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ASSESSMENT OF SUBUNIT-DEPENDENT DIRECT GATING AND ALLOSTERIC MODULATORY EFFECTS OF CARISOPRODOL AT GABA_A RECEPTORS

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Abstract

Carisoprodol is a widely prescribed muscle relaxant, abuse of which has grown considerably in recent years. It directly activates and allosterically modulates $\alpha 1\beta 2\gamma 2$ GABA_ARs, although the site(s) of action are unknown. To gain insight into the actions of carisoprodol, subunit-dependent effects of this drug were assessed. Whole-cell patch clamp recordings were obtained from HEK293 cells expressing $\alpha 1\beta 2$, $\alpha 1\beta 3$ or $\alpha x\beta z\gamma 2$ (where $x = 1-6$ and $z = 1-3$) GABA_ARs, and in receptors incorporating the δ subunit (modeling extrasynaptic receptors). The ability to directly gate and allosterically potentiate GABA-gated currents was observed for all configurations. Presence or absence of the $\gamma 2$ subunit did not affect the ability of carisoprodol to directly gate or allosterically modulate the receptor. Presence of the $\beta 1$ subunit conferred highest efficacy for direct activation relative to maximum GABA currents, while presence of the $\beta 2$ subunit conferred highest efficacy for allosteric modulation of the GABA response. With regard to α subunits, carisoprodol was most efficacious at enhancing the actions of GABA in receptors incorporating the $\alpha 1$ subunit. The ability to directly gate the receptor was generally comparable regardless of the α subunit isoform, although receptors incorporating the $\alpha 3$ subunit showed significantly reduced direct gating efficacy and affinity. In extrasynaptic ($\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$) receptors, carisoprodol had greater efficacy than GABA as a direct gating agonist. In addition, carisoprodol allosterically potentiated both EC₂₀ and saturating GABA concentrations in these receptors. In assessing voltage-dependence, we found direct gating and inhibitory effects were insensitive to membrane

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voltage, whereas allosteric modulatory effects were affected by membrane voltage. Our findings demonstrate direct and allosteric effects of carisoprodol at synaptic and extrasynaptic GABA_ARs and that subunit isoform influences these effects.

Keywords

GABA_A receptor; carisoprodol; muscle relaxant; drug abuse

1. Introduction

γ -Aminobutyric acid type A receptors (GABA_ARs) are ion channel-coupled, multi-subunit proteins that serve as the primary mediators of inhibitory neurotransmission in the adult central nervous system (CNS). Functional receptors are composed of individual subunits arranged in a pentameric manner. In mammals, the various subunits and their isoforms have been divided into the following classes: α (1–6), β (1–3), γ (1–3), ρ , δ , ϵ , π , and θ (Huang et al., 2006). Subunit architecture is highly conserved among GABA_ARs with each subunit composed of an extracellular amino-terminal, four transmembrane (TM) domains, a large intracellular loop, and an extracellular carboxyl-terminal. Subunit composition determines channel conductance, kinetics, and gating properties of the receptor (Olsen and Sieghart, 2008). Synaptic GABA_ARs are responsible for phasic changes in GABA-mediated postsynaptic inhibition. Extrasynaptic receptors, which typically incorporate a δ subunit (Mortensen et al., 2010), exert a tonic inhibitory influence on neuronal membrane potential. Given their vital role in inhibitory signaling in the CNS, GABA_ARs are the targets of several clinically relevant compounds. These compounds include benzodiazepines, barbiturates, general and inhalational anesthetics, and certain centrally-acting muscle relaxants.

Carisoprodol is a centrally-acting muscle relaxant indicated in the alleviation of acute musculoskeletal conditions (Toth and Urtis, 2004). With a single dose of 350 mg, effects of carisoprodol begin within 30 minutes of administration and plasma concentrations reach 4–7 $\mu\text{g/mL}$ in 2 to 4 hours (Littrell et al., 1993). The dangers associated with carisoprodol abuse, such as psychomotor impairment and severe withdrawal that may lead to seizures and death, are well-documented (Adams et al., 1975; Elder, 1991; Littrell et al., 1993; Reeves and Parker, 2003; Rust et al., 1993; Zacny et al., 2011, 2012). Precipitated withdrawal studies in mice have demonstrated tolerance to carisoprodol develops in as few as four doses (Gatch et al., 2012). In recent years, the ready availability of carisoprodol via internet pharmacies has led to increased recreational use of carisoprodol. Because of the increasing misuse of carisoprodol and associated adverse effects, the United States Drug Enforcement Administration placed carisoprodol into Schedule IV of the Controlled Substances Act in January 2012.

The illicit effects of carisoprodol are generally attributed to the actions of its primary metabolite, meprobamate—a federally controlled substance with barbiturate-like activity at GABA_ARs (Rho et al., 1997). While conversion to meprobamate likely contributes to the therapeutic and illicit effects of carisoprodol, the pharmacological and physiological profiles

of carisoprodol are not entirely consistent with that of its metabolite, supporting the possibility that carisoprodol may have effects independent of meprobamate.

The full spectrum of potential targets of carisoprodol has not been established. Adverse effects may be associated with serotonergic-like effects (Bramness et al., 2004). In animal studies, the NMDA receptor antagonist dizocilpine partially substitutes for the discriminative stimulus effects of carisoprodol (M. Gatch, personal communication). Considerable evidence implicates the GABA_AR in its effects. As noted, the metabolite of carisoprodol has barbiturate-like effects at GABA_ARs (Rho et al., 1997). We have demonstrated carisoprodol itself allosterically modulates and directly activates human $\alpha 1\beta 2\gamma 2$ GABA_ARs, and its actions are not mediated via reported sites of action for benzodiazepines or barbiturates (Gonzalez et al., 2009b). Although receptors of $\alpha 1\beta 2\gamma 2$ subunit composition are the prevalent configuration in the brain, a vast array of GABA_AR configurations have been shown to exist throughout the CNS, with each configuration contributing to specific physiological and pharmacological responses (Olsen and Sieghart, 2008). For example, benzodiazepines mediate sedative and anticonvulsant effects through $\alpha 1$ -containing GABA_ARs, anxiolytic effects primarily through $\alpha 2$ -expressing receptors, and myorelaxant effects via receptors expressing $\alpha 3$ and $\alpha 5$ subunits (Tan et al., 2010). It has also been demonstrated that abuse and dependence potential of benzodiazepines and barbiturates are related to their subunit-selective interactions with GABA_ARs (Ator, 2005; Ito et al., 1996; Licata and Rowlett, 2008; Wafford, 2005). Because dependence and withdrawal symptoms are associated with carisoprodol use, a better understanding of its molecular mechanism is needed. Thus, we assessed direct and allosteric actions of carisoprodol in varying configurations of synaptic and extrasynaptic GABA_A receptors. Given the clinical and adverse effects associated with carisoprodol, we hypothesized it would interact with a number of GABA_ARs in addition to $\alpha 1\beta 2\gamma 2$, likely those expressing $\alpha 2$ and/or $\alpha 3$ and/or $\alpha 5$ subunits. Our results indicate that the actions of carisoprodol are influenced by subunit isoforms and that direct and allosteric effects are likely mediated via distinct domains.

