

Different Residues in the GABA_A Receptor Benzodiazepine Binding Pocket Mediate Benzodiazepine Efficacy and Binding^S

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ABSTRACT

Benzodiazepines (BZDs) exert their therapeutic actions by binding to the GABA_A receptor (GABA_AR) and allosterically modulating GABA-induced 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. In this study, we measured the effects of individually mutating 22 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 laevis* oocytes and analyzed using a two-electrode voltage clamp. GABA EC₅₀, BZD EC₅₀, and BZD maximal potentiation were measured. These data, combined with previous radioligand

binding data describing the mutations' effects on BZD apparent binding affinities (*J Neurosci* **28**:3490–3499, 2008; *J Med Chem* **51**:7243–7252, 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.

Introduction

Benzodiazepines (BZDs) are commonly used in the treatment of sleep disorders, anxiety, muscle spasms, seizure disorders, and some forms of depression (Möhler et al., 2002). They exert their therapeutic actions by binding to the GABA_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 5HT₃ receptor, glycine receptor, and nicotinic acetylcholine receptor (Ortells and Lunt, 1995). The most common GABA_A receptor subtype found in the brain comprises α_1 , β_2 , and γ_2 subunits in a ratio of 2 α :2 β : γ (Chang et al., 1996; Farrar et al., 1999; Baumann

et al., 2002; 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 it is formed by six noncontiguous regions historically designated loops A to F (Fig. 1B) (Sigel and Buhr, 1997; Boileau et al., 1998; Boileau et al., 2002).

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 effect on I_{GABA} (BZD antagonists). Although multiple studies have identified residues that are involved in mediating the apparent binding affinity (K_d) of BZD-site ligands, including typical [1,4]benzodiazepines (Wieland and Lüddens, 1994; Kucken et al., 2000; Boileau et al., 2002; Derry et al., 2004), cyclopyrrolones [e.g., eszopiclone (ESZ)] (Davies et al., 2000; Hanson and Czajkowski, 2008), and imidazopyridines [e.g., zolpidem (ZPM)] (Buhr et al., 1996, 1997; Buhr and Sigel, 1997; Schaerer et al., 1998; Hanson et al., 2008), much less is known about the 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).

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ABBREVIATIONS: BZD, benzodiazepine; GABA_AR, GABA_A receptor; ESZ, eszopiclone; Ro 15-1788, flumazenil; FZM, flurazepam; ZPM, zolpidem; DMCM, 3-carbomethoxy-4-ethyl-6,7-dimethoxy- β -carboline; I_{GABA} , GABA-induced chloride current; WT, wild type; ANOVA, analysis of variance; Ro 15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate.

In general, it is believed that BZDs exert their allosteric effects by either shifting the GABA_AR closed to open-state channel equilibrium (Downing et al., 2005; Rüschen and Forman, 2005; Campo-Soria et al., 2006) or altering the receptor's microscopic binding affinity for GABA (Twyman et al., 1989; Rogers et al., 1994; Lavoie and Twyman, 1996; Mellor and Randall, 1997; Thompson et al., 1999; Goldschien-Ohm et al., 2010). 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 probably have different roles in this process. Some residues might directly interact with the ligand and contribute to its binding affinity, and some might stabilize binding site structure, whereas others might 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.

In a previous study, we identified residues within the BZD binding site that were important for high-affinity binding of

flumazenil (Ro 15-1788), ESZ, and zolpidem (ZPM) (Hanson et al., 2008; Hanson and Czajkowski, 2008). In this study, we tested the hypothesis that residues in the BZD binding site are also crucial for determining BZD efficacy. We measured the effects of 22 single cysteine mutations (Fig. 1D), which were made throughout the BZD binding site, on the abilities of flurazepam (FZM), ESZ, and ZPM to potentiate I_{GABA} (BZD EC₅₀ values and maximal potentiation 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, which suggests 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, which suggests that they play a role in mediating high-affinity BZD binding and the initial structural rearrangements in the sites 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.

Materials and Methods

Site-Directed Mutagenesis. Rat cDNAs encoding the GABA_AR 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 et al., 2008; Hanson and Czajkowski, 2008) using recombinant polymerase chain reaction and verified by double-stranded DNA sequencing.

Expression in *Xenopus laevis* Oocytes. Expression of wild-type (WT) and mutant GABA_AR_s was performed as described previously (Hanson and Czajkowski, 2008). Capped cRNA from NotI-digested cDNA was in vitro transcribed using the mMessage mMachine T7 kit (Ambion, Austin, TX). *Xenopus laevis* oocytes were harvested and prepared as described previously (Boileau et al., 1998). Oocytes were injected within 24 h of treatment with 27 nl of cRNA (1–15 pg/nl per subunit) in the ratio 1:1:10 ($\alpha/\beta/\gamma$) (Boileau et al., 2002) and stored at 16°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.2) supplemented with 100 μ g/ml bovine serum albumin until used for electrophysiological recordings.

