Effects of the GABA_B Receptor-Positive Modulators CGP7930 and rac-BHFF in Baclofen- and γ -Hydroxybutyrate-Discriminating Pigeons

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ABSTRACT

In vivo effects of GABA_B receptor-positive modulators suggest them to have therapeutic potential to treat central nervous system disorders such as anxiety and drug abuse. Although these effects are thought to be mediated by positive modulation of GABA_B receptors, such modulation has been examined primarily in vitro. This study further examined the in vivo properties of the GABA_B 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-3*H*-benzofuran-2-one (rac-BHFF). In pigeons discriminating baclofen from saline, γ -hydroxybutyrate (GHB) produced 100% baclofen-appropriate responding, and the GABA_B antagonist 3-aminopropyl(dimethoxymethyl) phosphinic acid (CGP35348) blocked the effects of both drugs. CGP7930 and rac-BHFF produced at most 41 and 74% baclofen-appropriate responding, respectively, and enhanced the discriminative stimulus effects of baclofen, but not of GHB. In pigeons discriminating

GHB from saline, CGP7930 and rac-BHFF produced at most 1 and 49% GHB-appropriate responding, respectively, and enhanced the effects of baclofen, but not of GHB. Enhancement of the discriminative stimulus effects of baclofen by rac-BHFF and CGP7930 is further evidence of their effectiveness as GABA_B receptor-positive modulators in vivo. Furthermore, lack of complete substitution of the positive modulators rac-BHFF and CGP7930 for baclofen and GHB suggests that their discriminative stimulus effects differ from those of GABA_B receptor agonists. Finally, together with converging evidence that the GABA_B receptor populations mediating the effects of baclofen and GHB are not identical, the present findings suggest that these populations differ in their susceptibility to positive modulatory effects. Such differences could allow for more selective therapeutic targeting of the GABA_B system.

Introduction

Allosteric modulators bind to regions on the receptor that are different from the orthosteric site where the endogenous ligand binds and act by enhancing or attenuating the response elicited by the endogenous transmitter or orthosteric agonist (Jensen and Spalding, 2004). By altering only activated receptors, allosteric modulators may have a broader

therapeutic window than ligands that alter the activity of all receptors. Allosteric modulators have been identified for many receptors, including GABA_A and GABA_B receptors. The GABA_A receptor is part of a chloride ionophore and has modulatory sites for benzodiazepines and other compounds. The GABA_B receptor is a G protein-coupled heterodimer composed of two subunits, GABA_{B1}, where GABA and other GABA_B receptor ligands bind, and GABA_{B2}, where allosteric modulators have been proposed to act (Pin et al., 2004). Because GABA_B receptors are thought to be involved in psychiatric disorders (Kerr and Ong, 1995; Markou et al., 2004; Pilc and Nowak, 2005; Frankowska et al., 2007; Addolorato et al., 2009), modulation of these receptors could provide new treatments.

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ABBREVIATIONS: 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-3*H*-benzofuran-2-one; CGP35348, 3-aminopropyl(dimethoxymethyl)phosphinic acid; GHB, γ -hydroxybutyrate; GS39783, *N,N'*-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine; BHF177, *N*-[(1*R*,2*R*,4*S*)-bicyclo[2.2.1]hept-2-yl]-2-methyl-5-[4-(trifluoromethyl)phenyl]-4-pyrimidinamine; COR627, methyl-2-(1-adamantanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate; COR628, methyl-2-(cyclohexanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate; CL, confidence level; NCS-382, (2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6*H*-benzo[*a*][7]annulen-6-ylidene ethanoic acid.

Several compounds have been shown to have positive GABA_B modulatory activity in vitro [2,6-di-*tert*-butyl-4-(3-hydroxy-2,2-dimethylpropyl)phenol (CGP7930) (Urwylers et al., 2001; Adams and Lawrence, 2007), *N,N'*-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) (Urwylers et al., 2003), (*R,S*)-5,7-di-*tert*-butyl-3-hydroxy-3-trifluoromethyl-3*H*-benzofuran-2-one (rac-BHFF) (Malherbe et al., 2008), *N*-[(1*R*,2*R*,4*S*)-bicyclo[2.2.1]hept-2-yl]-2-methyl-5-[4-(trifluoromethyl)phenyl]-4-pyrimidinamine (BHF177) (Guery et al., 2007; Maccioni et al., 2009), methyl-2-(1-adamantanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR627), and methyl-2-(cyclohexanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR628) (Castelli et al., 2012)], evidenced by enhancing GABA, and, for all compounds except BHF177, also by enhancing the GABA_B receptor agonist baclofen. In vivo results suggest positive GABA_B receptor modulators to have anxiolytic and antidepressant-like properties in elevated-maze and forced-swimming tests, respectively (Cryan et al., 2004; Frankowska et al., 2007; Jacobson and Cryan, 2008). In addition, they reduce self-administration of alcohol (Orrù et al., 2005; Liang et al., 2006; Maccioni et al., 2008, 2009), cocaine (Filip et al., 2007), and nicotine (Mombereau et al., 2007; Paterson et al., 2008). Although these effects are thought to be mediated by positive modulation of GABA_B receptors, such modulation has been examined almost exclusively in vitro. Examination of positive modulating properties in vivo may help to further elucidate the mechanism by which these compounds exert their potential therapeutic effects.