2. Materials and Methods

2.1 Cell Culture and Transfection

Both stably- and transiently-expressing GABA_A receptors HEK293 cells were used in the present study. Human embryonic kidney 293 (HEK293) cells were transfected with human GABA_A $\alpha 1$ – $\alpha 6$; human $\beta 1$ – 2 ; and human $\gamma 2$ s (short isoform) cDNA in a 1:1:5 ratio using *TransIT*[®]-293 (Mirus Bio, Madison, WI) and used for recording 24–48 h later. The $\gamma 2$ s subunit will be referred to as $\gamma 2$ from this point forward. Human GABA_A $\alpha 1$ subunit cDNA was generously provided by Neil Harrison (Columbia University Medical Center, New York). In resequencing the $\gamma 2$ subunit that was used in many of these studies, we detected a mutation (D to N at position 115). We mutated this residue to the native form (QuikChange[®] Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA), confirmed the mutation via sequencing, and conducted a number of studies to assess possible impact on carisoprodol activity. We observed no effect and thus data with that mutation are included. HEK293 stably expressing human $\alpha 1\beta 2\gamma 2$ or $\alpha 2\beta 2\gamma 2$ GABA_ARs

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were also used. A complete description of the preparation and maintenance of these stable cell lines has been published previously (Hawkinson et al., 1996). For studies assessing carisoprodol effects in a model of extrasynaptic receptors (rat $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ subunits) a transfection ratio of 2:1:0.25 for $\alpha:\beta:\delta$ plasmids was used, as this transfection ratio reliably results in receptors expressing the native 2 α :2 β :1 δ stoichiometry (Wagoner and Czajkowski, 2010). The rat GABA_A $\alpha 4$ subunit cDNA was purchased from Genescript (Piscataway, New Jersey). As rat extrasynaptic GABA_ARs have been shown to have similar physiology and pharmacology to human extrasynaptic GABA_ARs (Adkins et al., 2001; Feng et al., 2004; Feng and Macdonald, 2010; Mortensen et al., 2010), use in these studies is appropriate. For all studies, cells were plated on glass coverslips coated with poly-L-lysine in 35-mm culture dishes. Cells were incubated and maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

2.2 Electrophysiology

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Whole-cell patch clamp electrophysiology was used to assess GABA- or carisoprodol-activated Cl⁻ currents. Except for studies examining voltage-dependence (below), electrophysiology experiments were conducted at room temperature (22–25°C) with the membrane potential clamped at –60 mV. Patch pipettes of borosilicate glass (1B150F; World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC; Sutter Instrument Company, Novato, CA) to a tip resistance of 4–6 M Ω . Patch pipettes were filled with a solution consisting of 140 mM CsCl, 10 mM EGTA-Na⁺, 10 mM HEPES-Na⁺, and 4 mM Mg²⁺-ATP, pH 7.2. Coverslips containing cultured cells were placed in the recording chamber on the stage of an inverted light microscope and superfused continuously with an external solution consisting of 125 mM NaCl, 20 mM HEPES, 3 mM CaCl₂, 5.5 mM KCl, 0.8 mM MgCl₂, and 10 mM glucose, pH 7.3. Agonist-induced Cl⁻ currents were obtained with an Axopatch 200B amplifier with a rate of 50 samples per second (Molecular Devices, Sunnyvale, CA) equipped with a CV-203BU headstage. Currents were low-pass filtered at 5 kHz, monitored simultaneously on an oscilloscope and a chart recorder (Gould TA240; Gould Instrument Systems Inc., Cleveland, OH), and stored on a computer using an on-line data acquisition system (pCLAMP 6.0; Axon Instruments) for subsequent off-line analysis.

2.3 Chemicals and solutions

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Carisoprodol, pentobarbital, THIP (4,5,6,7-tetrahydroisoxazolo(5,4-c)pyridin-3-ol), salts and buffers were purchased from Sigma Aldrich (St. Louis, MO). GABA was obtained from Acros Organics (New Jersey, US). GABA stock solution (500 mM) was prepared in de-ionized water. A stock solution (1 or 3M) of carisoprodol and THIP (1M) was made in DMSO. Final concentrations of DMSO in working solutions were 0.3% or less, a concentration that does not affect GABA-gated current. Diazepam and furosemide were utilized from a stock solution prepared in water. All stock solutions were stored at –20°C. On experimental days, drug-containing solutions were prepared from stock by serial dilution into external solution.

2.4 Experimental protocol

GABA (with or without carisoprodol) or carisoprodol were applied to each cell by gravity flow using a Y-shaped tube positioned adjacent to the cell. The modulatory effects of carisoprodol on GABA-gated currents were assessed using an EC₂₀ gating concentration of GABA as the control. This gating concentration was selected to ensure there was a sufficient range to observe the full allosteric potential of carisoprodol. To ensure the gating concentration was approximately an EC₂₀, control responses were compared to the maximal GABA-gated current for each individual cell. Carisoprodol was tested only if the gating concentration was within the EC_{15–25} range. Control responses were established by observing two consecutive agonist-activated currents that varied in amplitude by no more than $\pm 10\%$. In our analyses of the modulatory effects of carisoprodol, peak current amplitude was defined as the maximum current elicited by carisoprodol. In studies investigating direct gating effects, carisoprodol-gated currents were normalized to maximum GABA-mediated current. In recordings where an inhibitory component was observed (high CSP concentrations), a rebound current was sometimes followed the inhibitory phase. The channel state(s) from which these openings occur may vary (Feng et al., 2004; Williams et al., 1997; Woollorton et al., 1997), and thus peak current amplitude was taken during the active drug application phase. For $\alpha\beta\gamma$ configurations, incorporation of $\gamma 2$ subunit was confirmed in $\alpha 1$ -, $\alpha 2$ -, $\alpha 3$ - and $\alpha 5\beta 2\gamma 2$ GABA_ARs by demonstration of sensitivity of GABA (EC₅₀)-gated currents to allosteric potentiation by diazepam (1 μ M). Antagonism by furosemide (100 μ M) was used to confirm expression of $\alpha 4\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ GABA_ARs. Presence of the δ subunit in $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors was confirmed by loss of inhibition by 1 μ M Zn²⁺ on GABA-gated current. After establishing the control response, effects of the test drugs were determined.