Two-Electrode Voltage Clamp. Electrophysiological recordings were performed as described previously (Hanson and Czajkowski, 2008). Oocytes were held at –80 mV under a two-electrode voltage clamp while being continuously perfused with ND96 at a rate of 5 ml/min in a bath volume of 200 μ l. Borosilicate glass electrodes (0.4–1.0 m Ω) (Warner Instruments, Hamden, CT) were filled with 3 M KCl. Electrophysiological data were collected using GeneClamp 500 (Molecular Devices, Sunnyvale, CA) interfaced to a computer with a Digidata 1200 device (Molecular Devices). Recordings were made using the Whole Cell Program, version 3.6.7 (kindly provided by J. Dempster, University of Strathclyde, Glasgow, UK). Stock solutions of FZM (Sigma/RBI, Natick, MA) were dissolved in ND96 and diluted in ND96 for working concentrations. GABA (Sigma-Aldrich, St. Louis, MO) solutions were prepared fresh daily with ND96. Stock solutions of 3-carbomethoxy-4-ethyl-6,7-dimethoxy- β -carboline (DMCM; Sigma/RBI), ZPM (Sigma-Aldrich), and ESZ (kindly provided by Sepracor, Inc., Marlborough, MA) were prepared in dimethyl sulfoxide and subsequently diluted in ND96 for working

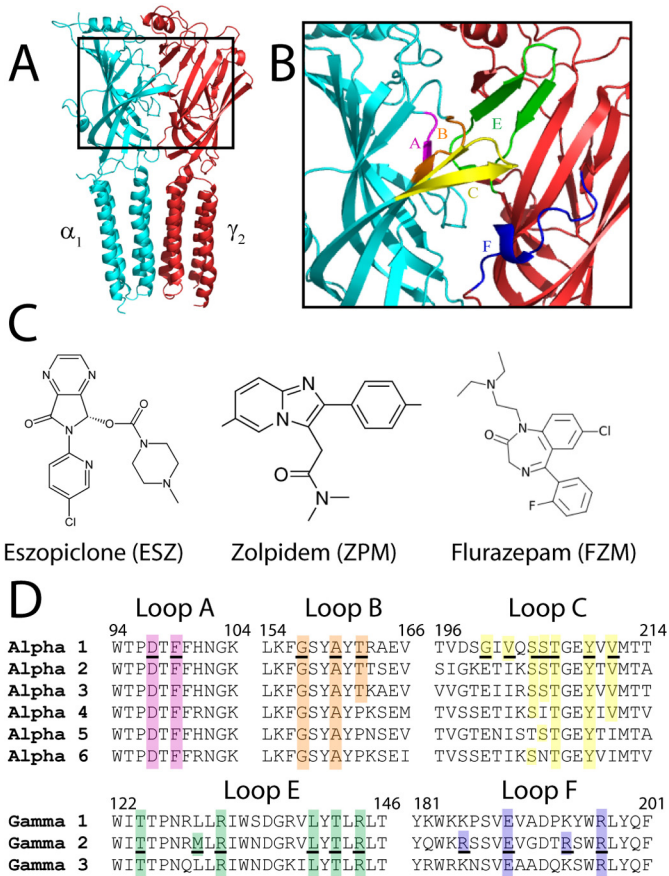


Fig. 1. The BZD binding site at the α_1/γ_2 interface of the GABA_AR and structures of BZD site ligands. A, homology model of the α_1/γ_2 interface perpendicular to the plane of the membrane. The α_1 subunit is in blue and the γ_2 subunit is in red. B, the region of the α_1/γ_2 interface that contains the BZD binding site is expanded, and BZD binding-site loop regions A to 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.

concentrations in which the final concentration of dimethyl sulfoxide ($\leq 2\%$) did not affect GABA_AR function.

Concentration-Response Analysis. GABA concentration-response curves were determined as described previously (Hanson and Czajkowski, 2008). Six to 12 concentrations of GABA were used to determine each GABA EC₅₀ value. Each current response was scaled to a low, nondesensitizing concentration of GABA 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: $I = I_{\max}/[1 + (EC_{50}/[A])^{n_H}]$, in which I is the peak response to a given drug concentration, I_{\max} is the maximal amplitude of current, EC₅₀ is the drug concentration that produces the half-maximal response, $[A]$ is drug concentration, and n_H is the Hill coefficient using Prism version 4.0 (GraphPad Software Inc., San Diego, CA). The EC₅₀ values in Table 1 for four mutants (γ R185C, γ E189C, γ R194C, and γ R197C) are from Hanson and Czajkowski (2008) with the associated errors in S.E.M., rather than the S.D. reported in the 2008 publication. Two values that were significantly different from WT values in Hanson and Czajkowski (2008) (γ R185C and γ R194C) are no longer significant in the present study because the GABA EC₅₀ value for WT receptors is slightly lower than in the previous report, and the full data set that was used for the analysis of variances (ANOVAs) in both studies are different.

BZD concentration responses (six to eight different concentrations) were measured at GABA EC₁₅. BZD modulation was defined as follows: $[(I_{\text{GABA+BZD}}/I_{\text{GABA}}) - 1]$, in which $I_{\text{GABA+BZD}}$ is the current response in the presence of GABA and BZD, and I_{GABA} is the current evoked by GABA alone (GABA EC₁₅). When measuring BZD concentration responses, each application of GABA + BZD is preceded by a brief pulse of GABA EC₁₅ 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 EC₁₅ pulses alone did not change ($< 3\%$), even for high concentrations of BZD, which indicates complete washout of the BZD. BZD concentration-response curves were fit with the equation $P = P_{\max}/(1 + (EC_{50}/[A])^{n_H})$, in which $[A]$ is the BZD concentration, EC₅₀

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_H is the Hill coefficient. The reported values for maximal 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 \pm S.E.M. Significant differences in EC₅₀ values and maximal BZD modulation values were determined by one-way ANOVA, followed by a post hoc Dunnett's test using Prism (version 4.0; GraphPad Software, San Diego, CA). Log (EC₅₀) values were used for statistical analyses.