There is initial evidence that positive GABA_B receptor modulators can enhance the effects of the GABA_B receptor agonist baclofen not only in vitro but also in vivo. CGP7930 and rac-BHFF increase loss of righting in mice induced by a subthreshold dose of the GABA_B receptor agonist baclofen without producing loss of righting when given alone (Carai et al., 2004; Malherbe et al., 2008), which shows that they could have positive modulating properties at GABA_B receptors in vivo. Recently, these properties were characterized in more detail by using shifts of dose-response curves for GABA_B receptor agonists to compare the relative potency and effectiveness of the positive modulators (Koek et al., 2010). These studies showed that rac-BHFF was approximately 3-fold more potent than CGP7930 in enhancing baclofen-induced loss of righting in mice. However, baclofen-induced hypothermia, which occurs at lower doses than loss of righting, was not altered by rac-BHFF and was enhanced by CGP7930 only at doses that produced hypothermia when given alone (Koek et al., 2010). Thus, rac-BHFF and CGP7930 act in vivo as positive modulators at GABA_B receptors that mediate loss of righting, but not at those mediating hypothermia. These findings seem to be consistent with recent in vitro observations that rac-BHFF and CGP7930 were markedly more effective at enhancing the effects of baclofen in cerebellum than in other brain regions (Hensler et al., 2012), because cerebellar GABA_B receptors are involved in motor coordination (Dar, 1996), whereas hypothalamic GABA_B receptors play a role in hypothermia (Pierau et al., 1997). The present study is part of an effort to examine whether enhancement by positive modulators at GABA_B receptors is limited to loss of righting produced by high doses of GABA_B agonists. To investigate whether these positive modulators also enhance effects produced by low doses of GABA_B agonists, the present

study examined whether rac-BHFF and CGP7930 enhance the discriminative stimulus effects of baclofen.

GABA_B receptors can be activated by baclofen, but also by other drugs, such as γ -hydroxybutyrate (GHB; Mathivet et al., 1997). However, the receptor mechanisms underlying the effects of baclofen and GHB do not seem to be identical. First, the GABA_B receptor antagonist 3-aminopropyl(diethoxymethyl)phosphinic acid (CGP35348) antagonizes the behavioral effects of GHB (discriminative stimulus effects, suppression of operant responding, catalepsy) less potently than those of baclofen (Koek et al., 2004, 2007b, 2009; Carter et al., 2006). Second, *N*-methyl-D-aspartate antagonists enhance the behavioral effects of GHB (discriminative stimulus effects, catalepsy), but not those of baclofen (Koek et al., 2007a; Koek and France, 2008). Preferential activity of GHB at GABA_B heteroreceptors on glutamatergic neurons and baclofen at GABA_B autoreceptors on GABAergic neurons could conceivably account for some of these differences (Carter et al., 2009). In vitro evidence suggests that CGP7930 potentiates activity at GABA_B autoreceptors, but not at heteroreceptors (Chen et al., 2006; Parker et al., 2008). This suggests the possibility that CGP7930 preferentially enhances the in vivo effects of baclofen compared with GHB. Indeed, CGP7930 was more effective in enhancing loss of righting induced by baclofen than loss of righting induced by GHB (Koek et al., 2010). To examine the generality of this differential enhancement, the present study compared the ability of CGP7930 and rac-BHFF to enhance the discriminative stimulus effects of baclofen with their ability to enhance the discriminative stimulus effects of GHB.

