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Different residues in the $GABA_A$ receptor benzodiazepine binding pocket mediate benzodiazepine efficacy and binding

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RUNNING TITLE PAGE

Running title: Defining residues that contribute to BZD binding and efficacy

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Nonstandard abbreviations:

GA BA: γ amino butyric acid
GA BA_AR: GABA type A receptor
E SZ: eszopiclone
F ZM: flurazepam
B ZD: benzodiazepine
ZP M: zolpidem
DM CM: 3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline
I_{GABA}: GABA mediated current

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ABSTRACT

Benzodiazepines (BZDs) exert their therapeutic actions by binding to the γ-aminobutyric acid type A receptor ($GABA_AR$) and allosterically modulating GABA-activated chloride currents (I_{GABA}). A variety of ligands with divergent structures bind to the BZD site and the structural mechanisms that couple their binding to potentiation of I_{GABA} are not well understood. Here, we measured the effects of individually mutating twenty-two residues throughout the BZD binding pocket on the abilities of eszopiclone, zolpidem and flurazepam to potentiate I_{GABA}. Wild-type and mutant $\alpha_1\beta_2\gamma_2$ $GABA_ARs$ were expressed in *Xenopus* oocytes and analyzed using two-electrode voltage clamp. GABA EC_{50}, BZD EC_{50} and BZD maximal potentiation were measured. This data, combined with previous radioligand binding data describing the mutations' effects on BZD apparent binding affinities (Hanson and Czajkowski, 2008; Hanson et al., 2008), were used to distinguish residues within the BZD pocket that contribute to BZD efficacy and BZD binding. We identified six residues whose mutation altered BZD maximal potentiation of I_{GABA} (BZD efficacy) without altering BZD binding apparent affinity, three residues whose mutation altered binding but had no effect on BZD efficacy, and four residues whose mutation affected both binding and efficacy. Moreover, depending on the BZD ligand, the effects of some mutations were different indicating that the structural mechanisms underlying the ability of BZD ligands with divergent structures to potentiate I_{GABA} are distinct.

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INTRODUCTION

Benzodiazepines (BZDs) are commonly used in the treatment of sleep disorders, anxiety, muscle spasms, seizure disorders, and some forms of depression (Mohler et al., 2002). They exert their therapeutic actions by binding to the γ-aminobutyric acid type A receptor ($GABA_AR$) and modulating GABA-induced chloride current (I_{GABA}). The $GABA_AR$ is a heteropentameric, ligand-gated ion channel and belongs to the cys-loop superfamily of receptors that includes the $5HT_3$ receptor, glycine receptor and nicotinic acetylcholine receptor (nAChR) (Ortells and Lunt, 1995). The most common $GABA_A$ receptor subtype found in the brain is comprised of α_1, β_2, and γ_2 subunits in a ratio of 2α:2β:γ (Baumann et al., 2002; Chang et al., 1996; Farrar et al., 1999; Sieghart and Sperk, 2002). The BZD binding site is located in the extracellular domain of the receptor at the interface of the α and γ subunits (Fig. 1A), and is formed by six noncontiguous regions historically designated loops A-F (Fig. 1B) (Boileau et al., 2002; Boileau et al., 1998; Sigel and Buhr, 1997).

Ligands that bind to the BZD site can act as negative modulators that inhibit I_{GABA} (BZD inverse agonists), as positive modulators that potentiate I_{GABA} (BZD agonists) or as zero modulators that bind yet have no affect on I_{GABA} (BZD antagonists). While multiple studies have identified residues that are involved in mediating the apparent binding affinity (K_d) of BZD-site ligands including classical, [1,4]benzodiazepines (Boileau et al., 2002; Derry et al., 2004; Kucken et al., 2000; Wieland and Luddens, 1994), cyclopyrrolones (e.g. eszopiclone)(Davies et al., 2000; Hanson and Czajkowski, 2008) and imidazopyridines (e.g. zolpidem) (Buhr et al., 1996; Buhr et al., 1997; Buhr and Sigel, 1997; Hanson et al., 2008; Schaerer et al., 1998), much less is known about the

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structural determinants that couple their binding to modulation of I_{GABA} and govern whether a BZD-site ligand is a positive modulator, zero modulator or negative modulator (i.e. BZD efficacy).

In general, it is believed that BZDs exert their allosteric effects by either shifting the $GABA_AR$ closed to open state channel equilibrium (Campo-Soria et al., 2006; Downing et al., 2005; Rusch and Forman, 2005) or by altering the receptor's microscopic binding affinity for GABA (Goldschen-Ohm et al., 2010; Lavoie and Twyman, 1996; Mellor and Randall, 1997; Rogers et al., 1994; Thompson et al., 1999; Twyman et al., 1989). Regardless of the mechanism, BZD binding to the receptor is the initial perturbation that triggers structural rearrangements in the protein that result in modulation of $GABA_AR$ function. Residues that line the BZD binding site pocket likely have different roles in this process. Some residues may directly interact with the ligand and contribute to its binding affinity, some may stabilize binding site structure, whereas others may mediate local conformational movements important for coupling BZD binding to modulation of I_{GABA}. Identifying the residues that are involved in these actions is critical for elucidating the structural mechanisms that govern the pharmacological effects of these drugs and will help predict the therapeutic effects of new drugs.

Previously, we identified residues within the BZD binding site that were important for high-affinity binding of flumazenil (Ro15-1788), eszopiclone (ESZ) and zolpidem (ZPM) (Hanson and Czajkowski, 2008; Hanson et al., 2008). Here, we tested the hypothesis that residues in the BZD binding site are also crucial for determining BZD efficacy. We measured the effects that 22 single cysteine mutations (Fig. 1D), made

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throughout the BZD binding site, had on the abilities of flurazepam (FZM), ESZ and ZPM to potentiate I_{GABA} (BZD EC_{50} values and maximum potentiations were measured). We focused on residues that have not been extensively examined previously and for which the effects of mutating the residue on BZD apparent binding affinities were known. We identified six residues whose mutation solely altered BZD efficacy suggesting that they are part of the allosteric pathway involved in coupling BZD binding to modulation of $GABA_AR$ function. We identified three residues that when mutated only altered BZD binding affinity suggesting that they are important for ligand docking. Four additional residues, in the α subunit, when mutated, decreased both the binding affinity and efficacy of the BZD ligands suggesting that they play roles in mediating high affinity BZD binding and the initial structural rearrangements in the site that help couple binding to modulation of I_{GABA} and likely contribute to the structural integrity of the binding site. Moreover, we provide evidence that the structural mechanisms underlying the ability of BZD ligands of diverse structure to modulate I_{GABA} are distinct.

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MATERIALS AND METHODS

Site directed mutagenesis. Rat cDNAs encoding the GABAR α_1, β_2 and γ_{2L} subunits in the pUNIV vector (Venkatachalan et al., 2007) were used. Cysteine mutations in the α_1 and γ_{2L} subunits were made previously (Hanson and Czajkowski, 2008; Hanson et al., 2008) using recombinant PCR and verified by double stranded DNA sequencing.