To study the voltage-dependence effect of carisoprodol in GABA_A receptors, $\alpha 1\beta 2\gamma 2$ transfected HEK293 cells were clamped at -60 mV and $+60$ mV and studied for carisoprodol direct gating, allosteric modulatory and blocking effects as described above.

2.5 Data Analysis

To ensure equipotent concentrations were used for gating, GABA concentration-response data were collected for all synaptic and extrasynaptic GABA_ARs tested, (Table 1 and 4). From these data, EC₂₀ and saturating GABA concentrations were calculated for each configuration and used in subsequent investigations of the allosteric and direct effects of carisoprodol, respectively.

Concentration-response profiles for the positive modulatory actions of carisoprodol were generated (Origin; OriginLab Corp., Northampton, MA) using the equation $I/I_{\max} = [\text{carisoprodol}]^n / ([\text{carisoprodol}]^n + \text{EC}_{50}^n)$, where I is the normalized current amplitude at a given concentration of carisoprodol, $I_{\max}$ is the maximum current induced by carisoprodol, EC₅₀ is the half-maximal effective concentration of carisoprodol, and n is the Hill coefficient. For concentration-response curves illustrating allosteric actions, a correction was applied to subtract direct gating effects. In some cases, the blocking actions of carisoprodol became notable at high concentrations; in these instances, curves were fitted to the data point corresponding to peak effect, and the curve was extrapolated.

All data are presented as mean values $\pm$ S.E.M. Statistical significance ($p < 0.05$) between control and test conditions was determined using Student's *t*-test (paired or unpaired) and one-way analysis of variance. Tukey-Kramer *post hoc* test for multiple comparisons was performed as needed.

3. Results

3.1 Influence of α subunit isoform of carisoprodol-mediated activity of the GABA_A receptor

3.1.1 α subunit influences on direct gating by carisoprodol—Table 1 provides EC₅₀ values for the synaptic GABA_A receptors studied in the present investigation. Both allosteric (assessed using an EC₂₀ GABA gating concentration) and direct effects of carisoprodol (1 μ M – 10 mM) were assessed in $\alpha x \beta 2 \gamma 2$ GABA_A receptors, where $x = \alpha$ subunit 1–6. Carisoprodol directly gated each of the configurations tested, evoking inward currents in the absence of GABA (Fig. 1A and 1B). Maximal carisoprodol-gated currents were of similar magnitude regardless of α subunit, with efficacies ranging from 13–43% of maximal GABA-gated current (Fig. 1B, Table 2). However, carisoprodol direct gating potency and efficacy was significantly less at $\alpha 3$ -containing receptors compared to other α isoform receptors (Table 2). As originally reported for $\alpha 1$ -containing receptors (Gonzalez et al., 2009b), attenuation of current amplitude at high concentrations, followed by rebound current, was consistently observed regardless of the α subunit.

3.1.2 α subunit influences on allosteric modulation by carisoprodol of GABA-gated current—We also assessed the ability of carisoprodol to allosterically modulate the response to GABA when α subunits were varied. Carisoprodol positively modulated the effects of an EC₂₀ concentration of GABA in all configurations tested (Fig. 2A and 2B). Carisoprodol potency was not significantly influenced by the α subunit isoform (Table 3) whereas the efficacy of carisoprodol modulation was influenced by the α subunit, being greatest in $\alpha 1$ -containing receptors (Fig. 2B and Table 3). As observed when evaluating direct gating effects of carisoprodol, allosteric enhancement began to diminish with higher concentrations of carisoprodol, and a notable rebound current was observed. Pharmacologically, the presence of carisoprodol increased the apparent affinity of GABA, resulting in a significant leftward shift in the GABA concentration-response profile without increasing maximal GABA current amplitude (Fig. 2E).

3.2 Influence of β subunit isoform of carisoprodol-mediated activity of the GABA_A receptor

3.2.1 β subunit influences on direct gating by carisoprodol—As we reported previously (Gonzalez et al., 2009a), application of carisoprodol to $\alpha 1 \beta 2 \gamma 2$ receptors elicited inward currents in the absence of GABA, with an efficacy approximately 43% of that observed in response to a saturating concentration of GABA (Fig. 1A). The ability to directly gate the receptor was also observed in $\alpha 1 \beta 1 \gamma 2$ GABA_ARs. Efficacy in $\beta 1$ -containing receptors was significantly greater than that observed in $\beta 2$ -containing receptors (Fig. 1C and Table 2) and approximated 70% of the magnitude of maximal GABA-gated current in $\alpha 1 \beta 1 \gamma 2$ GABA_ARs. Potency of carisoprodol's direct gating effect was similar in $\beta 1$ - and $\beta 2$ -expressing receptors (Table 2).

3.2.2 β subunit influences on allosteric modulation by carisoprodol of GABA-gated current—Carisoprodol modulated the GABA-gated currents of $\alpha 1\beta 1\gamma 2$ GABA_ARs in a manner previously described for $\alpha 1\beta 2\gamma 2$ GABA_ARs—potentiation in a concentration-dependent manner accompanied by inhibition and rebound currents at millimolar concentrations (Fig. 2A). The β subunit isoform affected both potency ($33.1 \pm 4 \mu\text{M}$ and $88.2 \pm 20 \mu\text{M}$, in $\beta 1$ - and $\beta 2$ -containing receptors, respectively) and efficacy (maximum efficacy was $225 \pm 14.6\%$ of control in $\beta 1$ -containing receptors, compared to $474.7 \pm 53.5\%$ in $\beta 2$ -containing receptors) of carisoprodol-mediated potentiation of GABA-gated currents (Fig. 2C and Table 3). This is in contrast to the pattern observed for the direct gating effects of carisoprodol, in which carisoprodol was more efficacious at receptors containing the $\beta 1$ isoform.

3.3 Direct gating and allosteric modulatory effects of carisoprodol do not require the γ subunit

In assessing the influence of the γ subunit on actions of carisoprodol, we found its efficacy as a direct agonist was unchanged with the presence of the γ subunit (Fig. 1A and 1D, Table 2). Peak current amplitude of carisoprodol-evoked currents was $45.9 \pm 3\%$ of the maximum GABA-gated current for $\alpha 1\beta 2$ receptors, whereas it was $43.6 \pm 4.3\%$ for $\alpha 1\beta 2\gamma 2$ receptors. Consistent with this, we observed the actions of carisoprodol were comparable regardless of whether the $\gamma 2\text{short}$ or $\gamma 2\text{long}$ isoform was expressed (data not shown).