Although GHB has been used extensively as a training drug in drug discrimination procedures (Carter et al., 2009), to date baclofen has been used as a training drug in only one study in rats (Carter et al., 2004). The present study is the first to establish a baclofen discrimination in pigeons. In this species, comparative data are available on the differential potency with which CGP35348 antagonizes the behavioral effects of baclofen and GHB (Koek et al., 2004, 2009) and on the discriminative stimulus effects of GHB as a function of training dose (Koek et al., 2006). The discriminative stimulus effects of GHB in pigeons are pharmacologically selective, because compounds pharmacologically unrelated to GHB (e.g., the GABA_A receptor-positive modulator diazepam and the μ opioid receptor agonist morphine) substitute only partially for GHB (Koek et al., 2006). In the present study, diazepam and morphine were tested to examine the pharmacological selectivity of the discriminative stimulus effects of baclofen in pigeons.

Materials and Methods

Animals. Seventeen adult white Carneau pigeons (*Columbia livia*; Palmetto, Sumter, SC) were individually housed under a 12-h light/dark cycle. They had free access to water and were maintained between 80 and 90% of their free-feeding weight by food (Purina Pigeon Checkers; Purina, St. Louis, MO) received during experimental sessions and supplemental postsession feedings (Purina Pigeon Checkers or mixed grain). The animals were maintained and the experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Apparatus. Experiments were conducted in sound-attenuating, ventilated chambers (BRS/LVE, Laurel, MD) equipped with two response keys that could be illuminated by a red light. After completion of each fixed ratio, the key light was extinguished for 4 s, during which time a white light illuminated the hopper where food (Purina Pigeon Checkers) was available. Chambers were connected by an interface (MED Associates, St. Albans, VT) to a computer that used MED-PC IV software (MED Associates) to monitor and control inputs and outputs and to record the data.

Procedure. One group of pigeons ($n = 9$) was trained to discriminate between baclofen and saline, and a different group of pigeons ($n = 8$) was trained to discriminate between GHB and saline. The discrimination training and testing procedure was similar to that described in detail previously (Koek et al., 2004). In brief, before each daily session, subjects received either the training dose of the training drug or saline (intramuscularly) and were immediately placed into the chamber; drug and vehicle training sessions occurred with equal frequency. Sessions started with a period of 15 min, during which the lights were off and key pecks had no programmed consequence. Subsequently, the left and the right keys were illuminated red, and 20 consecutive responses on the injection appropriate key resulted in 4-s access to food. Responses on the injection inappropriate key reset the fixed-ratio requirement on the injection appropriate key. The response period ended after 30 food presentations or 15 min, whichever occurred first. Initially, pigeons had to satisfy the following criteria for at least seven of nine consecutive sessions: $\geq 90\%$ of the total responses on the injection appropriate key and fewer than 20 responses on the injection inappropriate key before the first food presentation. 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 (a 15-min period, followed by a response period that ended after 30 food presentations or 15 min, whichever occurred first), except that food was available after completion of 20 consecutive responses on either key. Agonists (or vehicle) were given intramuscularly immediately before the session, antagonists were given intramuscularly 10 min before agonists, and positive modulators were given orally 45 min before agonists.

The dose of baclofen that was initially chosen for training was the dose (10 mg/kg) that produced the greatest amount of drug key responding in pigeons discriminating GHB from saline (Koek et al., 2004). The training dose of GHB was 178 mg/kg, shown in a previous study to be the highest dose without marked rate-decreasing effects (Koek et al., 2006).

Data Analysis. The mean percentage of responses on the training drug-appropriate key ± 1 S.E.M. was plotted as a function of dose. When an animal responded at a rate less than 20% of the saline control rate, discrimination data from that test were not included in the average. Mean percentages of responses on the training drug-appropriate key were calculated only when they were based on at least half the animals tested.

Dose-response curves that attained at least 80% training drug-appropriate responding were analyzed by nonlinear regression of individual values by means of Prism version 5.04 for Windows (GraphPad Software Inc., San Diego CA) by using the sigmoid equation: $\text{response} = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\log \text{ED}_{50} - \log(\text{dose})) \times \text{slope}})$, with bottom = 0 and top = 100. F ratio tests in Prism were used to compare dose-response curves with respect to their slopes. Parallel shifts of dose-response curves were examined by simultaneously fitting sigmoid models to the control and the shifted curves and expressing the $\log \text{ED}_{50}$ of the shifted curve as the sum of the $\log \text{ED}_{50}$ of the control curve and the log of the potency ratio, which yielded an estimate of the potency ratio and its 95% confidence limits (CL) (see EC_{50} shift equation in Prism). Shifts of dose-response curves were considered statistically significant if the 95% confidence interval of the potency ratio did not include 1. The statistical significance of differences between shifts was assessed by Student's t test, performed on the mean shifts and their standard

errors. Dose-response curves that attained a maximum between 50 and 80% training drug-appropriate responding were analyzed in the same manner, except that instead of fitting a sigmoid curve to all of the dose-response data, a straight line was fitted only to the data at doses with effects immediately below and above 50%, to estimate the ED_{50} and slope. Dose-response curves with a maximum between 25 and 50% were analyzed similarly by fitting a straight line to the data immediately below and above 25% to estimate the ED_{25} and slope. Possible deviations from the regression models were examined by the replicates test implemented in Prism. None of the dose-response data obtained in the present study deviated significantly from the regression models used, unless stated otherwise. Differences among drugs with respect to maximal effects were examined by one-factor analysis of variance followed by individual comparisons with the maximal effect of the training drug with Dunnett's test or by individual comparisons among all means with Newman-Keuls test (Prism).