Expression in *Xenopus laevis* oocytes. Expression of WT and mutant GABARs was performed as described previously (Hanson and Czajkowski, 2008). Capped cRNA from *Not*I- digested cDNA was *in vitro* transcribed using the mMessage mMachine T7 kit (Ambion, Austin, TX). *X. Laevis* oocytes were harvested and prepared as described previously (Boileau et al., 1998). Oocytes were injected within 24h of treatment with 27nl cRNA (1-15pg/nl/subunit) in the ratio 1:1:10 (α:β:γ) (Boileau et al., 2002) and stored at 16ºC in ND96 buffer (in mM: 96 NaCl, 2KCl, 1$MgCl_2$, 1.8$CaCl_2$, 5 HEPES, pH 7.2) supplemented with 100µg/ml BSA until used for electrophysiological recordings.

Two-electrode voltage clamp. Electrophysiological recordings were performed as described previously (Hanson and Czajkowski, 2008). Oocytes were held at –80mV under two-electrode voltage clamp while being continuously perfused with ND96 at a rate of 5ml/min in a bath volume of 200µl. Borosilicate glass electrodes (0.4-1.0 mΩ) (Warner Instruments, Hamden, CT) were filled with 3M KCl. Electrophysiological data were collected using GeneClamp 500 (Molecular Devices, Sunnyvale, CA) interfaced to a computer with a Digidata 1200 A.D device (Molecular Devices). Recordings were made using the Whole Cell Program, v.3.6.7 (kindly provided by J. Dempster, University of Strathclyde, Glasgow, UK). Stock solutions of FZM (RBI, Natick, MA) were

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dissolved in ND96 and diluted in ND96 for working concentrations. GABA (Sigma, St. Louis, MO) solutions were prepared fresh daily with ND96. Stock solutions of DMCM (3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline) (RBI, Natick, MA), ZPM (Sigma, St. Louis, MO) and ESZ (kindly provided by Sepracor, Inc.) were prepared in DMSO and subsequently diluted in ND96 for working concentrations where the final [DMSO] (≤ 2%) did not affect $GABA_AR$ function.

Concentration-response analysis. GABA concentration-response curves were determined as described previously (Hanson and Czajkowski, 2008). Six to twelve concentrations of GABA were used for each GABA EC_{50} value determination. Each current response was scaled to a low, non-desensitizing concentration of GABA (EC_{1-5}) applied just before the test concentration to correct for any drift in I_{GABA} responsiveness over the course of the experiment. Concentration-response data were fit by the following equation: $I = I_{max}/[1+EC_{50}/[A]^n)]$, where I is the peak response to a given drug concentration, I_{max} is the maximum amplitude of current, EC_{50} is the drug concentration that produces that half-maximal response, [A] is drug concentration, and n is the Hill coefficient using Prism V.4.0 (GraphPad Software, San Diego, CA). The EC_{50} values in Table 1 for four mutants (γR185C, γE189C, γR194C and γR197C) are from Hanson and Czajkowski, 2008 with the associated errors in SEM, as opposed to SD reported in the 2008 publication. Two values that were significantly different from wild-type (WT) values in the 2008 publication (γR185C and γR194C) are no longer significant in the present study because the GABA EC_{50} value for WT receptors in this study is slightly lower than in the previous report and the full data sets that was used for the ANOVAs in both studies are different.

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BZD concentration responses (6-8 different concentrations) were measured at GABA EC_{15}. BZD modulation was defined as follows: $[(I_{GABA+BZD}/I_{GABA})-1]$, where $I_{GABA+BZD}$ is the current response in the presence of GABA and BZD, and I_{GABA} is the current evoked by GABA alone (GABA EC_{15}). When measuring BZD concentration responses, each application of GABA + BZD is preceded by a brief pulse of EC_{15} GABA alone. Wash times between application of GABA + BZD and the following application of GABA alone were increased with every increase in BZD concentration. During the experiment, the magnitude of the currents elicited by the GABA EC_{15} pulses alone did not change (< 3%) even following high concentrations of BZD, indicating complete washout of the BZDs. BZD concentration response curves were fit with the equation $P = P_{max}/(1+(EC_{50}/A)^n)$, where A is the BZD concentration, EC_{50} is the concentration of BZD eliciting half maximal current potentiation, P_{max} is the maximal BZD potentiation of I_{GABA}, P is the potentiation amplitude and n the Hill coefficient. The reported values for maximum potentiation were determined from curve fitting the data.

Statistical analysis. All data are from at least three different oocytes from at least two different frogs. Data represent mean ± SEM. Significant differences in EC_{50} values and maximal BZD modulation values were determined by one-way ANOVA, followed by a post hoc Dunnett's test using Prism v.4.0 (GraphPad Software Inc., San Diego, CA). Log (EC_{50}) values were used for statistical analyses.

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RESULTS

We previously made 22 single cysteine mutations throughout the BZD binding site in loops A (D97C, F99C), B (G157C, A160C, T162C), and C (G200C, V202C, S204C, S205C, T206C, Y209C, V211C) of the α_1 subunit and loops E (T126C, M130C, R132C, L140C, T142C, R144C) and F (R185C, E189C, R194C, R197C) of the γ_2 subunit (Fig. 1) and examined the effects of these mutations on BZD binding using competitive radioligand binding experiments (see Table 2 for mut/WT K_i values) (Hanson and Czajkowski, 2008; Hanson et al., 2008). The mutations in the γ Loop F region had no effect on BZD apparent binding affinity, whereas at least one mutation in each of the α loops A and B and γ loop D altered the affinities of all of the ligands tested (Ro15-1788, ZPM and ESZ) suggesting that these regions are critical for the binding of a variety of structurally-diverse BZD-site ligands (Hanson et al., 2008). In contrast, a number of the mutations in α loop C and γ loop E altered the binding of some BZDs but not others suggesting that residues in these regions help define BZD selectivity (Hanson et al., 2008). Here, we tested the hypothesis that residues in the BZD binding site are not only important for BZD binding but also play a role in defining BZD efficacy. Cysteine mutant subunits were co-expressed with wild type (WT) subunits in *Xenopus laevis* oocytes to form $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors and analyzed using two-electrode voltage clamp. We examined the effects the mutations had on GABA-activated currents (I_{GABA}) and on FZM, ESZ and ZPM modulation of EC_{15} I_{GABA}.

Effects of cysteine substitutions on I_{GABA}

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All of the mutant subunits assembled into functional $GABA_A$Rs (Table 1). Seven out of the twelve cysteine substitutions in the α_1 subunit significantly increased GABA EC_{50} values (13-31 fold) as compared to WT receptors (18.1± 4.4μM; Table 1). In general, the mutations in the γ_2 subunit had smaller effects. γT126C and γM130C increased GABA EC_{50} approximately 3-fold, whereas γR144C decreased GABA EC_{50} 6-fold compared to WT receptors (Table 1).

