ receptors (Urwyler et al., 2005). The present results differ from a report that CGP35348 antagonized hypothermia produced by GBL, a prodrug of GHB, (Carai et al., 2008), and from a report that GHB-induced hypothermia was antagonized by the GABA$_B$ antagonist SCH50911 (van Nieuwenhuijzen and McGregor, 2009). These differences could be related in part to the use of GBL, which has pharmacokinetic properties different from GHB, and to the use of SCH50911, which may have pharmacological properties different from CGP35348. Be that as it may, these studies clearly indicate an important role for GABA$_B$ receptors in GHB-induced hypothermia. The present results with CGP35348 suggest that baclofen and GHB produce hypothermia through different GABA$_B$ receptor mechanisms.
rac-BHFF did not alter baclofen- and GHB-induced hypothermia, at doses that enhanced baclofen- and GHB-induced loss of righting. CGP7930 enhanced baclofen- and GHB-induced hypothermia, but only at a dose that produced hypothermia when given alone. In a recent study in mice, CGP7930 did not significantly affect body temperature except for a moderate increase of less than 1 °C at the highest dose tested, i.e., 300 mg/kg p.o. (Jacobson and Cryan, 2008). In the present study, CGP7930 decreased body temperature with similar potency when administered i.p. (ED\textsubscript{50} = 100 mg/kg) and when administered orally (ED\textsubscript{50} = 140 mg/kg). Thus, differences between the effects of CGP7930 in the study by Jacobson and Cryan (2008) and in the present study do not appear to be related to the route of administration, but may be related to mouse strain (C57BL/6J in the present study, OF1 in the prior study), because strains can differ in their sensitivity to the hypothermic effects of baclofen (Jacobson and Cryan, 2005). In the present study, the hypothermic effects of CGP7930 were not altered by doses of CGP35348 that antagonized the hypothermic effects of baclofen. This suggests that the hypothermic effects of CGP7930 do not result from enhancement of the effects of endogenous GABA at GABA\textsubscript{B} receptors. Instead, they could result from activation of the GABA\textsubscript{B} receptor by CGP7930 through a site different from the site where GABA acts, suggested by the observation that CGP7930 can directly activate the receptor (Binet et al., 2004). Thus, although the present results do not provide evidence that CGP7930 acts in vivo as a positive modulator at GABA\textsubscript{B} receptors mediating hypothermia, they are consistent with the possibility that CGP7930 behaves as an allosteric agonist at these receptors.
The NOS inhibitor L-NAME dose-dependently enhanced the hypothermic effects of baclofen and GHB in mice, without affecting body temperature when given alone. These results are in agreement with previous observations on the enhancement of baclofen-induced hypothermia by L-NAME in rats (Rawls et al., 2004; Rawls et al., 2006), and extend these results to mice, and to hypothermia induced by GHB. The mechanism underlying these synergistic effects has been suggested to involve GABA\textsubscript{B} receptor-mediated suppression of NO synthesis in brain regions that regulate body temperature, with NO production diminished further by L-NAME (Rawls et al., 2006). This mechanism may be involved not only in the hypothermic synergy of baclofen and L-NAME, but also in that of GHB and L-NAME, because L-NAME enhanced the hypothermic effects of baclofen and GHB in a similar manner. In contrast, the hypothermic effects of baclofen and GHB were differentially antagonized by CGP35348. These results suggest the possibility that baclofen and GHB produce hypothermia through different GABA\textsubscript{B} receptor populations that are similarly coupled to NO production.

CGP7930-induced hypothermia was not affected by L-NAME, suggesting CGP7930 to act through GABA\textsubscript{B} receptors not coupled to NO production. Although CGP7930 appears to be a selective GABA\textsubscript{B} receptor modulator (Urwyler et al., 2001), the possible involvement of other, non-GABA\textsubscript{B} receptors in its hypothermic effects can at present not be ruled out. Be that as it may, the finding that rac-BHFF did not produce hypothermic effects suggests rac-BHFF to be a more selective in vivo GABA\textsubscript{B} receptor modulator than CGP7930.
Taken together, the present results show that CGP7930 and rac-BHFF enhance baclofen- and GHB-induced loss of righting, but not hypothermia. Effects of GABA<sub>B</sub> agonists on motor coordination and on body temperature are likely mediated by different GABA<sub>B</sub> receptor populations, in brain regions such as motor cortex and cerebellum, and in the hypothalamus, respectively. There is evidence that the specific GABA<sub>B</sub> receptor populations that mediate ataxia and hypothermia are under differential genetic control (Jacobson and Cryan, 2005). Based on the results of the present experiments, it is tempting to speculate that the pharmacological properties of these receptor populations differ as well. Differential enhancement of GABA<sub>B</sub> receptor populations by positive modulators has been shown in vitro: presynaptic GABA<sub>B</sub> autoreceptors appear to be sensitive to CGP7930 and the CGP7930 analogue BSPP, whereas presynaptic GABA<sub>B</sub> heteroreceptors are not (Chen et al., 2006; Parker et al., 2008). Conceivably, such differential enhancement could also be involved in the in vivo effects of CGP7930 and rac-BHFF reported here.