Drug effects on response rate were examined by calculating for each dose the 95% confidence interval around the mean rate of responding (expressed as percentage of saline control). If this interval did not contain 100, the response rate was considered significantly different from control.

Drugs. Baclofen and diazepam were purchased from Sigma-Aldrich (St. Louis, MO). GHB and morphine sulfate were provided by the National Institute on Drug Abuse (Bethesda, MD). CGP7930 and rac-BHFF were synthesized by K. Cheng at the National Institute on Drug Abuse (Bethesda, MD), and CGP35348 was synthesized by J. Aguin at the University of Texas Health Science Center (San Antonio, TX). All compounds were dissolved in sterile water or saline, except diazepam, which was dissolved in sterile water with 70% Emulphor and 10% ethanol (by volume), 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 intramuscularly in a volume of 0.1 to 1 ml, except CGP7930 and rac-BHFF, which were administered orally in a volume of 5 ml/kg. Doses are expressed as the form of the compound listed above.

Results

Because 10 mg/kg baclofen had marked rate-decreasing effects in drug-naïve pigeons, for which little tolerance occurred within 20 sessions (data not shown), the training dose was decreased to 5.6 mg/kg. At this dose, none of the animals met the discrimination criterion within 50 sessions. Therefore, the training dose of baclofen was increased to 7.5 mg/kg, and all nine animals acquired the discrimination (median sessions to criterion 37, range 11–45, excluding sessions that were used to calculate criterion performance).

Under test conditions, baclofen dose-dependently increased responding on the baclofen-appropriate key from 0.9 to 2.2% after saline and other vehicles (data not shown) to a maximum of 93% at the training dose of 7.5 mg/kg (Fig. 1, top left). A sigmoid curve fitted to the dose-response data obtained with baclofen yielded an ED_{50} value of 3.7 mg/kg (95% CL: 2.9–4.7). GHB increased responding on the baclofen-appropriate key to a maximum similar to that of baclofen, with an ED_{50} value of 57 mg/kg (95% CL: 43–77) [16-fold (95% CL: 11–23) less potent than baclofen]. At a dose of 100 mg/kg, the GABA_B antagonist CGP35348 significantly shifted the dose-response curves of baclofen 5.3-fold to the right in a parallel manner (Table 1). The same dose of CGP35348 shifted the dose-response curve of GHB also to the right, but to a lesser extent [3.0-fold ($p = 0.10$ compared with

Fig. 1. Effects of the GABA_B receptor agonist baclofen and GHB, the GABA_B receptor antagonist CGP35348, the GABA_B receptor-positive modulators CGP7930 and rac-BHFF, the GABA_A receptor-positive modulator diazepam, and the μ receptor opioid morphine in pigeons ($n = 9$) trained to discriminate between 7.5 mg/kg baclofen and saline by using a two-key food-reinforced procedure. The mean ($\pm$ S.E.M.; if not shown, S.E.M. values are contained by the symbol) percentage of responses on the baclofen-appropriate key is plotted as a function of dose ($n = 5 - 7$ per dose, except for doses of baclofen, which were tested in eight animals). For dose-response data that attained the 80% level, nonlinear regression was used to obtain the best-fitting sigmoid curve; for other dose-response data, the individual points were connected. Numbers in the insets are doses in mg/kg. Numbers in parentheses are mean rates of responding (expressed as percentage control) that were significantly ($p < 0.05$) different from control.