Effects of cysteine substitutions on FZM modulation of I_{GABA}

We measured the effects the mutations had on the abilities of three structurally different BZD-site positive modulators, FZM (1,4 benzodiazepine), ESZ (cyclopyrrolone) and ZPM (imidazopyridine) to potentiate GABA (EC_{15}) currents. Current traces and dose response curves for BZD potentiation of I_{GABA} are depicted in Figures 2 and 3, respectively. At saturating BZD concentrations (i.e. when the BZD binding site is fully occupied), the effects of the mutations on BZD efficacy are being monitored. Eight out of the 22 mutations significantly decreased FZM maximal potentiation of I_{GABA} compared to WT receptors (pot = 2.3 ± 0.2; Fig. 4, Table 2). In the α_1 subunit, cysteine substitution of D97 and F99 in loop A; G157 and A160 in loop B; and Y209 in loop C significantly decreased FZM maximal potentiation. In the γ_2 subunit, cysteine substitution of T142 and R144 in loop E, and R197 in loop F also significantly decreased FZM maximal potentiation. Note, that αF99C and γR144C almost completely eliminated FZM potentiation of I_{GABA} and thus FZM EC_{50} values could not be determined.

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Effects of cysteine substitutions on ESZ modulation of I_{GABA}

The effects of the mutations on ESZ were also measured. Eight of the 22 mutations altered ESZ max potentiation of I_{GABA} as compared to WT receptors (pot = 2.8 ± 0.3) (Figs. 2, 3, 4; Table 2). In the α_1 subunit, D97C in loop A; G157C and A160C in loop B; and T206C and Y209C in loop C significantly decreased ESZ maximal potentiation. ESZ inhibited I_{GABA} and became a negative modulator at αF99C containing receptors (Figs. 2B, 3B). As reported in Hanson et al. (2008), the specific binding of [^{3}H]Ro15-1788, [^{3}H]flunitrazepam or [^{3}H]Ro15-4513 to alpha D97C- and Y209C-mutant receptors was not detectable using a filtration-based radioligand binding assay (Table 2). The inability to detect radioligand binding is likely due to inherent limitations of filtration binding assays, which preclude measuring binding when the affinity of the radioligand is much above 100nM. Given that we can measure BZD modulation of I_{GABA} for these mutant receptors, these drugs bind to the mutant receptors, likely with lower apparent affinity. The rightward shifts in the BZD concentration responses are consistent with this idea. In the γ_2 subunit, mutations at R144 in loop E and R197 in loop F significantly reduced ESZ maximal potentiation. While αA160C significantly reduced ESZ potentiation of I_{GABA} (i.e. ESZ efficacy), this mutation had little to no effect on ESZ apparent binding affinity (K_i, Table 2).

Effects of cysteine substitutions on ZPM modulation of I_{GABA}

The effects of the mutations on ZPM modulation of I_{GABA} were also examined. Nine out of the 22 mutations altered ZPM max potentiation of I_{GABA} (Figs 2, 3, 4, Table 2). αF99C in loop A, αG157C and αA160C in loop B, αT206C and αY209C in loop C,

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γR144C in loop E, and γR197C in loop F significantly decreased ZPM potentiation when compared to WT receptors (pot = 2.8 ± 0.3). Interestingly, αV211C (loop C) and γE189C (loop F) significantly increased ZPM potentiation of I_{GABA} (1.8 and 2.3 fold, respectively; Figs. 3C and 4C). Previously, we reported that γE189C had no effect on ZPM potentiation (Hanson and Czajkowski, 2008; Hanson et al., 2008). The differences in results are likely due to using higher concentrations of ZPM used in this study. While αA160C, αT206C, αV211C, γR144C, γE189C and γR197C significantly altered ZPM potentiation of I_{GABA} (efficacy), the mutations had little to no effect on ZPM apparent binding affinity (K_i, Table 2).

Effects of cysteine substitutions on DMCM modulation of I_{GABA}

For a subset of mutations (αF99C, αG157C, αA160C, αT206C, αY209C and γR144C), we also examined the ability of DMCM (3-carbomethoxy-4-ethyl-6,7-dimethoxy-β-carboline) to inhibit GABA (EC_{15}) currents. DMCM is a BZD site inverse agonist. None of the mutations tested significantly altered DMCM inhibition of I_{GABA} (WT, DMCM inh = 0.55 ± 0.04, n = 3, Fig. 5) indicating that the effects of the mutations on BZD positive modulator actions are specific. DMCM inhibition of one mutant, γR144C, was decreased compared to WT but this did not reach significance. Since only γ-containing $GABA_A$Rs are modulated by DMCM, the near WT inhibition of I_{GABA} by DMCM also indicates that the mutations do not impair subunit assembly or incorporation into functional αβγ $GABA_A$Rs.

Changes in BZD modulation of I_{GABA} are not correlated to changes in GABA EC_{50}

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Some mutations caused significant changes in GABA EC_{50} raising the possibility that the changes in BZD potentiation observed are linked to the GABA EC_{50} alterations. BZD positive modulators enhance $GABA_AR$ current by decreasing GABA EC_{50} and shifting the GABA dose response curve to the left. If a mutation only shifted the GABA dose response curve to the right, one would expect that the mutation would increase FZM, ESZ and ZPM potentiation and that inhibition by a negative modulator, such as DMCM, would decrease if a fixed GABA concentration was being used to elicit the responses. In our experiments, BZD modulation of I_{GABA} was measured at the same effective GABA concentration (EC_{15}) for each of the mutant and wild-type receptors, which should mitigate GABA EC_{50} effects on BZD modulation. Moreover, for many of the mutations, their effects on GABA EC_{50} and BZD potentiation were not correlated (Supplementary Fig. 1). Some mutations significantly altered BZD potentiation without affecting GABA EC_{50} (γT142, γE189 and γR197) whereas others affected GABA EC_{50} without changing BZD potentiation (γT126C, γM130C, αS205C, αV211C). Additionally, while the αF99C, αAG157C, αA160C, αT06C, αY209C, γR144C mutations altered GABA EC_{50}, inhibition of I_{GABA} by DMCM was not significantly altered (Fig. 5). Taken together, these data indicate that the observed changes in GABA EC_{50} are not causative for the observed alterations in the efficacies of BZD site positive modulators (Fig. 4).

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DISCUSSION

We identified four residues in the BZD binding pocket that specifically contribute to BZD-site agonist efficacy: in loop B, A160; in loop C, T206; in loop E, R144; and in loop F, R197 (Fig. 6, Top row). Mutating these residues significantly disrupted the abilities of ZPM, ESZ and FZM to potentiate I_{GABA} but had little to no effect on high affinity binding (Table 2) (Hanson et al., 2008). Consistent with the mutations having little effect on binding, these residues are largely localized at the periphery of the binding pocket (Fig. 6C) and thus, are in an ideal position to propagate local movements in the BZD binding pocket outward to more distant regions of the protein involved in modulating I_{GABA}. We also identified two residues (αV211 and γE189) that when mutated significantly increased ZPM potentiation of I_{GABA} without affecting FZM or ESZ potentiation indicating that the residues involved in coupling high affinity BZD binding to potentiation of I_{GABA} can be different depending upon the type of BZD-site ligand bound. This is consistent with our previous data, where we demonstrated that structural determinants for high affinity binding of ESZ and ZPM are different (Hanson and Czajkowski, 2008; Hanson et al., 2008). One can envision that depending on the orientation of the BZD in the binding pocket and its contact residues that some of the residues involved in the initial coupling of binding to potentiation of I_{GABA} may differ. ZPM binding is largely dependent on shape recognition and *in silico* docking has revealed that ZPM can adopt multiple orientations in the site (Hanson et al., 2008). Mutating γE189 or αV211 may cause ZPM to preferentially adopt a position that has a higher efficacy.