In summary, the positive GABA<sub>B</sub> receptor modulators CGP7930 and rac-BHFF enhanced baclofen- and GHB-induced loss of righting, but not hypothermia, suggesting that they act in vivo as positive modulators at some, but not all, GABA<sub>B</sub> receptors. If different GABA<sub>B</sub> receptor populations differ in their susceptibility to positive modulatory effects, this could allow a more selective therapeutic interference with the GABA<sub>B</sub> system.
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Footnotes

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Address correspondence to: Wouter Koek, Ph.D., Departments of Psychiatry and Pharmacology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, Mail Code 7792, San Antonio, Texas 78229-3900. E-mail:
koek@uthscsa.edu
Figure legends

Fig. 1 Effects of the GABA_B receptor agonist baclofen, GHB, and the GABA_B receptor positive modulators CGP7930 and rac-BHFF on righting in C57BL/6J mice (n=8 per dose). Symbols in the left panels represent the percentage of animals showing loss of righting at different times after i.p. administration of baclofen or GHB. Symbols in the right panels represent mean±S.E.M righting scores, totaled for each animal across the six different times after injection. Open symbols in the upper right panel represent data obtained when baclofen and GHB were given together with 320 mg/kg of the GABA_B receptor antagonist CGP35348.

Fig. 2 Effects of the GABA_B receptor positive modulators CGP7930 (upper panels) and rac-BHFF (middle panels), administered i.p., on loss of righting produced by baclofen (upper and middle left panels) and GHB (upper and middle right panels). Symbols in the upper and middle panels represent mean±S.E.M righting scores, totaled for each animal (n=8 mice per dose) across the six different times after injection. Filled triangles in the upper and middle panels indicate data obtained in the presence of 320 mg/kg CGP35348. Lower left panel: Schild-like plot of dose ratios for baclofen and GHB, calculated from the ED_{50} values of the dose-response curves shown in the upper and middle panels, as a function of the dose of the positive modulator.

Fig. 3 Effects of baclofen, GHB, CGP7930, and rac-BHFF on body temperature in C57BL/6J mice. Symbols represent mean±S.E.M. (n=4-6 mice per dose). Gray-filled
symbols in the left and middle panels represent data that are significantly different from vehicle control (Dunnett’s test, P<0.05). AUC values shown in the right panels were calculated for each dose from the time response data shown in the left and middle panels. Open symbols in the upper right panel represent data obtained when baclofen and GHB were given together with 320 mg/kg of the GABA<sub>B</sub> receptor antagonist CGP35348.

Fig. 4 Effects of CGP7930 (upper panels) and rac-BHFF (lower panels) on hypothermia induced by baclofen (left panels) and GHB (right panels). Symbols represent mean±S.E.M. AUC values (n=4-6 mice per dose).

Fig. 5 Effects of CGP35348 (upper panels) and L-NAME (lower panels) on hypothermia induced by CGP7930 (left panels), baclofen (middle panels), and GHB (right panels). Symbols represent mean±S.E.M. AUC values (n=4-6 mice per dose).
Figure 1

**Graphs showing loss of righting response to baclofen and GHB**

- **Baclofen (mg/kg)**
  - Vehicle
  - 17.8
  - 32
  - 56

- **GHB (mg/kg)**
  - Vehicle
  - 320
  - 560
  - 1000
  - 1780

**Y-axis:** Loss of righting (% animals)
**X-axis:** Time (min)

**Graph showing dose-response relationship for baclofen and GHB**

- **Baclofen**
  - Dose range: 0 to 3200 mg/kg

- **GHB**
  - Dose range: 0 to 3200 mg/kg

**Legend:**
- Vehicle
- Dose values (mg/kg)

**Data points and error bars indicated.**
**Figure 4**

**CGP7930**

- **Vehicle**
- **32 mg/kg**
- **100 mg/kg**
- **320 mg/kg**

**Hypothermia (AUC 0-120)**

**baclofen (mg/kg)**

- 1
- 1.78
- 3.2
- 5.6
- 10

**GHB (mg/kg)**

- 56
- 100
- 178
- 320
- 560

**rac-BHFF**

- **Vehicle**
- **32 mg/kg**
- **100 mg/kg**

**Hypothermia (AUC 0-120)**

**baclofen (mg/kg)**

- 1
- 1.78
- 3.2
- 5.6
- 10

**GHB (mg/kg)**

- 56
- 100
- 178
- 320
- 560
Figure 5

**CGP35348 (mg/kg)**
- Vehicle
- 32
- 100
- 320

**I-NAME (mg/kg)**
- Vehicle
- 10
- 32
- 100