TABLE 1
Shifts of the dose-response curves of baclofen and GHB, measured as dose ratios (95% confidence limits), produced by the GABA_B antagonist CGP35348 and the GABA_B receptor-positive modulators CGP7930 and rac-BHFF in different groups of pigeons trained to discriminate either baclofen or GHB from saline

Pretreatment	Treatment	Discrimination	
		Baclofen vs. Saline	GHB vs. Saline
CGP35348, 100 mg/kg	Baclofen	5.3 (3.2- 8.8)*, $P = 0.10$	N.D.
CGP35348, 100 mg/kg	GHB	3.0 (1.9-4.7)*	N.D.
CGP35348, 320 mg/kg	GHB	3.8 (2.6-5.5)*	2.8 (2.0-3.9)*
CGP7930, 100 mg/kg	Baclofen	3.9 (2.5-6.1)*, $P = 0.004$	N.D.
CGP7930, 100 mg/kg	GHB	1.4 (0.8-2.4)	N.D.
CGP7930, 320 mg/kg	Baclofen	N.D.	2.0 (1.2-3.3)*, $P = 0.04$
CGP7930, 320 mg/kg	GHB	N.D.	1.0 (0.6-1.6)
rac-BHFF, 32 mg/kg	Baclofen	3.4 (2.0-5.8)*, $P = 0.02$	N.D.
rac-BHFF, 32 mg/kg	GHB	1.4 (0.8-2.4)	N.D.
rac-BHFF, 56 mg/kg	Baclofen	N.D.	2.3 (1.2-4.3)*, $P = 0.30$
rac-BHFF, 56 mg/kg	GHB	N.D.	1.5 (0.9-2.4)

N.D., not done.
* Dose ratio significantly different from 1.

the 5.3-fold shift of the baclofen dose-response curve)]. Increasing the dose of CGP35348 to 320 mg/kg shifted the GHB curve somewhat further to the right (3.8-fold), but still less than the extent to which 100 mg/kg CGP35348 shifted the baclofen curve (5.3-fold). Thus, CGP35348 appeared to be less potent to antagonize the baclofen-like discriminative stimulus effects of GHB than those of baclofen.

When given alone, both rac-BHFF and CGP7930 increased baclofen-appropriate responding, but did so with different potencies and to different maximum levels (Fig. 1, top right).

rac-BHFF increased baclofen-appropriate responding with an ED₅₀ value of 56 mg/kg to a maximum of 74%. CGP7930 produced at most 41% baclofen-appropriate responding [significantly lower than the maximal effects of baclofen, unlike GHB and rac-BHFF (Dunnett's test)] with a potency (ED₂₅ = 88 mg/kg) approximately 3-fold lower than rac-BHFF (estimated at the 25% effect level). Because of limited solubility, higher doses of CGP7930 and rac-BHFF could not be tested. CGP35348, at a dose (320 mg/kg) that antagonized the effects of the training dose of baclofen (baclofen-appropriate re-

sponding decreased from 93 to 19%; data not shown) and the effects of GHB (Fig. 1, top left), did not significantly alter the potency of rac-BHFF to produce baclofen-appropriate responding ($p > 0.20$). However, CGP35348 seemed to shift the dose-response curve of CGP7930 to the right and down ($p = 0.10$).

When given together with baclofen and GHB, rac-BHFF and CGP7930 significantly enhanced the discriminative stimulus effects of baclofen, but not the baclofen-like discriminative stimulus effects of GHB (Fig. 1, bottom left). rac-BHFF and CGP7930 shifted the baclofen dose-response curve to the left in a parallel manner. Because the shifts observed with 32 mg/kg rac-BHFF and 100 mg/kg CGP7930 were similar [3.4-fold (95% CL: 2.0–5.8) and 3.9-fold (95% CL: 2.5–6.1), respectively], rac-BHFF was approximately 3-fold more potent than CGP7930 in enhancing the discriminative stimulus effects of baclofen. In contrast, rac-BHFF and CGP7930 did not significantly alter the potency with which GHB produced baclofen-like discriminative stimulus effects: both rac-BHFF and CGP7930 produced a shift [1.4-fold (95% CL: 0.8–2.4)] that was not significantly different from 1.

The GABA_A-positive modulator diazepam and the μ opioid receptor agonist morphine dose-dependently increased baclofen-appropriate responding (Fig. 1, bottom right). However, their maximal effects (58 and 43%, respectively) were lower than observed with baclofen. The rate of responding was decreased to 19% of control by 17.8 mg/kg diazepam (data not shown) and to 32% of control by the highest dose of morphine tested.

All eight animals trained with 178 mg/kg GHB acquired the discrimination (median session to criterion 12, range 9–30) and did so significantly faster ($P < 0.02$, Mann-Whitney test) than the animals trained with 7.5 mg/kg baclofen. GHB, baclofen, rac-BHFF, and CGP7930 had different maximal effects in the GHB-trained animals (Fig. 2, left). GHB increased drug-appropriate responding from 0.0 to 1.9% after saline and other vehicles (data not shown) to a maximum of 100% at the training dose and did so with an ED₅₀ value of 62 mg/kg (95% CL: 48–80). In contrast, baclofen increased responding on the GHB-appropriate key to a maximum of 38% and completely suppressed responding at 17.8 mg/kg (data not shown). When tested up to the solubility limit, rac-BHFF increased GHB-appropriate responding (maximum 49%) but CGP7930 did not (maximum 2%). The maximal effects of

baclofen and rac-BHFF were not significantly different, but both were significantly lower than the maximum effect of GHB and significantly higher than the maximum of CGP7930 ($P < 0.05$, Newman-Keuls test).