We also assessed the influence of the γ subunit on allosteric actions of carisoprodol. Micromolar concentrations of carisoprodol potentiated the GABA-gated currents of $\alpha 1\beta 2$ GABA_ARs in a concentration-dependent manner (Fig. 2A and 2D, Table 2). The patterns of potentiation and inhibition by carisoprodol at $\alpha 1\beta 2$ GABA_ARs were similar to those observed at $\alpha 1\beta 2\gamma 2$ GABA_ARs, shown here (Fig. 1A) and previously using a stable $\alpha 1\beta 2\gamma 2$ cell line (Gonzalez et al., 2009b). The estimated EC_{50} for carisoprodol at $\alpha 1\beta 2$ GABA_ARs was $87.4 \pm 16.4 \mu\text{M}$ compared to $88.2 \pm 20 \mu\text{M}$ for receptors containing the $\gamma 2$ subunit, with direct gating by carisoprodol likely contributing to maximal current amplitude elicited by higher concentrations of the drug. Maximum potentiation of control currents occurred with 1 mM carisoprodol for each configuration (Fig. 1D), with efficacies of $346.7 \pm 67.6\%$ and $474.7 \pm 53.5\%$ for $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs, respectively. Thus, the γ subunit did not significantly influence the potency or efficacy of carisoprodol as an allosteric modulator (Table 2). At millimolar concentrations, rebound currents were observed upon termination of drug application, and co-application of 3 mM carisoprodol elicited an inhibitory effect on GABA-gated currents during drug application. Together with the direct gating studies, these findings demonstrate the γ subunit is not essential for carisoprodol-mediated regulation of GABA_AR function.

3.4 Effects of carisoprodol in “extrasynaptic” receptors

3.4.1 δ subunit influences on direct gating by carisoprodol—To assess carisoprodol mediated-activity in a model of extrasynaptic GABA_A receptors, we studied its effects in HEK293 expressing rat $\alpha 1$ or $\alpha 4$, $\beta 3$ and δ GABA_A receptor subunits. Carisoprodol was more efficacious than GABA in directly gating $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ extrasynaptic receptors, with the latter receptor having the highest sensitivity to CSP (Fig. 3,

Table 4). We also compared the efficacy of carisoprodol to that of THIP, which has high efficacy at extrasynaptic GABA_A receptors (Belelli et al., 2009; Brickley and Mody, 2012). As reported by others, we observed that THIP was a super agonist, with THIP-gated currents being 135.6 ± 7.2 and $153.4 \pm 10.7\%$ of the amplitude of currents obtained with saturating GABA in $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors, respectively. Carisoprodol was thus roughly 90% as efficacious as THIP in directly gating $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors (Fig. 3). Direct gating efficacy by carisoprodol in rat $\alpha 1\beta 2$, $\alpha 1\beta 3$, and $\alpha 1\beta 3\gamma 2$ configurations was similar, indicating neither the $\beta 2/3$ isoforms nor the γ subunit significantly affects direct activation by carisoprodol. Effects in the rat $\alpha 1\beta 2$, $\alpha 1\beta 3$ and $\alpha 1\beta 3\gamma 2$ receptors were also generally comparable to what we observed across a range of human GABA_A receptors (see Fig. 1). Thus, species differences do not appear to be a significant influence with regard to direct gating effects of carisoprodol, as has been observed for other GABAergic ligands such as pentobarbital, neurosteroids, THIP and general anesthetics (Adkins et al., 2001; Feng et al., 2004; Feng and Macdonald, 2010; Mortensen et al., 2010).

3.4.2 δ subunit influences on allosteric modulation by carisoprodol of GABA-gated current—We also determined the ability of carisoprodol to allosterically modulate GABA-gated current in extrasynaptic receptors. In contrast to what was observed with direct gating, the δ subunit did not have a significant effect on allosteric modulatory effects of carisoprodol (Fig. 4). These results also confirm carisoprodol is most efficacious on receptors expressing $\beta 2$ compared to $\beta 1$ or $\beta 3$ subunits for its allosteric modulatory effects (Table 4). Interestingly, like pentobarbital, neurosteroids, and general anesthetics (Feng et al., 2004; Meera et al., 2009; Wohlfarth et al., 2002), carisoprodol potentiated maximal GABA currents of $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ GABA_A receptors, but not of $\alpha 1\beta 3$ or $\alpha 1\beta 3\gamma 2$ receptors (Fig. 5). These results demonstrate carisoprodol has distinct gating properties on extrasynaptic GABA_A receptors compared to synaptic receptors.

3.5 Effect of membrane voltage on direct gating and allosteric modulatory effects of carisoprodol

As voltage sensitivity may sometimes provide insight into possible site of action of a ligand, we determined whether direct gating, allosteric modulatory and/or blocking actions of carisoprodol are voltage-dependent, by assessing these actions in $\alpha 1\beta 2\gamma 2$ GABA_A receptors with the membrane voltage clamped at -60 mV and $+60$ mV. GABA-gated and CSP-gated current at 0 mV was essentially undetectable, consistent with the theoretical reversal potential of 0.4 mV, as predicted from the Nernst equation. As reported by others (O'Toole and Jenkins, 2012), we observed outward rectification in response to lower concentrations of GABA (current amplitude at $+60$ mV was 110% of that recorded at -60 mV), whereas no significant rectification was observed at saturating GABA. To assess voltage-dependence of the direct gating effects of CSP, we chose a concentration of 1 mM, which elicits a robust current, but nominal apparent inhibition. As shown in Figure 6 (A, B), the direct gating effect of CSP was not impacted by membrane voltage. Interestingly, allosteric potentiation of GABA currents by carisoprodol was affected by cell membrane polarity; in these studies, 100 μ M CSP enhanced GABA current to $285.3 \pm 17.7\%$ of control at -60 mV, whereas the extend of potentiation was $204.8 \pm 5.3\%$ at $+60$ mV (Fig. 6C, 6D). To assess voltage-dependence of blocking by carisoprodol, we compared the decrease in current amplitude in

response to 5 mM CSP, compared to 3 mM CSP, at both -60 and $+60$ mV. The magnitude of current attenuation with 5 mM CSP was similar in both cases (Fig. 6E, 6F).

Discussion

Despite the clinical utility of carisoprodol in treatment of low back pain, its use is complicated by its abuse potential. Carisoprodol abuse, tolerance, and withdrawal are well-documented in the literature (Adams et al., 1975; Elder, 1991; Reeves and Parker, 2003; Rust et al., 1993). This abuse has become particularly apparent in recent years, due in part to ready access of carisoprodol via internet pharmacies. The abuse liability of carisoprodol has often been attributed to meprobamate—the primary metabolite of carisoprodol and a controlled substance at the federal level. Our previous research has demonstrated that carisoprodol itself, independent of its conversion to meprobamate, acts at GABA_ARs in a manner described for drugs of abuse that act via the GABAergic system (Gonzalez et al., 2009b). Whereas meprobamate has been federally scheduled for many years, such was not the case for carisoprodol. Effective January 2012, following extensive review of scientific research and data on abuse, law enforcement encounters, and other information, the United States Drug Enforcement Agency placed carisoprodol into Schedule IV of the Controlled Substances Act (Federal Register, vol 76, no 238, pp. 77330–77360, Dec 12, 2011).