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We also identified three residues (αG200, γM130 and γR132) that specifically mediate high affinity BZD agonist binding. In contrast to the residues discussed above, mutating these residues had no significant effects on BZD agonist efficacy but significantly altered their binding (Fig. 6, middle row). Consistent with the mutations affecting binding and not efficacy, αG200, γM130 and γR132 are located on β strands (Fig. 6F) that line the core of the BZD binding pocket. Previous mutagenesis studies have demonstrated the importance of αG200 and γM130 in BZD binding. The glycine at position 200 is only found in the $GABA_AR$ α_1 subunit isoform, $\alpha_{2\text{-}6}$ subunits have a glutamate at aligned positions (Fig. 1D). Schaerer et al. showed that replacing α_1G200 with glutamate decreases ZPM binding affinity (Schaerer et al., 1998). Mutating α_6E200 to its α_1 counterpart in a background of 3 other point mutations confers ZPM binding to the BZD insensitive α_6 subunit (Wieland and Luddens, 1994). Mutating γ_2M130 to a variety of different residues also alters ZPM binding (Buhr and Sigel, 1997) and replacement of the aligned lysine in the γ_1 subunit (Fig. 1D) with a methionine increases the binding affinity of a variety of classical BZDs (Wingrove et al., 1997).

Here, we also identified residues that are important for both high affinity BZD agonist binding and BZD efficacy: αD97and αF99 in loop A, αG157 in loop B and αY209 in loop C. Introducing cysteines at these positions decreased ZPM and ESZ binding and decreased the efficacy of FZM, ZPM and ESZ potentiation of I_{GABA} (Fig. 6, bottom row, Table 2). The binding of ZPM and ESZ to αD97C and αY209C containing receptors was so disrupted, their binding affinities could not be reliably measured (Hanson et al., 2008). These residues are located in the back of the BZD binding pocket in loop A (D97 and F99), the side of the pocket in loop B (G157) and at the base of loop

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C facing directly into the binding site (Y209) (Fig. 6I). αD97and αF99 in loop A are located near αH101. αH101 has been previously shown to be important for binding of ZPM (Wieland and Luddens, 1994; Wieland et al., 1992), zopiclone (the racemate of ESZ) (Davies et al., 1998), flunitrazepam (Berezhnoy et al., 2004), and diazepam (Berezhnoy et al., 2004; Davies et al., 2000). Mutation of αH101 to arginine has also been shown to alter BZD efficacy (Benson et al., 1998). Previous studies have also identified αG157 in loop B and αY209 in loop C as important determinants for BZD binding (Amin et al., 1997; Tan et al., 2007b). Interestingly, all of the residues we have identified that are important for both high affinity BZD agonist binding and BZD efficacy are located in the α subunit and are conserved in all α subunit isoforms (Fig. 1D). Residues in the α subunit are likely to play critical roles in BZD efficacy since a single α subunit contributes to forming both a GABA and BZD binding site at the β-α and α-γ interfaces, respectively. Thus, BZD induced movements may be directly propagated through the α subunit from the BZD site to the GABA binding site. Previous studies have demonstrated that BZDs cause movements at the GABA binding site interface (Kloda and Czajkowski, 2007).

Interestingly, mutating αF99 to cysteine caused ESZ to switch from a potent positive modulator to a negative modulator (Fig. 2B) and had similar effects on the BZD agonist diazepam, making it a weak negative modulator (Tan et al., 2007a). It is not unprecedented that a single mutation can alter a BZD's action from enhancement to inhibition of I_{GABA}. The γT142S mutation, as well as mutations of αH101, cause the inverse agonist Ro15-4513 and the antagonist flumazenil to become BZD agonists and potentiate I_{GABA} (Benson et al., 1998; Mihic et al., 1994). How these mutations result in

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switches in a BZD's actions is not clear. Many structurally diverse ligands bind to the BZD binding site indicating the site can accommodate a variety of ligands. We speculate that the mutations may alter the positioning of the drug in the site and/or positioning of nearby residues, which then induces different downstream allosteric rearrangements.

Previously, we identified residues and regions in the γ_2 subunit, outside of the BZD binding pocket, that were critical for coupling BZD agonist binding to potentiation of I_{GABA} actions but were not involved in coupling DMCM binding to inhibition of I_{GABA} (Boileau and Czajkowski, 1999; Hanson and Czajkowski, 2008; Kloda and Czajkowski, 2007). Here, none of the mutations we tested significantly altered the inhibitory abilities of DMCM (Fig. 5) demonstrating, even at the level of the BZD binding site, that the structural mechanisms underlying the coupling of DMCM binding to inhibition of I_{GABA} are different than those underlying BZD agonist modulation.

The benzodiazepine (BZD) binding site of the $GABA_A$ receptor is pharmacologically complex. Structurally diverse ligands can bind to it and elicit a range of actions from potentiation of I_{GABA} to inhibition. Residues that line the BZD binding site pocket likely play different roles in mediating these actions. Here, we have identified specific residues that contribute to BZD binding affinity, other residues that contribute to BZD efficacy and others that mediate both binding and efficacy. Moreover, we show that local structural elements important for coupling BZD binding to modulation I_{GABA} are not only different for BZD positive modulators versus negative modulators but are also different for structurally diverse BZD positive modulators indicating that, at the level of the binding site, there is not a single common set of BZD induced movements that underlies BZD positive modulation. We envision that depending on how a BZD

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occupies the site (e.g. the orientation of the BZD in the site and its interactions with the receptor), its binding elicits distinct motions within the site, which then can induce different downstream allosteric rearrangements. It has been demonstrated for G-protein coupled receptors that even structurally similar agonists interacting with the same orthosteric site can bind to and activate the receptor via different structural mechanisms. (Ghanouni et al., 2001; Swaminath et al., 2005; Swaminath et al., 2004). In summary, the data in this study provide substantial new insights into the structural determinants important for BZD allosteric modulation of $GABA_A$ receptor function. Our results, which identify residues within the BZD binding site that encode BZD efficacy versus affinity, will aid in the design of more efficacious and selective drugs.