In the GHB-trained animals, the dose-response curve of GHB was shifted to the right by the GABA_B receptor antagonist CGP35348, but was not altered by the GABA_B-positive modulators rac-BHFF and CGP7930 (Fig. 2, right). At a dose of 320 mg/kg, CGP35348 significantly shifted the dose-response curve of GHB 2.8-fold to the right (Table 1). At the highest dose that did not produce any GHB-appropriate responding when administered alone, neither rac-BHFF (56 mg/kg) nor CGP7930 (320 mg/kg) significantly altered the potency of GHB (dose ratio: 1.5 and 1.0, respectively), but both significantly enhanced the potency of baclofen (dose ratio: 2.3 and 2.0, respectively) without significantly altering its maximum effect (ranging from 38 to 60%). Both modulators shifted the baclofen curve more than the GHB curve, and the difference between the magnitude of these shifts was statistically significant for CGP7930, but not for rac-BHFF.

Discussion

The main finding of the present study is that the positive GABA_B receptor modulators CGP7930 and rac-BHFF enhanced the discriminative stimulus effects of baclofen, but not of GHB. Although GABA_B receptors probably mediate effects that GHB has in common with baclofen, there is growing evidence that the GABA_B receptor mechanisms underlying these behavioral effects of baclofen and GHB are not identical. This evidence is extended by the present finding that the selective GABA_B receptor antagonist CGP35348 antagonized the discriminative stimulus effects of baclofen and GHB, but tended to more potently antagonize the discriminative stimulus effects of baclofen than those of GHB, consistent with previous findings of differential antagonism (Koek et al., 2004, 2007b, 2009; Carter et al., 2006). Thus, the differential enhancement of the discriminative stimulus effects of baclofen and GHB by CGP7930 and rac-BHFF observed here suggests that CGP7930 and rac-BHFF act as positive modulators at GABA_B receptors mediating the discriminative stimulus effects of baclofen, but not at GABA_B receptors mediating the discriminative stimulus effects of GHB. Such differential susceptibility of GABA_B receptor popu-

Fig. 2. Effects of the GABA_B receptor agonist baclofen and GHB, the GABA_B receptor antagonist CGP35348, and the GABA_B receptor-positive modulators CGP7930 and rac-BHFF in pigeons ($n = 8$) trained to discriminate between 178 mg/kg GHB and saline by using a two-key food-reinforced procedure. The mean ($\pm$ S.E.M.; if not shown, S.E.M. values are contained by the symbol) percentage of responses on the GHB-appropriate key is plotted as a function of dose ($n = 6 - 7$ per dose, except for doses of GHB, which were tested in eight animals). For dose-response data that crossed the 80% level, nonlinear regression was used to obtain the best-fitting sigmoid curve; for other dose-response data, the individual points were connected. Numbers in the insets are doses in mg/kg. Mean rates of responding (not shown) were not significantly different from control.

lations to positive modulatory effects possibly allows for a more selective therapeutic manipulation of the GABA_B system.

Drug discrimination has proven to be useful for studying mechanisms of drug action because it can provide sensitive and pharmacologically selective assays of *in vivo* effects (Colpaert, 1999). The results of this study, the first to establish baclofen as a discriminative stimulus in pigeons, are consistent with results in rats indicating that baclofen produces its pharmacologically selective discriminative stimulus effects by agonist activity at GABA_B receptors (Carter et al., 2004). Using the highest dose of baclofen [and GHB, see Koek et al. (2006)] that did not suppress responding, the baclofen discrimination was acquired less rapidly than the GHB discrimination, consistent with results obtained in rats (Carter et al., 2003, 2004). Also like in rats, the discriminative stimulus effects of baclofen in pigeons were pharmacologically selective in that the GABA_B agonist GHB produced full baclofen-appropriate responding, whereas the GABA_A receptor-positive modulator diazepam and the μ opioid receptor agonist morphine substituted only partially for baclofen. Antagonism of the discriminative stimulus effects of baclofen by the GABA_B receptor antagonist CGP35348 in rats (Carter et al., 2004) and pigeons (present study) is further evidence that the discriminative stimulus effects of baclofen are mediated by agonist actions at GABA_B receptors, thus providing a suitable assay for examining the effects of GABA_B receptor-positive modulators *in vivo*.