The abuse and dependence potential of many GABAergic drugs are related to their subunit-selective interactions with GABA_ARs (Ator, 2005; Ator et al., 2010; Ito et al., 1996; Licata and Rowlett, 2008; Wafford, 2005). Thus, we assessed whether the actions of carisoprodol are subunit-dependent, potentially underlying its physiological effects and abuse liability. The role of the γ subunit has been established for several modulators of GABA_AR function. The most prominent example is the benzodiazepine class of drugs, which require the presence of a γ subunit to potentiate GABA-gated currents (Pritchett et al., 1989). The γ subunit does not play an essential role in mediating the actions of carisoprodol at GABA_ARs because the modulatory and agonistic effects of the drug were not significantly different between $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ configurations. These findings support our earlier assertion that carisoprodol does not act at the benzodiazepine site of the receptor (Gonzalez et al., 2009a) and led us to pursue the α and β subunits as critical mediators of the actions of carisoprodol.

In the current study, the influence of β subunit isoforms was examined via comparison of effects elicited from $\beta 1$ -, $\beta 2$ - and $\beta 3$ -containing receptors. Whereas the actions of GABAergic compounds such as etomidate, loreclezole, and furosemide are dependent upon incorporation of $\beta 2$ or $\beta 3$ subunits (Korpi et al., 2002; Korpi et al., 1995; Olsen, 2014; Stewart et al., 2013), carisoprodol-mediated effects were observed at $\alpha 1\beta 1\gamma 2$ GABA_ARs, suggesting critical domains for carisoprodol activity are located within regions conserved between the $\beta 1$ and $\beta 2$ isoforms. For allosteric modulation, carisoprodol was significantly more efficacious at $\beta 2$ -containing receptors. Expression of the $\beta 2$ isoform in the brain is extensive (Sigel and Steinmann, 2012), suggesting the majority of receptor configurations are susceptible to modulation by carisoprodol. In contrast, direct activation by carisoprodol was significantly greater at $\beta 1$ -containing receptors. This disparity suggests allosteric modulation and direct gating by carisoprodol may be mediated by distinct sites of the β subunit.

There has been considerable progress in recent years in understanding the involvement of specific GABA_A receptor α subunits in both desired and adverse pharmacologic actions (for review, see (Rudolph and Mohler, 2006). α 1-expressing GABA_A receptors are critical for the sedative effects of benzodiazepines (McKernan et al., 2000) whereas α 2- and/or α 3-expressing receptors underlie their anxiolytic actions (Atack et al., 2005; Dias et al., 2005). In addition, the efficacy of benzodiazepines at α 1-containing GABA_ARs may predict their abuse potential (Ator, 2005; Licata and Rowlett, 2008). Inhibitory GABAergic interneurons in the ventral tegmental area (VTA) specifically express α 1-containing GABA_ARs (Tan et al., 2010). Potentiation of α 1-containing GABA_ARs on these interneurons causes disinhibition of VTA dopaminergic neurons, resulting in increased dopamine release in the nucleus accumbens. Addictive drugs increase dopamine levels in the mesolimbic system (Luscher and Ungless, 2006). As physical dependence is more likely to develop with drugs that interact with a broader collection of GABA_AR subtypes (Licata and Rowlett, 2008), the high efficacy of carisoprodol for both direct and allosteric effects on α 1-containing receptors, coupled with its ability to interact with GABA_AR subtypes, is consistent with its high abuse potential (Rudolph and Knoflach, 2011).

The therapeutic goal of carisoprodol use is alleviation of low back pain, which results from centrally-mediated muscle relaxation. Others have demonstrated muscle relaxation is associated preferentially with α 2- and α 3-expressing receptors (Crestani et al., 2001; Griebel et al., 2003; Licata et al., 2005; Licata et al., 2009). Although carisoprodol did not selectively interact with these subunits, it had notable allosteric modulatory effects on both α 2- and α 3-expressing receptors and almost similar potency for direct activation for α 2-containing receptors as compared to most abundant configuration of human CNS, α 1-containing receptors (Olsen and Sieghart, 2008). Taken together, the pharmacological profile of carisoprodol is consistent with its clinical effects and its potential for abuse. A molecule that selectively interacts with α 2/ α 3 GABA_A receptor subunits would seem promising as a muscle relaxant with reduced abuse potential and sedative effects (Rudolph and Knoflach, 2011).

Unlike benzodiazepines, carisoprodol also has the ability to directly gate the GABA_AR. Misuse of agents that directly gate the receptor is more likely to result in death than those that only allosterically enhance receptor activity, and overdose of carisoprodol is responsible for a substantial number of both unintentional and intentional deaths (Fass, 2010). We observed that carisoprodol elicited significant and concentration-dependent direct gating effects at the GABA_AR. According to case reports, blood or plasma concentrations of carisoprodol as low as 140 μ M have proven to be fatal (McIntyre et al., 2012; Robertson and Marinetti, 2003). Interestingly, this concentration correlates well to the onset of direct activation for the majority of receptor subunit complexes tested in the present study.

These studies are the first to explore subunit-dependent actions of carisoprodol, and thus little is known about possible domains that are key for its actions. One fundamental question is whether the site for allosteric and direct gating effects is the same. The general anesthetics propofol and etomidate also both allosterically enhance and directly gate the receptor, and evidence suggests a single site of action confers both properties (Forman, 2011; Ruesch et al., 2012). In contrast, the ability of neurosteroids to directly gate and allosterically modulate

the receptor is mediated via distinct sites (Hosie et al., 2006). Our data are most consistent with the contention that the two actions of carisoprodol are mediated via distinct sites. First, we observed greatest direct gating efficacy in $\beta 1$ -expressing receptors, whereas efficacy for allosteric modulation was greatest in $\beta 2$ -expressing receptors. Similarly, we observed decreased potency and efficacy for direct gating capacity in $\alpha 3$ -expressing receptors, whereas allosteric effects of those receptors were similar to other α subunit-expressing receptors.