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AUTHORSHIP CONTRIBUTION

Participated in research design: Morlock and Czajkowski
Conducted experiments: Morlock
Contributed new reagents or analytic tools:
Performed data analysis: Morlock and Czajkowski
Wrote or contributed to the writing of the manuscript: Morlock and Czajkowski
Other:

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. The BZD binding site at the α_1/γ_2 interface of the $GABA_AR$ and structures of BZD site ligands. (A) Homology model of the α_1/γ_2 interface perpendicular to the plane of the membrane. The α_1 subunit is in blue and the γ_2 subunit is in red. (B) The region of the α_1/γ_2 interface that contains the BZD binding site is expanded and BZD binding site loop regions A-F are each highlighted in a different color. (C) Structures of BZD ligands ESZ, ZPM and FZM. (D) Sequence alignments of the extracellular domain of α_{1-6} and γ_{1-3} rat $GABA_AR$ subunit isoforms with BZD binding site loops are shown. Loop regions are colored as in (B). Residues mutated in this study are underlined and residues highlighted in color are identical. Numbering refers to α_1 and γ_2 residues.

Figure 2. Effects of the mutations on BZD maximal potentiation. Representative current traces showing maximal potentiation of GABA EC_{15} current from oocytes expressing WT and mutant receptors by (A) FZM, (B) ESZ or (C) ZPM. In all cases, BZDs were at concentrations that elicited maximal responses. I bar in panel A indicates potentiation of I_{GABA}. Note, in panel B, for αF99Cβγ receptors, ESZ inhibited I_{GABA}.

Figure 3. BZD concentration response curves from WT and mutant $GABA_ARs$ for (A) FZM, (B) ESZ and (C) ZPM. BZD potentiation was calculated as $[(I_{GABA+BZD}/I_{GABA})-1]$. Data represent mean ± SEM. Data were fit by nonlinear regression as described in Materials and Methods. Dashed lines are curve fits from WT receptors. BZD EC_{50} values and BZD maximal potentiation values are reported in Table 2.

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Figure 4. Mutations throughout the BZD binding site affect BZD efficacy. Maximal potentiation of GABA EC_{15} current from WT and mutant receptors by (A) FZM, (B) ESZ or (C) ZPM is plotted. BZD potentiation was calculated as $[(I_{GABA+BZD}/I_{GABA})-1]$. Data are mean ± SEM from at least three oocytes from two or more batches. Dashed lines indicate WT levels of potentiation. Black bars indicate values that are significantly different from WT (*, $p < 0.05$; **, $p < 0.01$).

Figure 5. DMCM modulation of WT and mutant $GABA_A$ receptors. Inhibition of EC_{15} GABA by 1μM DMCM for WT and mutant receptors is plotted. Inhibition of GABA current was calculated as $[(I_{GABA+DMCM}/I_{GABA})-1]$. Data are mean ± SEM from at least three oocytes from two or more batches. The dashed line indicates the level of WT inhibition. None of the mutations significantly altered DMCM inhibition of I_{GABA}. (B) Representative current traces from oocytes expressing WT αβγ and αF99Cβγ receptors in response to EC_{15} GABA and EC_{15} GABA + 1μM DMCM.

Figure 6. Summary of data highlighting residues important for BZD efficacy (A, B, C), BZD binding (D, E, F), and BZD binding and efficacy (G, H, I). Panels A, D and G plot the percent change in maximum potentiation for FZM , ESZ and ZPM [((mutant max potentiation-WT max potentiation)/WT max potentiation)(100)], respectively. Negative values represent a decrease in potentiation, while positive values indicate an increase. Panels B, E, and H plot changes in binding affinity [log (mut K_i/WT K_i)]. K_i values for FZM, ESZ and ZPM are from (Hanson and Czajkowski, 2008; Hanson et al., 2008) and were determined by displacement of [^{3}H]Ro15-1788 binding. Negative values indicate

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increased affinity, positive values indicate decreased affinity. Panels C, F and I are homology models with residues involved in BZD efficacy (C), BZD binding (F) or BZD binding and efficacy (I) shown in sticks. α subunit is blue, γ is red. Loop C is labeled. Values statistically different from WT are indicated (*, $p < 0.05$; **, $p < 0.01$). ND, binding of [^{3}H]Ro15-1788 was not detectable thus K_i values for FZM, ESZ and ZPM were not determined. †, no binding data available

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Table 1. Summary of GABA dose-response data for WT and mutant $\alpha_1\beta_2\gamma_2$ $GABA_A$Rs

	receptor	EC_{50} (μM)	n_H	n	I_{max} range (μA)
	WT αβγ	18.4 ± 4.4	1.50 ± 0.09	5	8.4 - 11.6
Loop A	αD97Cβγ	485 ± 67**	0.66 ± 0.10**	5	2.3 - 4.0
	αF99Cβγ	391 ± 94*	0.75 ± 0.06**	7	2.7 - 3.1
B	αG157Cβγ	408 ± 93*	0.65 ± 0.03**	8	1.4 - 4.1
	αA160Cβγ	560 ± 138**	0.60 ± 0.04**	6	3.3 - 5.0
	αT162Cβγ	39.8 ± 13.2	0.89 ± 0.12**	4	7.6 - 9.3
C	αG200Cβγ	33.0 ± 11.0	1.03 ± 0.02**	3	4.4 - 8.0
	αV202Cβγ	40.0 ± 22.5	0.99 ± 0.06**	3	6.0 - 7.2
	αS204Cβγ	22.0 ±4.7	1.04 ± 0.03**	3	2.7 - 8.8
	αS205Cβγ	230 ± 126*	1.08 ± 0.20*	3	2.6 - 7.7
	αT206Cβγ	268 ± 58.0*	0.59 ± 0.05**	3	1.9 - 2.8
	αY209Cβγ	319 ± 170*	0.68 ± 0.13**	3	3.5 - 8.8
	αV211Cβγ	80.3 ± 27.5	1.11 ± 0.19*	3	3.2 - 11.4
E	αβγT126C	49.1 ± 7.8**	1.13 ± 0.26	4	4.3 - 8.2
	αβγM130C	45.9 ± 7.5*	1.43 ± 0.02	3	2.6 - 3.5
	αβγR132C	15.4 ± 3.1	1.64 ± 0.04	3	5.8 - 9.2
	αβγL140C	22.2 ± 7.1	1.28 ± 0.21	3	4.4 - 11.0
	αβγT142C	11.9 ± 0.2	1.61 ± 0.12	4	10.6 - 13.7
	αβγR144C	3.0 ± 0.7*	1.55 ± 0.13	3	7.6 - 16.4
F	αβγR185C[a]	10.7 ± 2.0	1.52 ± 0.16	3	11.2-16.0
	αβγE189C[a]	16.6 ± 3.1	1.50 ± 0.03	4	5.2-11.2
	αβγR194C[a]	11.0 ± 2.4	1.41 ± 0.10	3	12.4-17.2
	αβγR197C[a]	17.4 ± 2.3	1.17 ± 0.04	4	9.7-12.0

Data are mean ± SEM for n experiments. n_H values are calculated Hill coefficients. I_{max} range is the lowest and highest maximal GABA current amplitude measured for each of the receptors. [a] Values are from (Hanson and Czajkowski, 2008) with errors in SEM not SD as previously reported. Values significantly different from wild type $\alpha_1\beta_2\gamma_2$ are indicated (*, $p < 0.05$, **, $p < 0.01$). In Hanson and Czajkowski (2008), GABA EC_{50} values for γR185C and γR194C were decreased 2.5-fold compared to WT and were statistically different. Here, these values are no longer significant due to a slight decrease in the WT EC_{50} value reported here and due to differences in the data sets analyzed by ANOVA.