When given alone, the GABA_B receptor-positive modulators CGP7930 and rac-BHFF both increased baclofen-appropriate responding. CGP7930 was approximately 3-fold less potent than rac-BHFF, and its maximal effects were significantly lower than those of baclofen, unlike rac-BHFF, which produced maximal effects that did not differ significantly from baclofen. These results are consistent with recent *in vitro* data (Hensler et al., 2012) showing the following: 1) CGP7930 and rac-BHFF alone stimulated [³⁵S]GTP γ S binding in rat cortex and hippocampus, like baclofen; 2) CGP7930 was approximately 3-fold less potent than rac-BHFF and was less efficacious; and 3) rac-BHFF stimulated [³⁵S]GTP γ S binding in rat hippocampus to levels similar to those observed with baclofen. Hensler et al. (2012) also found that combining CGP35348 with rac-BHFF enhanced the effects on [³⁵S]GTP γ S binding, consistent with the modulation of partial agonist activity of CGP35348 by rac-BHFF. In contrast with these *in vitro* findings, CGP35348 did not significantly alter the effects of rac-BHFF on baclofen-appropriate responding. These and other observations [lack of effects of rac-BHFF on CGP35348-induced hypothermia in mice (W. Koek, unpublished observation)] suggest that enhancement of *in vitro* effects produced by combining CGP35348 with rac-BHFF may have limited significance for the *in vivo* effects of these combinations.

Although combining CGP35348 with rac-BHFF does not always seem to enhance the effects of rac-BHFF, CGP35348 clearly did not antagonize the effects of rac-BHFF examined in the present study. Because CGP35348 acts at the endogenous ligand binding site of the GABA_B receptor, where baclofen also acts, this lack of antagonism suggests that baclofen-appropriate responding produced by rac-BHFF is not caused by enhancing endogenous GABA at these receptor sites. Instead, the effects of rac-BHFF on baclofen-appropriate responding could involve receptor activation through

other sites on the GABA_B receptor, consistent with *in vitro* evidence that rac-BHFF can activate GABA_B receptors in the absence of GABA (Malherbe et al., 2008; Hensler et al., 2012). Under conditions that significantly decreased the overall response rate, CGP35348 seemed to attenuate the baclofen-like effects of CGP7930. This may suggest that these effects of CGP7930 not only involve direct activation of GABA_{B2} subunits (Binet et al., 2004; Malherbe et al., 2008), but possibly also enhancement of endogenous GABA at GABA_{B1} subunits.

rac-BHFF and CGP7930 enhanced the discriminative stimulus effects of baclofen in animals trained to discriminate baclofen from saline and in animals trained to discriminate GHB from saline. These findings are further evidence of the effectiveness of rac-BHFF and CGP7930 as GABA_B receptor-positive modulators *in vivo*. Previously, they were found to enhance baclofen-induced loss of righting, but not baclofen-induced hypothermia (Koek et al., 2010). The present results indicate that their effectiveness as positive modulators is not limited to loss of righting produced by high doses of baclofen, which probably involves cerebellar GABA_B receptors that are especially sensitive to positive modulation (Hensler et al., 2012). Instead, their effectiveness as positive modulators *in vivo* extends to discriminative stimulus effects of baclofen. Under the conditions of the present experiments, aimed at examining discriminative stimulus effects, no evidence was obtained that the modulators CGP7930 and rac-BHFF enhanced the effects of baclofen on response rate. However, in preliminary experiments in pigeons trained to respond for food using a procedure detailed elsewhere (Koek et al., 2009) both CGP7930 and rac-BHFF modulators enhanced the response rate-decreasing effects of baclofen (W. Koek, unpublished observations). The generality of the positive modulatory effects of CGP7930 and rac-BHFF *in vivo* increases the likelihood that these effects are involved in their therapeutic-like activity.