We also studied the actions of carisoprodol on δ subunit-containing extrasynaptic GABA_A receptors, which exert a tonic inhibitory tone as opposed to the phasic inhibition characteristic of synaptic receptors (Glykys and Mody, 2007; Herd et al., 2009). It should also be noted that $\alpha 5\beta 2\gamma 2$ receptors, in addition to existing at synapses (Serwanski et al., 2006), are present at extrasynaptic sites and have a prominent role in contributing to tonic inhibition (Caraiscos et al., 2004; Glykys and Mody, 2006). The involvement of extrasynaptic GABA_A receptor dysfunction in neurological disorders like depression (Maguire et al., 2005), schizophrenia (Maldonado-Aviles et al., 2009), and some forms of epilepsy (Cope et al., 2009) has made these receptors an emerging clinical target (Brickley and Mody, 2012). As GABA has high affinity but low efficacy at extrasynaptic GABA_A receptors, it is considered a partial agonist at these receptors (Bianchi and Macdonald, 2003). We found that, as reported for other ligands (Bianchi and Macdonald, 2003), carisoprodol has higher efficacy at extrasynaptic $\alpha 1\beta 3\delta$ and $\alpha 4\beta 3\delta$ receptors than does GABA, and carisoprodol was about 90% as efficacious as the full agonist THIP. The efficacy of general anesthetics and sedative/hypnotics in potentiation of GABA-gated currents at extrasynaptic $\alpha 6\beta 2\delta$ GABA_A receptors depends upon the ambient GABA concentration or GABA occupancy of the receptors (Houston et al., 2012). The general anesthetic propofol and sleep-inducing drug THIP potentiate only when the ambient GABA levels are low and cease to modulate at saturating GABA. On the other hand, the neurosteroid THDOC (3 α ,21-dihydroxy-5 α -pregnan-20-one) has stronger potentiating effects on saturated GABA currents compared to submaximal GABA currents (Houston et al., 2012; Wohlfarth et al., 2002). We found that carisoprodol potentiated at both sub-saturating and saturating GABA concentrations. This is also true for pentobarbital (Feng et al., 2004). The N-terminal domain of the δ subunit has been shown to be involved in pentobarbital potentiation of saturated GABA currents of $\alpha 1\beta 3\delta$ receptors (Feng and Macdonald, 2010). The profile of carisoprodol at both synaptic and extrasynaptic GABA_AR is similar to that of barbiturates, although equivalent domains for action at synaptic receptors are unlikely (Gonzalez et al., 2009b). Whether the N-terminal domains of the δ subunit that influence pentobarbital potentiation of saturated GABA current also influence carisoprodol sensitivity remains to be determined.

At high concentrations, carisoprodol also inhibits GABA_AR function; this is evidenced by decreasing current amplitude and/or rebound currents associated with termination of drug application. A similar phenomenon also exists for barbiturates and gamma-butyrolactones (Akk et al., 2004; Feng et al., 2004; Rho et al., 1996; Williams et al., 1997; Woollorton et al., 1997). This rebound current has been explained as reentry into an active state following desensitization (Woollorton et al., 1997), or as relief from ligand binding to a low affinity inhibitory site, which permits reopening of the channel (Akk et al., 2004; Feng et al., 2004;

Rho et al., 1996; Williams et al., 1997). In the case of carisoprodol, rebound from block seems most likely. We have reported in abstract form (Kumar and Dillon, 2014) that in wild type homomeric $\beta 3$ GABA receptors, which are spontaneously open, application of carisoprodol by itself blocks the spontaneously open channels. In homomeric receptors with introduction of a transmembrane domain 2 mutation (mutation of the 6' T to F), blockade of the spontaneously open current is eliminated, and carisoprodol instead activates a significant inward current. Thus rebound currents following high concentrations of carisoprodol appear to be due to CSP dissociation from a low affinity inhibitory site. We did not fully quantitate the inhibitory effect, as it likely play a nominal role in the therapeutic or adverse effects of carisoprodol, and its low affinity combined with drug solubility issues makes accurate quantitation of it difficult.

We also studied whether the actions of carisoprodol on GABA_A receptors display voltage-dependence. Neither direct gating nor blocking effects were affected by transmembrane voltage. However, we found allosteric modulatory effects of carisoprodol were significantly reduced at + 60 mV compared to -60 mV; a similar effect has been reported for the general anesthetics etomidate, propofol and isoflurane, (O'Toole and Jenkins, 2012). One might conclude the differential voltage-dependence of direct and allosteric effects supports the likelihood of two sites for these effects. Whereas collectively our data do support distinct sites, the differences in voltage-dependence for direct and allosteric effects do not necessarily lead to this conclusion. The reduced effect of carisoprodol at + 60 mV may not relate to voltage per se, but instead is likely related to the enhanced open probability (P_o) of the channel when clamped at positive potentials, as effects of allosteric potentiators are inversely related to P_o (O'Toole and Jenkins, 2012).

With regard to lack of voltage-dependence of the inhibitory effect, one might conclude a site of action in the channel is unlikely. Whereas the presence of voltage-dependence of block is consistent with a site of action in the channel, it is not a requirement for a channel blocking mechanism. Indeed, the prototypical GABA_A blocker, picrotoxin, does not display voltage-dependence (Newland and Cull-Candy, 1992; Yoon et al, 1993), and there is a considerable body of evidence that picrotoxin binds in the ion channel (see Bali and Akabas, 2007, and several references therein). The fact that mutation of the TM2 6' residue does impact carisoprodol-mediated inhibition (Kumar and Dillon, 2014) is consistent with a site of action within the channel, but definitive conclusions regarding the site for inhibitory effects of carisoprodol will require additional study.

The recent scheduling at the federal level of carisoprodol confirms the danger this drug poses when misused and abused. In the current study, we demonstrated carisoprodol preferentially interacts with selective GABA_AR subunits. The pharmacological profile of carisoprodol at GABA_ARs we have identified is consistent with the therapeutic effects of the drug, and its subunit-selective actions may underlie its potential for abuse. Moreover, the complex interactions of carisoprodol suggest it interacts with multiple sites on the receptor. To our knowledge, no other GABAergic ligand has a subunit-dependent profile equivalent to that of carisoprodol, suggesting this drug may be acting at a novel site.

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Abbreviations

GABA	γ -aminobutyric acid
GABA_A	type A GABA receptor
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol-bis (β -aminoethyl ether)
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid N, N, N', N'-tetra acetic acid

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Highlights

- We studied subunit-dependent actions of carisoprodol on GABA_A receptors.
- Direct gating and allosteric modulation effects are influenced by subunit isoforms.
- Direct gating and allosteric modulation is likely via distinct sites.
- The pharmacological profile of carisoprodol at GABA_ARs is consistent with its therapeutic effects.
- subunit-selective actions may underlie potential for abuse of carisoprodol.