Results

We previously made 22 single cysteine mutations throughout the BZD binding site in loops A (D97C and F99C), B (G157C, A160C, and T162C), and C (G200C, V202C, S204C, S205C, T206C, Y209C, and V211C) of the α_1 subunit and loops E (T126C, M130C, R132C, L140C, T142C, and R144C) and F (R185C, E189C, R194C, and R197C) of the γ_2 subunit (Fig. 1). We examined the effects of these mutations on BZD binding using competitive radioligand binding experiments (see Table 2 for Mut/WT K_i values) (Hanson et al., 2008; Hanson and Czajkowski, 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 (Ro 15-1788, 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, several 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

TABLE 1

Summary of GABA dose-response data for WT and mutant $\alpha_1\beta_2\gamma_2$ GABA_ARs

Data are mean \pm S.E.M. 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. The loop where each mutation is located is indicated. In Hanson and Czajkowski (2008), GABA EC₅₀ values for γ R185C and γ R194C were decreased 2.5-fold compared to WT and were statistically different. Here, these values are no longer significant because of a slight decrease in the WT EC₅₀ value reported here and because of differences in the data sets analyzed by ANOVA.

Loop	Receptor	EC ₅₀	n_H	n	I _{max} range
		μM			μA
	WT $\alpha\beta\gamma$	18.4 \pm 4.4	1.50 \pm 0.09	5	8.4–11.6
A	α D97C $\beta\gamma$	485 \pm 67**	0.66 \pm 0.10**	5	2.3–4.0
A	α F99C $\beta\gamma$	391 \pm 94*	0.75 \pm 0.06**	7	2.7–3.1
B	α G157C $\beta\gamma$	408 \pm 93*	0.65 \pm 0.03**	8	1.4–4.1
B	α A160C $\beta\gamma$	560 \pm 138**	0.60 \pm 0.04**	6	3.3–5.0
B	α T162C $\beta\gamma$	39.8 \pm 13.2	0.89 \pm 0.12**	4	7.6–9.3
C	α G200C $\beta\gamma$	33.0 \pm 11.0	1.03 \pm 0.02**	3	4.4–8.0
C	α V202C $\beta\gamma$	40.0 \pm 22.5	0.99 \pm 0.06**	3	6.0–7.2
C	α S204C $\beta\gamma$	22.0 \pm 4.7	1.04 \pm 0.03**	3	2.7–8.8
C	α S205C $\beta\gamma$	230 \pm 126*	1.08 \pm 0.20*	3	2.6–7.7
C	α T206C $\beta\gamma$	268 \pm 58.0*	0.59 \pm 0.05**	3	1.9–2.8
C	α Y209C $\beta\gamma$	319 \pm 170*	0.68 \pm 0.13**	3	3.5–8.8
C	α V211C $\beta\gamma$	80.3 \pm 27.5	1.11 \pm 0.19*	3	3.2–11.4
E	$\alpha\beta\gamma$ T126C	49.1 \pm 7.8**	1.13 \pm 0.26	4	4.3–8.2
E	$\alpha\beta\gamma$ M130C	45.9 \pm 7.5*	1.43 \pm 0.02	3	2.6–3.5
E	$\alpha\beta\gamma$ R132C	15.4 \pm 3.1	1.64 \pm 0.04	3	5.8–9.2
E	$\alpha\beta\gamma$ L140C	22.2 \pm 7.1	1.28 \pm 0.21	3	4.4–11.0
E	$\alpha\beta\gamma$ T142C	11.9 \pm 0.2	1.61 \pm 0.12	4	10.6–13.7
E	$\alpha\beta\gamma$ R144C	3.0 \pm 0.7*	1.55 \pm 0.13	3	7.6–16.4
F	$\alpha\beta\gamma$ R185C ^a	10.7 \pm 2.0	1.52 \pm 0.16	3	11.2–16.0
F	$\alpha\beta\gamma$ E189C ^a	16.6 \pm 3.1	1.50 \pm 0.03	4	5.2–11.2
F	$\alpha\beta\gamma$ R194C ^a	11.0 \pm 2.4	1.41 \pm 0.10	3	12.4–17.2
F	$\alpha\beta\gamma$ R197C ^a	17.4 \pm 2.3	1.17 \pm 0.04	4	9.7–12.0

^a Values are from Hanson and Czajkowski (2008) with errors in S.E.M. not S.D. as previously reported.

* $p < 0.05$, significantly different from wild-type $\alpha_1\beta_2\gamma_2$.

** $p < 0.01$, significantly different from wild-type $\alpha_1\beta_2\gamma_2$.

binding site are not only important for BZD binding but also play a role in defining BZD efficacy. Cysteine mutant subunits were coexpressed with WT subunits in *X. laevis* oocytes to form $\alpha_1\beta_2\gamma_2$ GABA_ARs and analyzed using two-electrode voltage clamp. We examined the effects the mutations had on I_{GABA} and on FZM, ESZ, and ZPM modulation of $EC_{15} I_{GABA}$.

Effects of Cysteine Substitutions on I_{GABA} . All of the mutant subunits assembled into functional GABA_ARs (Table 1). Seven of the 12 cysteine substitutions in the α_1 subunit significantly increased GABA EC_{50} values (13–31-fold) compared with WT receptors ($18.4 \pm 4.4 \mu\text{M}$) (Table 1). In general, the mutations in the γ_2 subunit had smaller effects. γ T126C and γ M130C increased GABA EC_{50} approximately 3-fold, whereas γ R144C decreased GABA EC_{50} 6-fold compared with 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 Figs. 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 monitored. Eight of the 22 mutations significantly decreased FZM maximal potentiation of I_{GABA} compared with WT receptors (potentiation = 2.3 ± 0.2) (Fig. 4; Table 2). In the α_1 subunit, cysteine substitution of Asp97 and Phe99 in loop A, Gly157 and Ala160 in loop B, and Tyr209 in loop C significantly decreased FZM maximal potentiation. In the γ_2 subunit, cysteine substitution of Thr142

and Arg144 in loop E and Arg197 in loop F also significantly decreased FZM maximal potentiation. Note that α F99C and γ R144C almost completely eliminated FZM potentiation of I_{GABA} , and thus FZM EC_{50} values could not be determined.