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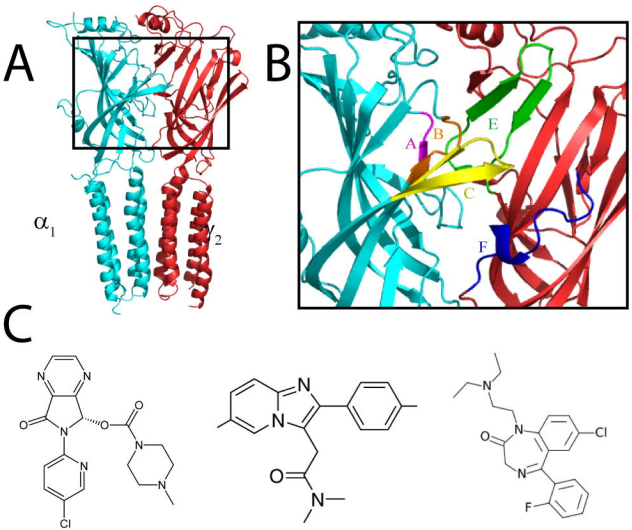
Table 2. Summary of BZD concentration response data and binding data for WT and mutant $\alpha_1\beta_2\gamma_2$ $GABA_A$Rs.

	receptor	Flurazepam Max potentiation	Flurazepam EC_{50} (nM)	n	Flurazepam mut/WT K_i[a]	Eszopiclone Max potentiation	Eszopiclone EC_{50} (nM)	n	Eszopiclone mut/WT K_i[b]	Zolpidem Max potentiation	Zolpidem EC_{50} (nM)	n	Zolpidem mut/WT K_i[b]
	WT αβγ	2.3 ± 0.2	442 ± 34	3	1.0	2.8 ± 0.3	55 ± 8.5	8	1.0	2.8 ± 0.2	76.9 ± 27.4	6	1.0
Loop A	αD97Cβγ	0.8 ± 0.2**	941 ± 140	3		1.1 ± 0.2**	166 ± 49	3	ND	2.5 ± 0.2	331 ± 18	3	ND
	αF99Cβγ	0.1 ± 0.0**		3		-0.5 ± 0.0**	1400 ± 290**	3	8.1**	0.7 ± 0.2**	3670 ± 1900**	3	2.9*
B	αG157Cβγ	0.9 ± 0.2**	1620 ± 760	4		0.7 ± 0.1**	1230 ± 140**	3	42**	1.3 ± 0.1*	2820 ± 610**	3	20**
	αA160Cβγ	1.0 ± 0.2**	368 ± 14	3		0.8 ± 0.1**	140 ± 47	3	1.2	1.4 ± 0.1*	118 ± 13	3	1.3
	αT162Cβγ	2.1 ± 0.1	500 ± 53	3		2.5 ± 0.5	131 ± 20	3	0.7	2.5 ± 0.3	410 ± 84	3	1.8
C	αG200Cβγ	2.0 ± 0.0	454 ± 83	3		3.7 ± 0.5	230 ± 8.1	4	2.4**	2.4 ± 0.5	996 ± 481**	3	9.7**
	αV202Cβγ	1.4 ± 0.4*	922 ±165	3		2.2 ± 0.4	522 ± 130**	3	5.0**	3.2 ± 0.3	2490 ± 250**	3	9.0**
	αS204Cβγ	1.5 ± 0.1	305 ± 43	3		1.7 ± 0.2	139 ± 39	3	1.2	3.6 ± 0.4	2100 ± 280**	3	7.0**
	αS205Cβγ	2.0 ± 0.1	366 ± 68	3		3.2 ± 0.2	75 ± 21	3	0.7	2.4 ± 0.2	143 ± 63.4	3	0.7
	αT206Cβγ	1.4 ± 0.3	946 ± 188	3		0.4 ± 0.0**	50.9 ± 19.1	3	0.02**	1.2 ± 0.2*	245 ± 42	3	1.2
	αY209Cβγ	0.7 ± 0.1**	2770 ± 649**	3		0.8 ± 0.2*	766 ± 250**	3	ND	0.9 ± 0.2**	14300 ± 2300**	3	ND
	αV211Cβγ	2.3 ± 0.1	511 ± 93	3		3.0 ± 0.4	115 ± 21	3	1.2	4.9 ± 0.1**	356 ± 117	3	1.1
E	αβγT126C	2.5 ± 0.3	280 ± 35.7	3		3.4 ± 0.2	14.4 ± 4.1	3	1.4	3.9 ± 0.2	9.6 ± 1.7	3	1.2
	αβγM130C	1.8 ± 0.2	305 ± 90	3		3.1 ± 0.5	24.5 ± 7.7	3	2.0*	3.0 ± 0.3	4.4 ± 0.4	3	0.3**
	αβγR132C	1.9 ± 0.1	264 ± 76.7	3		2.2 ± 0.3	12.5 ± 3.1	3	2.2*	3.3 ± 0.2	7.03 ± 1.28	3	0.6*
	αβγL140C	2.1 ± 0.1	330 ± 65	3		2.8 ± 0.2	12.6 ± 1.4*	4	0.9	3.7 ± 0.2	12.1 ± 2.7	3	1.0
	αβγT142C	1.0 ± 0.0**	561 ± 58	3		2.1 ± 0.5	148 ± 39**	3	10*	2.7 ± 0.3	980 ± 260**	3	21**
	αβγR144C	0.0 ± 0.0**		3		0.2 ± 0.0**		3	3.5**	1.0 ± 0.2**	22.5 ± 9.4	3	0.6
F	αβγR185C	1.7 ± 0.2	317 ± 82	3		2.4 ± 0.2	10.4 ± 1.5*	3	1.4	2.0 ± 0.4	10.0 ± 2.2	3	1.1
	αβγE189C	1.7 ± 0.2	645 ± 78	3	1.3	3.1 ± 0.2	12.2 ± 3.0*	4		6.3 ± 0.4**	1838 ± 636**	5	1.8[a]
	αβγR194C	1.4 ± 0.1	332 ± 53	3		1.7 ± 0.4	12.8 ± 3.4	3	1.3	1.7 ± 0.8	21.2 ± 8.3	3	1.0
	αβγR197C	0.6 ± 0.3**	494 ± 48	3	0.4*	0.4 ± 0.0**	6.0 ± 2.1*	3		0.8 ± 0.2**	13.8 ± 2.3	4	0.4[a]

Data are mean ± SEM for n experiments. Maximal potentiation is calculated as $[(I_{GABA+BZD}/I_{GABA})-1]$. The values for BZD binding affinities (K_i) were determined previously and the ratio of mutant to WT binding affinity is shown. [a] Values from (Hanson and Czajkowski, 2008). [b] Values from (Hanson et al., 2008). ND, not detectable. Values significantly different from wild type $\alpha_1\beta_2\gamma_2$ are indicated (*, $p < 0.05$, **, $p < 0.01$).