Figure 1. Influence of GABA_AR subunit isoforms on direct activation by carisoprodol
A, representative traces demonstrating carisoprodol activates human $\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_1\beta_1\gamma_2$ and $\alpha_1\beta_2$ GABA_ARs in a concentration-dependent manner. Carisoprodol-activated currents are presented relative to the maximum current elicited by GABA (100 μM for $\alpha_1\beta_2$ and 1 mM GABA for $\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$ and $\alpha_1\beta_1\gamma_2$). At millimolar concentrations, rebound currents were observed upon termination of drug application. **B**, concentration-response curves for carisoprodol-mediated currents for human α_1 -, α_2 -, α_3 -, α_4 -, α_5 -, and $\alpha_6\beta_2\gamma_2$ GABA_ARs. α_3 - subunit expressing receptors displayed significantly less potency and

efficacy to carisoprodol. Initial peak currents and not rebound currents elicited by carisoprodol were normalized to maximum GABA mediated currents. **C**, concentration-response curves for carisoprodol-mediated currents recorded from $\alpha 1\beta 2\gamma 2$ and $\alpha 1\beta 1\gamma 2$ GABA_ARs. Carisoprodol was significantly more efficacious at $\beta 1$ -containing receptors. **D**, concentration-response curves for carisoprodol-mediated currents recorded from $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs. There were no significant differences between the two configurations at each concentration tested ($p > 0.05$). Each data point represents the mean $\pm$ S.E.M. of minimum of eight cells. *, $p < 0.05$; **, $p < 0.01$.

Figure 2. Influence of GABA_AR subunit isoforms on allosteric modulation by carisoprodol
A, representative traces demonstrating the potentiation of GABA-gated (EC₂₀) currents from human $\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, $\alpha_1\beta_1\gamma_2$ and $\alpha_1\beta_2$ GABA_ARs by carisoprodol. **B**, concentration-response curves for the allosteric modulation of GABA-gated currents from human α_1 -, α_2 -, α_3 -, α_4 -, α_5 -, and $\alpha_6\beta_2\gamma_2$ GABA_ARs by carisoprodol. Peak current elicited by carisoprodol plus GABA (EC₂₀) normalized to GABA (EC₂₀) mediated currents. Carisoprodol was significantly more efficacious at α_1 -containing receptors. **C**, concentration-response curves for the allosteric modulation of GABA-gated currents

recorded from $\alpha 1\beta 1\gamma 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs by carisoprodol. Carisoprodol was significantly more efficacious at $\beta 2$ -containing receptors. **D**, concentration-response curves for the allosteric modulation of GABA-gated currents recorded from $\alpha 1\beta 2$ and $\alpha 1\beta 2\gamma 2$ GABA_ARs by carisoprodol. There were no significant differences between the two configurations at each concentration tested ($p > 0.05$). **E**, GABA concentration response in human $\alpha 1\beta 2\gamma 2$ GABA_A receptors in the absence (open square) or presence (filled square) of 100 μ M carisoprodol. Carisoprodol significantly decreased the GABA EC₅₀ (from 35.5 ± 0.64 to 8.2 ± 0.26 μ M ($n = 6$ and 4 , respectively)). The mean carisoprodol direct gating effect at 100 μ M (2.8 % of maximal GABA-gated current) was subtracted from the summary data. Each data point represents the mean $\pm$ S.E.M. of minimum of three cells. *, $p < 0.05$.

Figure 3. Direct gating action of carisoprodol on extrasynaptic GABA_A receptors

A, representative traces demonstrating carisoprodol (CSP, 1 or 3 mM) activation of $\alpha_1\beta_2$, $\alpha_1\beta_3$ and $\alpha_1\beta_3\gamma_2$ compared to δ -expressing extrasynaptic $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs.

GABA concentrations are saturating at each receptor. At 3 mM and above, the amplitude of CSP-gated current in $\alpha_4\beta_3\delta$ decreases significantly, followed by a rebound current. **B**,

concentration-response curves for carisoprodol-mediated currents in $\alpha_1\beta_3$, $\alpha_1\beta_3\gamma_2$, $\alpha_1\beta_2$ and extrasynaptic $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs. The δ subunit-containing extrasynaptic

GABA_ARs showed highest efficacy for carisoprodol. **C**, effect of the super agonist THIP in $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs, compared to GABA. **D**, summary data demonstrating carisoprodol's efficacy on δ -expressing extrasynaptic $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs

compared to THIP. Carisoprodol is approximately 90% and 88% efficacious as THIP on $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs, respectively. Each data point represents the mean $\pm$ S.E.M. of minimum of four cells. #, $p < 0.01$.

Figure 4. Allosteric modulation of extrasynaptic GABA_A receptors by carisoprodol

A, representative traces demonstrating the potentiation by carisoprodol (300 μM) of GABA-gated (EC_{20}) currents in $\alpha_1\beta_3$, $\alpha_1\beta_3\gamma_2$ and $\alpha_1\beta_2$ compared to δ -expressing extrasynaptic $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs. **B**, concentration-response curves showing mean allosteric carisoprodol modulation of GABA-gated currents in $\alpha_1\beta_3$, $\alpha_1\beta_3\gamma_2$, $\alpha_1\beta_2$ and extrasynaptic $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs. GABA_ARs containing β_2 subunits showed highest efficacy for carisoprodol. Each data point represents the mean $\pm$ S.E.M. of a minimum of four cells. *, $p < 0.05$; **, $p < 0.01$.

Figure 5. Potentiation of saturating GABA current by sub-gating concentration of carisoprodol in extrasynaptic GABA_A receptors

A, representative traces demonstrating the potentiation of saturating GABA currents by carisoprodol and pentobarbital in extrasynaptic $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs. **B**, bar graph summarizing carisoprodol's significant potentiation of maximal GABA currents in extrasynaptic $\alpha_1\beta_3\delta$ and $\alpha_4\beta_3\delta$ GABA_ARs, as seen with pentobarbital. This effect was not seen in $\alpha_1\beta_3$ and $\alpha_1\beta_3\gamma_2$ GABA_ARs. Each data point represents the mean $\pm$ S.E.M. of a minimum of four cells. **, $p < 0.01$.

Figure 6. Voltage-dependent effect of carisoprodol actions on GABA_A receptors

A, C and E, representative traces from $\alpha 1\beta 2\gamma 2$ GABA_ARs transfected HEK293 cells clamped at -60 mV and $+60$ mV for carisoprodol's direct gating, blocking and allosteric modulatory effects. **B, D and F**, bar graph summarizing carisoprodol direct gating, blocking and allosteric modulatory actions, respectively, at -60 mV and $+60$ mV. Carisoprodol direct gating and blocking effects are independent of voltage. However, allosteric potentiation of GABA currents by carisoprodol ($100\ \mu\text{M}$) was significantly reduced compared to the level of potentiation observed at -60 mV. Each data point represents the mean $\pm$ S.E.M. of minimum of four cells. *, $p < 0.01$.