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 maximal potentiation of I_{GABA} compared with WT receptors (potentiation = 2.8 ± 0.3) (Figs. 2–4; 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 α F99C-containing receptors (Figs. 2B and 3B). As reported in Hanson et al. (2008), the specific binding of [³H]Ro 15-1788, [³H]flunitrazepam, or [³H]ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (Ro 15-4513) to α D97C- and Y209C-mutant receptors was not detectable using a filtration-based radioligand binding assay (Table 2). The inability to detect radioligand binding is probably due to inherent limitations of filtration binding assays, which preclude measuring binding when the affinity of the radioligand is higher than 100 nM. Given that we can measure BZD modulation of I_{GABA} for these mutant receptors, these drugs bind to the mutant receptors, probably with lower apparent affinity. The rightward shifts in the BZD concentration responses are consistent with this idea. In the γ_2 subunit, mutations at Arg144 in loop E and Arg197 in loop F significantly reduced ESZ maximal potentiation. Although α A160C 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).

TABLE 2

Summary of BZD concentration response data and binding data for WT and mutant $\alpha_1\beta_2\gamma_2$ GABA_ARs

Data are mean \pm S.E.M. for *n* experiments. The loop where each mutation is located is indicated. 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. Values significantly different from wild type $\alpha_1\beta_2\gamma_2$ are indicated.

Loop	Receptor	Flurazepam				Eszopiclone				Zolpidem			
		Max Potentiation	EC_{50}	<i>n</i>	Mut/WT K_i^a	Max Potentiation	EC_{50}	<i>n</i>	Mut/WT K_i^b	Max Potentiation	EC_{50}	<i>n</i>	Mut/WT K_i^b
		<i>nM</i>				<i>nM</i>				<i>nM</i>			
	WT $\alpha\beta\gamma$	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
A	α D97C $\beta\gamma$	$0.8 \pm 0.2^{**}$	941 ± 140	3		$1.1 \pm 0.2^{**}$	166 ± 49	3	N.D.	2.5 ± 0.2	331 ± 18	3	N.D.
A	α F99C $\beta\gamma$	$0.1 \pm 0.0^{**}$		3		$-0.5 \pm 0.0^{**}$	$1400 \pm 290^{**}$	3	8.1^{**}	$0.7 \pm 0.2^{**}$	$3670 \pm 1900^{**}$	3	2.9^*
B	α G157C $\beta\gamma$	$0.9 \pm 0.2^{**}$	1620 ± 760	4		$0.7 \pm 0.1^{**}$	$1230 \pm 140^{**}$	3	42^{**}	$1.3 \pm 0.1^*$	$2820 \pm 610^{**}$	3	20^{**}
B	α A160C $\beta\gamma$	$1.0 \pm 0.2^{**}$	368 ± 14	3		$0.8 \pm 0.1^{**}$	140 ± 47	3	1.2	$1.4 \pm 0.1^*$	118 ± 13	3	1.3
B	α T162C $\beta\gamma$	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	α G200C $\beta\gamma$	2.0 ± 0.0	454 ± 83	3		3.7 ± 0.5	230 ± 8.1	4	2.4^{**}	2.4 ± 0.5	$996 \pm 481^{**}$	3	9.7^{**}
C	α V202C $\beta\gamma$	$1.4 \pm 0.4^*$	922 ± 165	3		2.2 ± 0.4	$522 \pm 130^{**}$	3	5.0^{**}	3.2 ± 0.3	$2490 \pm 250^{**}$	3	9.0^{**}
C	α S204C $\beta\gamma$	1.5 ± 0.1	305 ± 43	3		1.7 ± 0.2	139 ± 39	3	1.2	3.6 ± 0.4	$2100 \pm 280^{**}$	3	7.0^{**}
C	α S205C $\beta\gamma$	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
C	α T206C $\beta\gamma$	1.4 ± 0.3	946 ± 188	3		$0.4 \pm 0.0^{**}$	50.9 ± 19.1	3	0.02^{**}	$1.2 \pm 0.2^*$	245 ± 42	3	1.2
C	α Y209C $\beta\gamma$	$0.7 \pm 0.1^{**}$	$2770 \pm 649^{**}$	3		$0.8 \pm 0.2^*$	$766 \pm 250^{**}$	3	N.D.	$0.9 \pm 0.2^{**}$	$14,300 \pm 2300^{**}$	3	N.D.
C	α V211C $\beta\gamma$	2.3 ± 0.1	511 ± 93	3		3.0 ± 0.4	115 ± 21	3	1.2	$4.9 \pm 0.1^{**}$	356 ± 117	3	1.1
E	$\alpha\beta\gamma$ T126C	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
E	$\alpha\beta\gamma$ M130C	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^{**}
E	$\alpha\beta\gamma$ R132C	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^*
E	$\alpha\beta\gamma$ L140C	2.1 ± 0.1	330 ± 65	3		2.8 ± 0.2	$12.6 \pm 1.4^*$	4	0.9	3.7 ± 0.2	12.1 ± 2.7	3	1.0
E	$\alpha\beta\gamma$ T142C	$1.0 \pm 0.0^{**}$	561 ± 58	3		2.1 ± 0.5	$148 \pm 39^{**}$	3	10^*	2.7 ± 0.3	$980 \pm 260^{**}$	3	21^{**}
E	$\alpha\beta\gamma$ R144C	$0.0 \pm 0.0^{**}$		3		$0.2 \pm 0.0^{**}$		3	3.5^{**}	$1.0 \pm 0.2^{**}$	22.5 ± 9.4	3	0.6
F	$\alpha\beta\gamma$ R185C	1.7 ± 0.2	317 ± 82	3		2.4 ± 0.2	$10.4 \pm 1.5^*$	3	1.4	2.0 ± 0.4	10.0 ± 2.2	3	1.1
F	$\alpha\beta\gamma$ E189C	1.7 ± 0.2	645 ± 78	3	1.3	3.1 ± 0.2	$12.2 \pm 3.0^*$	4		$6.3 \pm 0.4^{**}$	$1838 \pm 636^{**}$	5	1.8^a
F	$\alpha\beta\gamma$ R194C	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
F	$\alpha\beta\gamma$ R197C	$0.6 \pm 0.3^{**}$	494 ± 48	3	0.4^*	$0.4 \pm 0.0^{**}$	$6.0 \pm 2.1^*$	3		$0.8 \pm 0.2^{**}$	13.8 ± 2.3	4	0.4^a