Figure 1



D

	Loop A										Loop B												Loop C											
	94									104	154											166	196											214
Alpha 1	W	T	P	D	T	F	F	H	N	G	K	L	K	F	G	S	Y	A	Y	T	R	A	E	V	T	V	D	S	G	I	V	Q	S	S	T	G	E	Y	V	V	M	T	T
Alpha 2	W	T	P	D	T	F	F	H	N	G	K	L	K	F	G	S	Y	A	Y	T	T	S	E	V	S	I	G	K	E	T	I	K	S	S	T	G	E	Y	T	V	M	T	A
Alpha 3	W	T	P	D	T	F	F	H	N	G	K	L	K	F	G	S	Y	A	Y	T	K	A	E	V	V	V	G	T	E	I	I	R	S	S	T	G	E	Y	V	V	M	T	T
Alpha 4	W	T	P	D	T	F	F	R	N	G	K	L	K	F	G	S	Y	A	Y	P	K	S	E	M	T	V	S	S	E	T	I	K	S	I	T	G	E	Y	I	V	M	T	V
Alpha 5	W	T	P	D	T	F	F	H	N	G	K	L	K	F	G	S	Y	A	Y	P	N	S	E	V	T	V	G	T	E	N	I	S	T	S	T	G	E	Y	T	I	M	T	A
Alpha 6	W	T	P	D	T	F	F	R	N	G	K	L	K	F	G	S	Y	A	Y	P	K	S	E	I	T	V	S	S	E	T	I	K	S	N	T	G	E	Y	V	I	M	T	V

	Loop E																								Loop F																					
	122																							146	181																							201
Gamma 1	W	I	T	T	P	N	R	L	L	R	I	W	S	D	G	R	V	L	Y	T	L	R	L	T	Y	K	W	K	K	P	S	V	E	V	A	D	P	K	Y	W	R	L	Y	Q	F
Gamma 2	W	I	T	T	P	N	R	M	L	R	I	W	N	D	G	R	V	L	Y	T	L	R	L	T	Y	Q	W	K	R	S	S	V	E	V	G	D	T	R	S	W	R	L	Y	Q	F
Gamma 3	W	I	T	T	P	N	Q	L	L	R	I	W	N	D	G	K	I	L	Y	T	L	R	L	T	Y	R	W	R	K	N	S	V	E	A	A	D	Q	K	S	W	R	L	Y	Q	F

Figure 2

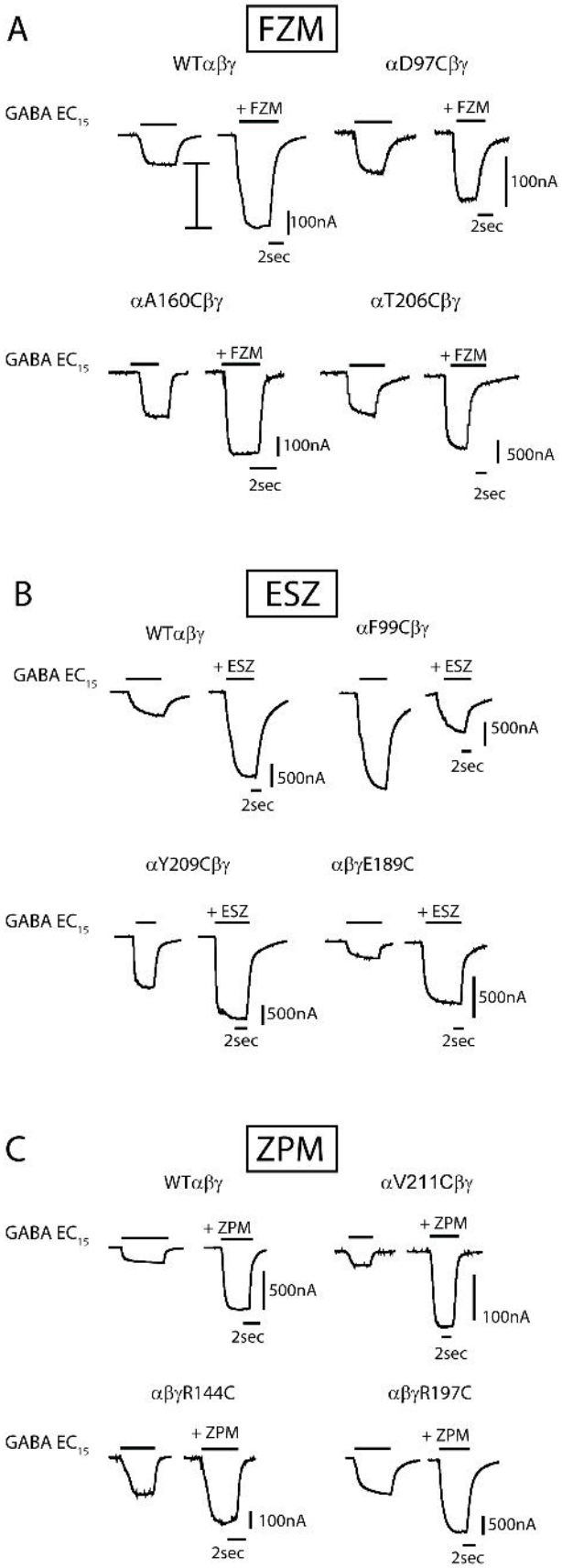

A
FZM
WTαβγ
αD97Cβγ
GABA EC15
+ FZM
100nA
2sec
αA160Cβγ
αT206Cβγ
500nA
B
ESZ
WTαβγ
αF99Cβγ
+ ESZ
αY209Cβγ
αβγE189C
C
ZPM
WTαβγ
αV211Cβγ
+ ZPM
αβγR144C
αβγR197C

Figure 3

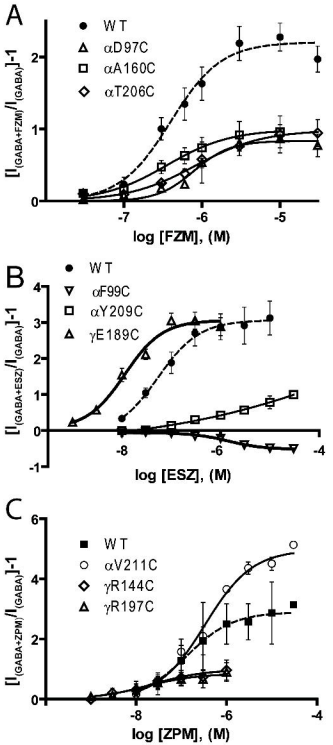

A
WT
αD97C
αA160C
αT206C
[I(GABA+FZM)/I(GABA)]-1
log [FZM], (M)
B
WT
αF99C
αY209C
γE189C
[I(GABA+ESZ)/I(GABA)]-1
log [ESZ], (M)
C
WT
αV211C
γR144C
γR197C
[I(GABA+ZPM)/I(GABA)]-1
log [ZPM], (M)