The positive modulators enhanced the discriminative stimulus effects of baclofen, but not GHB. This differential enhancement, which suggests that the baclofen-enhancing effects of the GABA_B receptor modulators do not result from the baclofen-appropriate responding they produce when given alone, is consistent with the finding that CGP7930 and rac-BHFF enhanced the effects of baclofen on righting more extensively than those of GHB (Koek et al., 2010). Effects of allosteric modulators can be agonist dependent (e.g., Kenakin, 2009). Thus, the present and previous results could be explained by assuming that the same GABA_B receptors mediate effects of baclofen and GHB, and that rac-BHFF and CGP7930 enhance effects at these receptors in an agonist-dependent manner. However, there is growing evidence that the GABA_B receptor mechanisms underlying the effects of baclofen and GHB are not identical. First, differential enhancement of behavioral effects (discriminative stimulus effects, catalepsy) of baclofen and GHB by *N*-methyl-D-aspartate antagonists suggests that the GABA_B receptors involved in these effects of baclofen and GHB are not identical (Koek et al., 2007a; Koek and France, 2008). Second, differential antagonism of behavioral effects of baclofen and GHB by CGP35348, reported previously (Koek et al., 2004, 2007b, 2009; Carter et al., 2006) and also observed in the present study, is further evidence that different GABA_B receptor populations mediate these effects of baclofen and GHB. Note, however, that GHB interacts not only with GABA_B receptors,

but also with specific GHB binding sites, which can be investigated with the selective radioligand [³H](2*E*)-(5-hydroxy-5,7,8,9-tetrahydro-6*H*-benzo[*a*][7]annulen-6-ylidene ethanoic acid (NCS-382) (Mehta et al., 2001). At these sites, which seem to be G protein-coupled receptors (Snead, 1977), NCS-382 is thought to act as an antagonist. Previously, the discriminative stimulus effects of GHB were found to be antagonized by the selective GABA_B receptor antagonist CGP35348, but not by NCS-382 (Koek et al., 2006), suggesting that these effects are not mediated by specific GHB receptors, but involve GABA_B receptors. In the present study, the discriminative stimulus effects of GHB were completely antagonized by CGP35348, consistent with previous findings (Koek et al., 2006). Because this antagonism was complete, it seems unlikely that receptors other than GABA_B receptors are involved in these effects of GHB. Thus, the different potency with which CGP35348 completely antagonized the discriminative stimulus and other behavioral effects of GHB and baclofen, reported previously (Koek et al., 2004, 2007b, 2009; Carter et al., 2006) and also observed in the present study, suggests that different GABA_B receptor populations mediate these effects of baclofen and GHB. Therefore, the differential enhancement of effects of baclofen and GHB by the GABA_B receptor modulators rac-BHFF and CGP7930 may not involve agonist-dependent enhancement of a single population of GABA_B receptors, but may result from preferential modulation of different GABA_B receptor populations. Consistent with this latter possibility, *in vitro* evidence shows that CGP7930 preferentially potentiates activity at GABA_B autoreceptors, but not at GABA_B heteroreceptors (Chen et al., 2006; Parker et al., 2008). Such receptor heterogeneity would allow more selective manipulation of the GABA_B system.

GABA_B receptor-positive modulators are thought to have advantages as potential medications for anxiety, depression, and drug addiction (Cryan et al., 2004; Frankowska et al., 2007; Jacobson and Cryan, 2008; Vlachou and Markou, 2010), because they may have a better side effect profile than GABA_B receptor agonists, based on the notion that selective enhancement of activated receptors has effects that differ from indiscriminate activation of all receptors. Unlike baclofen, GABA_B receptor-positive modulators do not seem to interfere with motor coordination (Cryan et al., 2004; Jacobson and Cryan, 2008), do not produce loss of righting (Carai et al., 2004; Malherbe et al., 2008; Koek et al., 2010) and, with the possible exception of CGP7930 (Koek et al., 2010), do not induce hypothermia (Jacobson and Cryan, 2008; Malherbe et al., 2008; Koek et al., 2010). In the present study, CGP7930 substituted at most partially for baclofen and did not substitute for GHB, which is evidence that its discriminative stimulus effects are different from those of baclofen and GHB. In contrast, rac-BHFF produced a level of drug-appropriate responding in baclofen-trained animals near the level observed with the training drug, and, like baclofen, substituted partially for GHB. Thus, the discriminative stimulus effects of rac-BHFF, unlike CGP7930, may be similar to those of baclofen. A more comprehensive characterization of the discriminative stimulus properties of rac-BHFF awaits additional studies, possibly using rac-BHFF as training drug.

In summary, the positive GABA_B receptor modulators CGP7930 and rac-BHFF enhanced the discriminative stimulus effects of baclofen, but not those of GHB. Together with evidence that the GABA_B receptor populations involved in

the *in vivo* effects of baclofen and GHB are not identical, the present findings suggest these populations differ in their susceptibility to positive modulatory effects. Such differential susceptibility could allow for more selective therapeutic targeting of the GABA_B system.

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Authorship Contributions

Participated in research design: Koek.

Contributed new reagents or analytic tools: Cheng and Rice.

Performed data analysis: Koek.

Wrote or contributed to the writing of the manuscript: Koek and France.

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