Mut, mutation; N.D., not detectable.

^a Values from Hanson and Czajkowski (2008).

^b Values from Hanson et al. (2008).

* $p < 0.05$ significantly different from wild-type $\alpha_1\beta_2\gamma_2$.

** $p < 0.01$ significantly different from wild-type $\alpha_1\beta_2\gamma_2$.

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 of the 22 mutations altered the ZPM maximal potentiation of I_{GABA} (Figs. 2–4; Table 2). α F99C in loop A, α G157C and α A160C in loop B, α T206C and α Y209C in loop C, γ R144C in loop E, and

γ R197C in loop F significantly decreased ZPM potentiation compared with WT receptors (potentiation = 2.8 ± 0.3). It is interesting to note that α V211C (loop C) and γ E189C (loop F) significantly increased ZPM potentiation of I_{GABA} (1.8- and 2.3-fold, respectively; Figs. 3C and 4C). In a previous study, we reported that γ E189C had no effect on ZPM potentiation (Hanson et al., 2008; Hanson and Czajkowski, 2008). The differences in results are probably due to the higher concentrations of ZPM used in this study. Although α A160C, α T206C, α V211C, γ R144C, γ E189C, and γ R197C significantly altered ZPM potentiation of I_{GABA} (efficacy), the mutations had little to no effect on ZPM apparent binding affinity (K_i ; Table 2).

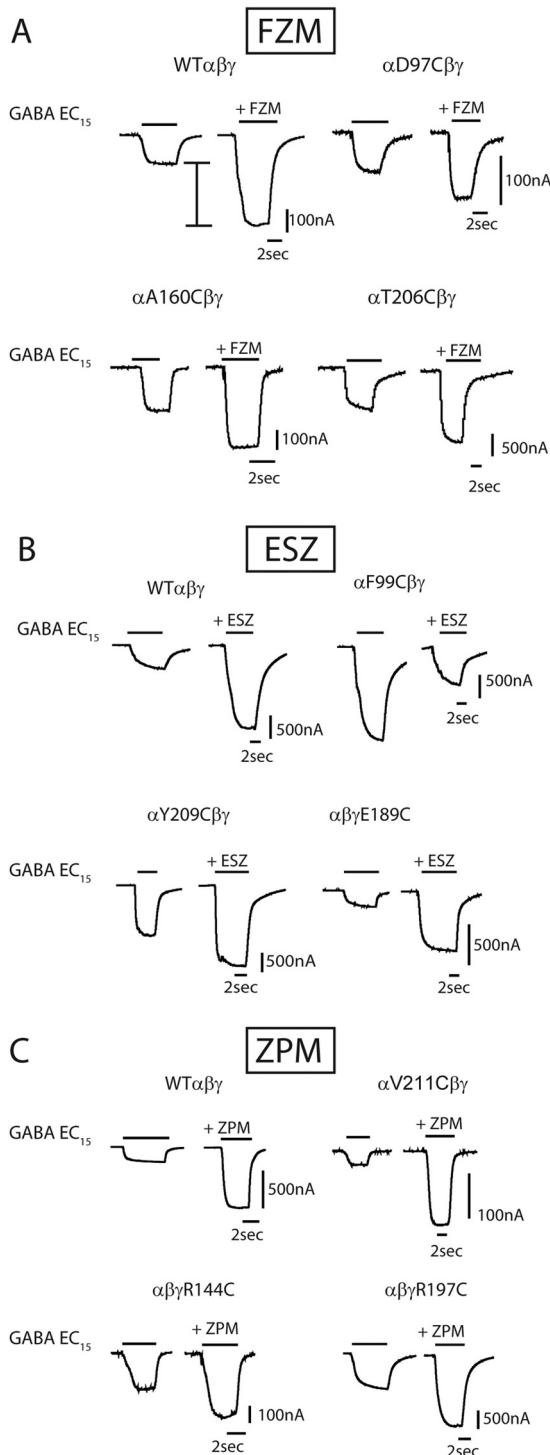


Fig. 2. Effects of the mutations on BZD maximal potentiation. Representative current traces showing maximal potentiation of GABA EC_{15} current from oocytes expressing WT and mutant receptors by FZM (A), ESZ (B), or ZPM (C). In all cases, BZDs were at concentrations that elicited maximal responses. I bar in A indicates potentiation of I_{GABA} . Note: in B, for α F99C $\beta\gamma$ receptors, ESZ inhibited I_{GABA} .