Figure 4

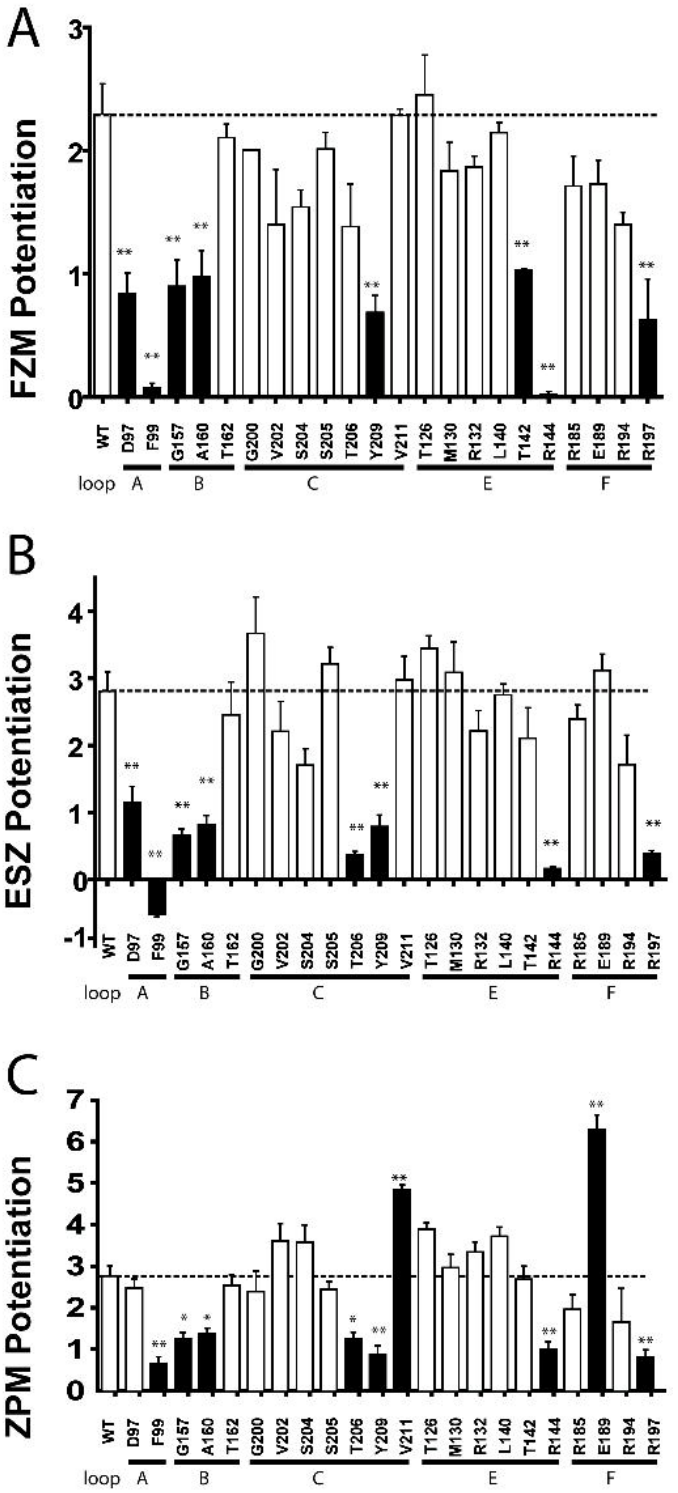

A
FZM Potentiation
B
ESZ Potentiation
C
ZPM Potentiation
WT
D97
F99
G157
A160
T162
G200
V202
S204
S205
T206
Y209
V211
T126
M130
R132
L140
T142
R144
R185
E189
R194
R197
loop
A
B
C
E
F

Figure 5

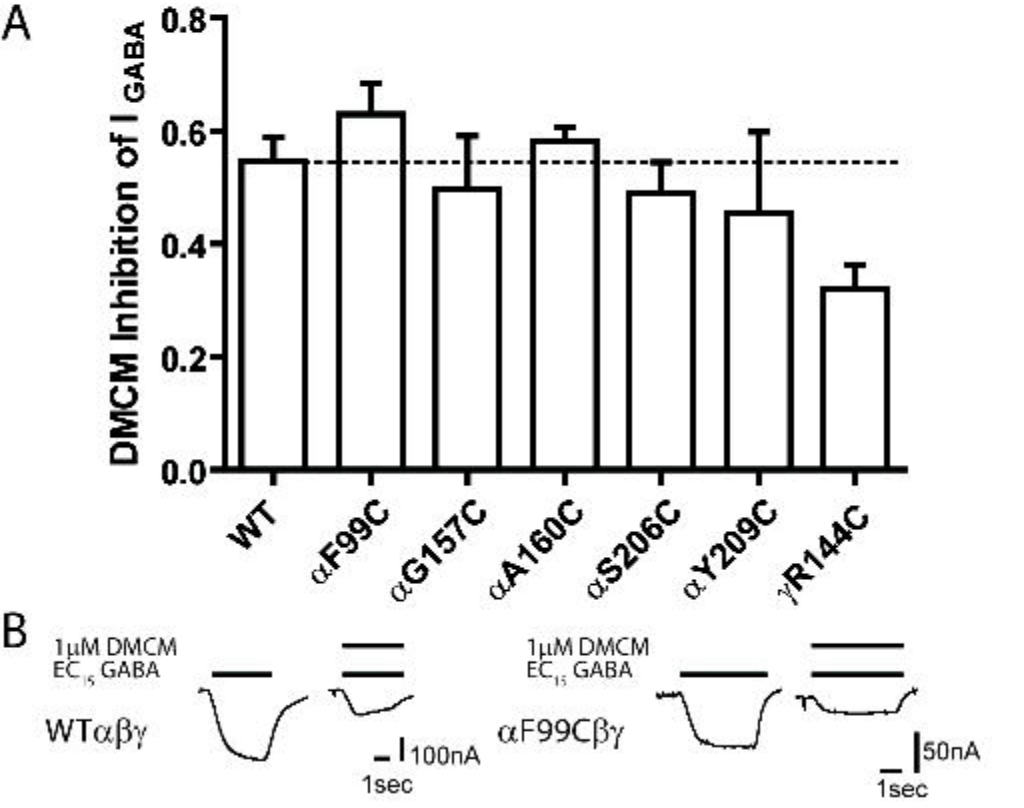

A
DMCM Inhibition of I GABA
0.8
0.6
0.4
0.2
0.0
WT
αF99C
αG157C
αA160C
αS206C
αY209C
γR144C
B
1μM DMCM
EC15 GABA
WTαβγ
100nA
1sec
1μM DMCM
EC15 GABA
αF99Cβγ
50nA
1sec

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Figure 6