Table 1
GABA sensitivity of different GABA_A receptors subunit configurations

GABA EC₅₀ values and Hill coefficients were calculated from each receptor configuration as mean $\pm$ S.E.M. from n cells.

Receptor Configuration	EC ₅₀ (μ M)	Hill Coefficient	n
$\alpha 1\beta 2$	14.0 ± 1.01	1.32 ± 0.11	4
$\alpha 1\beta 2\gamma 2$	35.5 ± 0.64	1.32 ± 0.03	6
$\alpha 2\beta 2\gamma 2$	48.4 ± 5.71	1.09 ± 0.12	9
$\alpha 3\beta 2\gamma 2$	34.8 ± 2.09	1.04 ± 0.06	8
$\alpha 4\beta 2\gamma 2$	4.48 ± 0.29	1.36 ± 0.11	6
$\alpha 5\beta 2\gamma 2$	2.50 ± 0.20	1.11 ± 0.09	6
$\alpha 6\beta 2\gamma 2$	0.47 ± 0.06	1.02 ± 0.12	8
$\alpha 1\beta 1\gamma 2$	6.6 ± 1.07	1.17 ± 0.09	6

Table 2
Comparison of potency and efficacy of carisoprodol as a direct gating agonist at different GABA_A receptor subunit configurations

Carisoprodol EC₅₀ was determined relative to maximum current elicited by carisoprodol. Carisoprodol efficacy is normalized to maximum GABA gated currents. The potency and efficacy of carisoprodol was significantly less at α 3-containing receptors relative to other α - subunit isoforms of GABA_A receptors. Each data point represents the mean $\pm$ S.E.M from n cells.

Receptor Configuration	EC ₅₀ (μ M)	Efficacy (%)	n
α 1 β 2	679.3 $\pm$ 24.3	45.9 $\pm$ 3.0	9
α 1 β 2 γ 2	685.5 $\pm$ 30.4	42.6 $\pm$ 4.3	31
α 2 β 2 γ 2	829.4 $\pm$ 50.2	30.1 $\pm$ 1.4	9
α 3 β 2 γ 2	1867.6 $\pm$ 92.4*	13.4 $\pm$ 1.4**	29
α 4 β 2 γ 2	757.7 $\pm$ 61.3	37.3 $\pm$ 5.0	8
α 5 β 2 γ 2	834.7 $\pm$ 60.3	37.7 $\pm$ 3.5	9
α 6 β 2 γ 2	294.3 $\pm$ 28.1	36.8 $\pm$ 4.7	12
α 1 β 1 γ 2	728.8 $\pm$ 58.1	69.7 $\pm$ 5.4*	12

Significant differences compared to α 1 β 2 γ 2 receptor are denoted by *, $p < 0.05$;

**, $p < 0.01$.

Table 3
Comparison of potency and efficacy of allosteric effects of carisoprodol at different GABA_A receptor subunit configurations

EC₅₀ for allosteric modulation by carisoprodol was calculated in the presence of GABA (EC₂₀). Efficacy was calculated as maximum current elicited by carisoprodol plus GABA (EC₂₀) normalized to GABA (EC₂₀) currents. Carisoprodol was significantly more efficacious at receptors incorporating the $\alpha 1$ subunit compared to receptors expressing any other α subunit isoform. Potency was not affected by α subunit isoform, but was enhanced in $\beta 1$ vs. $\beta 2$ expressing receptors. Each data point represents the mean $\pm$ S.E.M. from n cells.

Receptor Configuration	EC ₅₀ (μ M)	Efficacy (%)	n
$\alpha 1\beta 2$	87.4 $\pm$ 16.4	346.7 $\pm$ 67.6	3
$\alpha 1\beta 2\gamma 2$	88.2 $\pm$ 19.9	474.7 $\pm$ 53.5	7
$\alpha 2\beta 2\gamma 2$	64.9 $\pm$ 19.1	198.5 $\pm$ 9.5*	6
$\alpha 3\beta 2\gamma 2$	63.7 $\pm$ 16.5	242.7 $\pm$ 35.1*	4
$\alpha 4\beta 2\gamma 2$	72.3 $\pm$ 10.1	240.0 $\pm$ 16.4*	11
$\alpha 5\beta 2\gamma 2$	90.8 $\pm$ 14.7	247.6 $\pm$ 23.0*	5
$\alpha 6\beta 2\gamma 2$	79.8 $\pm$ 13.8	232.6 $\pm$ 26.9*	9
$\alpha 1\beta 1\gamma 2$	33.1 $\pm$ 4.0*	225.6 $\pm$ 14.6*	6

Significant differences compared to $\alpha 1\beta 2\gamma 2$ receptors are denoted by *, $p < 0.05$.

Table 4
Comparison of carisoprodol effects on δ subunit-containing extrasynaptic receptors with synaptic GABA_A receptors

Carisoprodol direct gating EC₅₀ was determined relative to maximum current elicited by carisoprodol and efficacy is normalized to maximum GABA gated currents. EC₅₀ for allosteric modulation was calculated in the presence of GABA (EC₂₀) and efficacy was calculated as maximum current elicited by carisoprodol plus GABA (EC₂₀) normalized to GABA (EC₂₀) currents. Carisoprodol was supra-efficacious at δ containing extrasynaptic GABA_A receptors for direct gating effects and significantly more efficacious at synaptic receptors incorporating the β 2 subunit for allosteric modulatory effects. Each data point represents the mean $\pm$ S.E.M. from n cells.

Receptor Configuration	GABA EC ₅₀		CSP gating		CSP modulation	
	(μ M)	n	Efficacy	EC ₅₀	n	EC ₅₀
α 1 β 2	1.15 $\pm$ 0.08	4	44.6 $\pm$ 4.85	670 $\pm$ 22.2	6	442.2 $\pm$ 27.7 **
α 1 β 3	0.67 $\pm$ 0.06	6	50.8 $\pm$ 6.24	606 $\pm$ 25.3	9	234.5 $\pm$ 18.9
α 1 β 3 γ 2	04.3 $\pm$ 0.68	6	44.8 $\pm$ 3.23	403 $\pm$ 30.5	4	200.4 $\pm$ 09.5
α 1 β 3 δ	1.37 $\pm$ 0.09	9	128.7 $\pm$ 12.3 **	801 $\pm$ 24.2	9	241.5 $\pm$ 25.1
						109 $\pm$ 11.4

Significant differences compared receptors studied are denoted by **, p< 0.01.