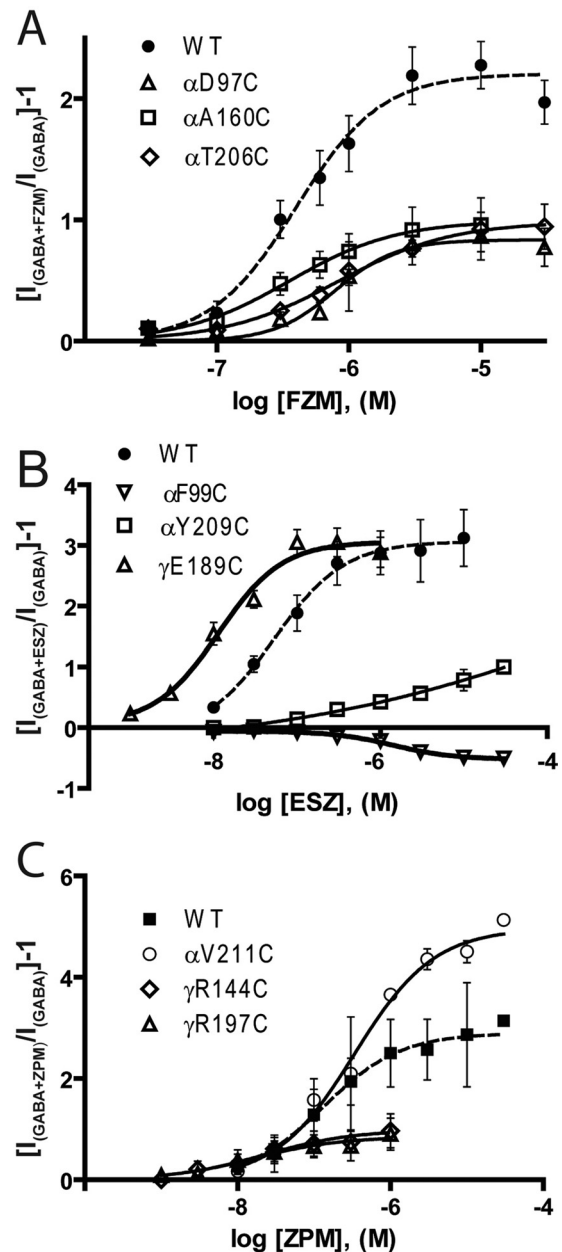


Fig. 3. BZD concentration-response curves from WT and mutant GABA $_A$ Rs for FZM (A), ESZ (B), and ZPM (C). BZD potentiation was calculated as $[(I_{GABA+BZD}/I_{GABA}) - 1]$. Data represent mean \pm S.E.M. Data were fit by nonlinear regression, as described under *Materials and Methods*. Dashed lines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

Effects of Cysteine Substitutions on DMCM Modulation of I_{GABA} . For a subset of mutations (α F99C, α G157C, α A160C, α T206C, α Y209C, and γ R144C), we also examined the ability of DMCM 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 inhibition = 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, γ R144C, was decreased compared with WT, but this did not reach significance. Because only γ -containing GABA_ARs are modulated by DMCM, the near WT inhibition of I_{GABA} by DMCM also indicates that the mu-

tations do not impair subunit assembly or incorporation into functional $\alpha\beta\gamma$ GABA_ARs.

Changes in BZD Modulation of I_{GABA} Are Not Correlated to Changes in GABA EC_{50} . Some mutations caused significant changes in GABA EC_{50} , raising the possibility that the changes observed in BZD potentiation 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 were 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 (Supplemental Fig. 1). Some mutations significantly altered BZD potentiation without affecting GABA EC_{50} (γ Thr142, γ Glu189, and γ Arg197.), whereas others affected GABA EC_{50} without changing BZD potentiation (γ T126C, γ M130C, α S205C, and α V211C). In addition, although the α F99C, α AG157C, α A160C, α T06C, α Y209C, and γ R144C 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).

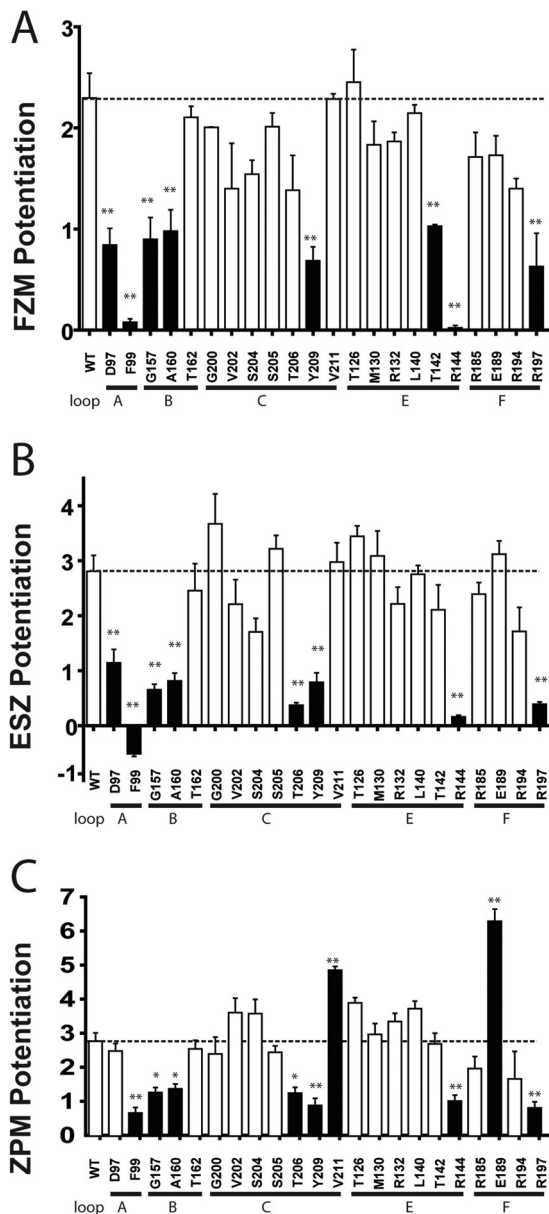


Fig. 4. Mutations throughout the BZD binding site affect BZD efficacy. Maximal potentiation of GABA EC_{15} current from WT and mutant receptors by FZM (A), ESZ (B), or ZPM (C) is plotted. BZD potentiation was calculated as $[(I_{GABA+BZD}/I_{GABA}) - 1]$. Data are mean \pm S.E.M. from at least three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

Discussion

We identified four residues in the BZD binding pocket that specifically contribute to BZD-site agonist efficacy: loop B, Ala160; loop C, Thr206; loop E, Arg144; and loop F, Arg197 (Fig. 6, top row). Our data suggest that mutation of these residues significantly disrupted the abilities of ZPM, ESZ,

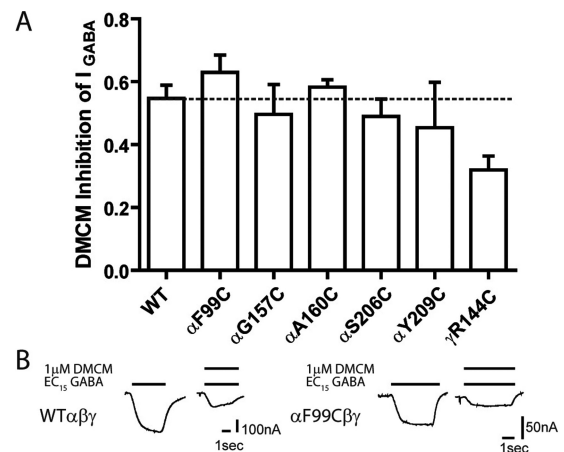


Fig. 5. DMCM modulation of WT and mutant GABA_A receptors. A, inhibition of EC_{15} GABA by 1μ M DMCM for WT and mutant receptors is plotted. Inhibition of GABA current was calculated as $[(I_{GABA+DMCM}/I_{GABA}) - 1]$. Data are mean \pm S.E.M. from at least three oocytes from two or more batches. The 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 $\alpha\beta\gamma$ and α F99C $\beta\gamma$ receptors in response to EC_{15} GABA and EC_{15} GABA + 1μ M DMCM.

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 (α Val211 and

γ Glu189) 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 on the type of BZD-site ligand bound. This result is consistent with our previous data, where we demonstrated that structural determinants for high-affinity binding of ESZ and ZPM are different (Hanson et al., 2008; Hanson and

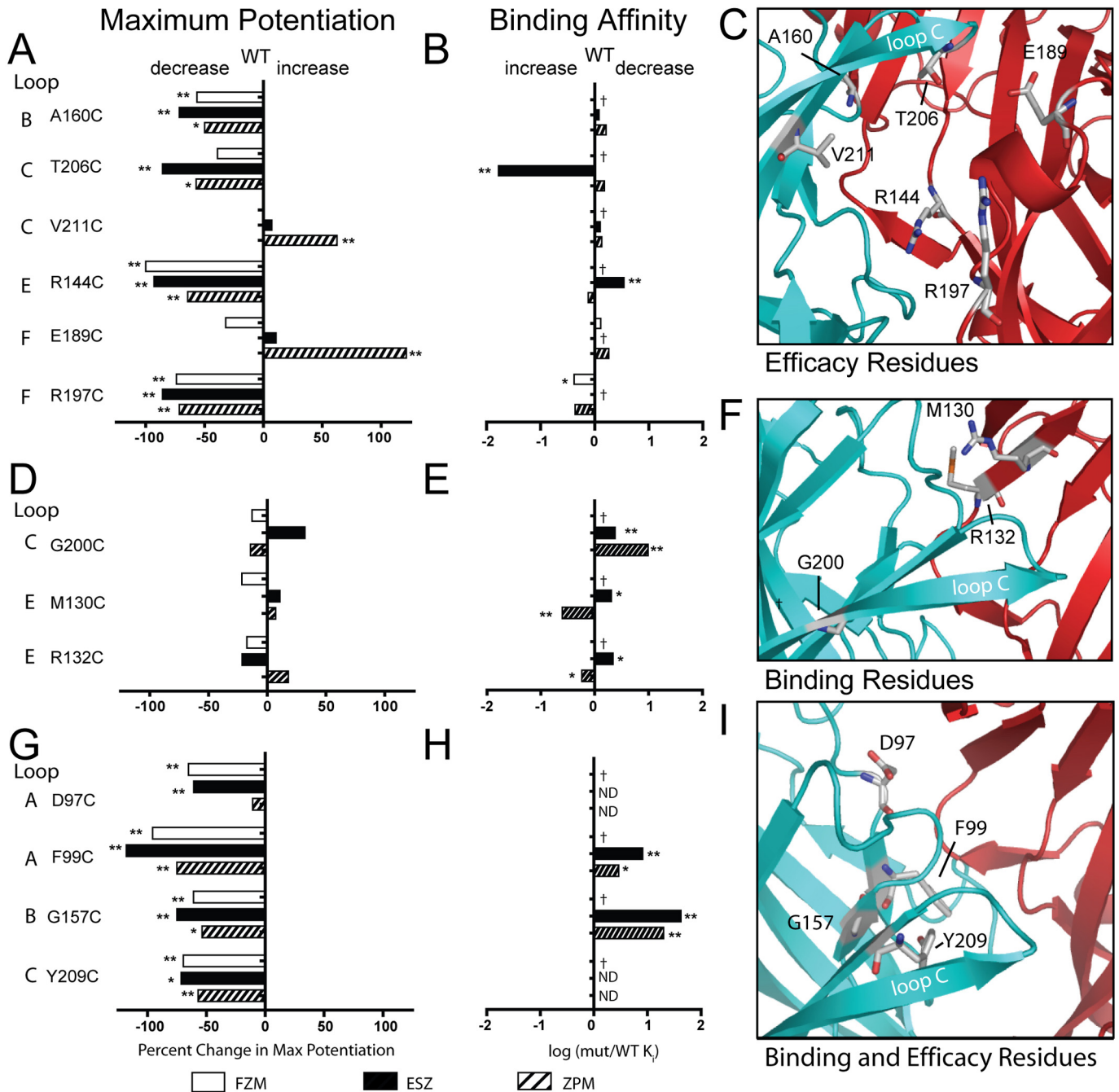


Fig. 6. Summary of data highlighting residues important for BZD efficacy (A–C), BZD binding (D–F), and BZD binding and efficacy (G–I). A, D, and G plot the percentage change in maximal potentiation for FZM, ESZ, and ZPM [(mutant max potentiation – WT max potentiation)/WT max potentiation](100), respectively. Negative values represent a decrease in potentiation, whereas positive values indicate an increase. B, E, and H plot changes in binding affinity [$\log(\text{mut } K_i/\text{WT } K_i)$]. K_i values for FZM, ESZ, and ZPM are from Hanson et al. (2008) and Hanson and Czajkowski (2008) and were determined by displacement of [^3H]Ro 15-1788 binding. Negative values indicate increased affinity, and positive values indicate decreased affinity. 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 and γ subunit is red. Loop C is labeled. Values statistically different from WT are indicated (*, $p < 0.05$; **, $p < 0.01$). ND, binding of [^3H]Ro 15-1788 was not detectable, thus K_i values for FZM, ESZ, and ZPM were not determined. †, no binding data available; Max, maximal; mut, mutant.

Czajkowski, 2008). We can envision that, depending on the orientation of the BZD in the binding pocket and its contact residues, some of the residues involved in the initial coupling of binding to potentiation of I_{GABA} might 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). Mutation of γ Glu189 or γ Val211 might cause ZPM to preferentially adopt a position that has a greater efficacy.

We also identified three residues (α Gly200, γ Met130, and γ Arg132) that specifically mediate high-affinity BZD agonist binding. Mutation of 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, α Gly200, γ Met130, and γ Arg132 are located on β strands (Fig. 6F) that line the core of the BZD binding pocket. Previous mutagenesis studies have demonstrated the importance of α Gly200 and γ Met130 in BZD binding. The glycine at position 200 is found only in the GABA_AR α_1 -subunit isoform, α_{2-6} subunits have a glutamate at aligned positions (Fig. 1D). Schaerer et al. (1998) showed that replacing α_1 Gly200 with glutamate decreases ZPM binding affinity. Mutation of α_6 Glu200 to its α_1 counterpart in a background of three other point mutations confers ZPM binding to the BZD-insensitive α_6 subunit (Wieland and Lüddens, 1994). Mutation of γ_2 Met130 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 typical BZDs (Wingrove et al., 1997).

In this study, we also identified residues that are important for both high-affinity BZD agonist binding and BZD efficacy: α Asp97 and α Phe99 in loop A, α Gly157 in loop B, and α Tyr209 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 α D97C and α Y209C-containing receptors was so disrupted that 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 (Asp97 and Phe99), the side of the pocket in loop B (Gly157), and at the base of loop C facing directly into the binding site (Tyr209) (Fig. 6I). α Asp97 and α Phe99 in loop A are located near α His101, which has been previously shown to be important for binding of ZPM (Wieland et al., 1992; Wieland and Lüddens, 1994), zopiclone (the racemate of ESZ) (Davies et al., 1998), flunitrazepam (Bereznoy et al., 2004), and diazepam (Davies et al., 2000; Bereznoy et al., 2004). Mutation of α His101 to arginine has also been shown to alter BZD efficacy (Benson et al., 1998). Previous studies have also identified α Gly157 in loop B and α Tyr209 in loop C as important determinants for BZD binding (Amin et al., 1997; Tan et al., 2007b). 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 because 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 might 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; Sancar and Czajkowski, 2011).

It is interesting to note that mutating α Phe99 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 BZD action from enhancement to inhibition of I_{GABA} . The γ T142S mutation and mutations of α His101 cause the inverse agonist Ro 15-4513 and the antagonist flumazenil to become BZD agonists and potentiate I_{GABA} (Mihic et al., 1994; Benson et al., 1998). How these mutations result in switches in BZD actions is not clear. Many structurally diverse ligands bind to the BZD binding site, which indicates that the site can accommodate a variety of ligands. We speculate that the mutations might alter the positioning of the drug in the site and/or positioning of nearby residues, which then induces different downstream allosteric rearrangements.

In previous studies, 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; Kloda and Czajkowski, 2007; Hanson and Czajkowski, 2008). Here, none of the mutations we tested significantly altered the inhibitory abilities of DMCM (Fig. 5), which demonstrates that, even at the level of the BZD binding site, the structural mechanisms underlying the coupling of DMCM binding to inhibition of I_{GABA} are different from those underlying BZD agonist modulation.

The 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 probably play different roles in mediating these actions. In this study, 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 they are also different for structurally diverse BZD-positive modulators, which indicates 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 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 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., 2004, 2005). 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 Contributions

Participated in research design: Morlock and Czajkowski.

Conducted experiments: Morlock.

Performed data analysis: Morlock and Czajkowski.

Wrote or contributed to the writing of the manuscript: Morlock and Czajkowski.

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