Structural Requirements for Eszopiclone and Zolpidem Binding to the γ-Aminobutyric Acid Type-A ($GABA_A$) Receptor Are Different

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The sleep-aids zolpidem and eszopiclone exert their effects by binding to and modulating γ-aminobutyric acid type-A receptors ($GABA_A$Rs), but little is known about the structural requirements for their actions. We made 24 cysteine mutations in the benzodiazepine (BZD) binding site of $\alpha_1\beta_2\gamma_2$ $GABA_A$Rs and measured zolpidem, eszopiclone, and BZD-site antagonist binding. Mutations in $\gamma_2$loop D and $\alpha_1$loops A and B altered the affinity of all ligands tested, indicating that these loops are important for BZD pocket structural integrity. In contrast, $\gamma_2$loop E and $\alpha_1$loop C mutations differentially affected ligand affinity, suggesting that these loops are important for ligand selectivity. In agreement with our mutagenesis data, eszopiclone docking yielded a single model stabilized by several hydrogen bonds. Zolpidem docking yielded three equally populated orientations with few polar interactions, suggesting that unlike eszopiclone, zolpidem relies more on shape recognition of the binding pocket than on specific residue interactions and may explain why zolpidem is highly α_1- and γ_2-subunit selective.

Introduction

Insomnia is associated with increased morbidity and mortality resulting from accidents, cardiovascular disease, and psychiatric disorders.[1] Approximately 10% of the population suffers from insomnia,[2] with an estimated 2.5% using medications to aid sleep each year.[1] Past pharmacological treatments have included barbiturates and benzodiazepines (BZDs[a]), both of which promote sleep by binding to and allosterically modulating $GABA_A$ receptors ($GABA_A$Rs) in the central nervous system. These drugs however, have several unwanted side effects including alteration of sleep architecture, nightmares, agitation, confusion, lethargy, withdrawal, and a risk of dependence and abuse.[3] The newest generation of sleep-aid drugs, the non-BZD hypnotics, was developed to overcome some of these disadvantages. These drugs, which include zolpidem (ZPM) and eszopiclone (ESZ) (Figure 1), act through a similar neural mechanism as classical BZDs in that they bind to the same site in the $GABA_A$R but differ significantly in their chemical structures and neuropharmacological profiles. Unlike classical BZDs, the non-BZDs have minimal impact on cognitive function and psychomotor performance while facilitating more restorative sleep stages, thus inducing a pattern and quality of sleep similar to that of natural sleep.[4,5] Moreover, patients taking non-BZDs are less likely to exhibit tolerance, physical dependence, or withdrawal.[4−7]

Unlike classical BZDs, the sedative/hypnotic effect of ZPM occurs at much lower doses than the other pharmacological effects attributed to BZD-site action such as muscle relaxation and anticonvulsant activity.[7] This likely results from its selective binding to a specific $GABA_A$R subtype. The $GABA_A$R is a pentameric ligand-gated ion channel that can be formed by several different subunits (e.g., α, β, γ, etc.) and subunit isoforms (e.g., α_1, α_2, α_3, etc.). Receptors composed of different subunits have different kinetics, cellular distributions, and pharmacological profiles. Classical BZDs bind equally well to $GABA_A$Rs containing all of the α subunit isoforms except α_4 and α_6.[8,9] In contrast, ZPM has high affinity for receptors containing the α_1 subunit, low affinity for α_2- and α_3-containing receptors, and no significant affinity for α_5-containing receptors (Table 1).[8−10] The sedative actions of BZDs have been shown to be mediated by α_1-containing $GABA_A$Rs, whereas BZD effects such as anxiolysis are mediated by other α subunit isoforms.[11] This helps explain why ZPM is useful as a sedative/hypnotic but is not a clinically efficacious anxiolytic, whereas classical BZDs such as diazepam are effective at treating anxiety but their use is accompanied by a myriad of adverse side effects. Interestingly, the non-BZD ESZ (and its racemate zopiclone) has similar affinity for $GABA_A$Rs containing α_1, α_2, α_3, and α_5 subunits (Table 1)[10,12] and yet when taken for extended periods does not induce the adverse side effects associated with classical BZD treatment.[6] Thus, the neuropharmacological properties of ESZ must stem from more than just α-subunit selectivity.

The BZD binding site is located on the extracellular surface of the $GABA_A$R at the interface of the α and γ subunits and is formed by residues located in at least six noncontiguous regions (historically designated loops A−F) (Figure 1).[13] Although several studies have made significant strides in uncovering the specific amino acid residues that contribute to the binding of classical BZDs (e.g., flunitrazepam and diazepam) (Figure 1),[13] complete descriptions of the residues that preferentially contribute to the binding of non-BZD ligands and the orientation of these ligands within the BZD site are relatively unknown.

In this study, we used site-directed mutagenesis, radioligand binding, and molecular docking to compare the structural requirements for ZPM and ESZ binding to α_1-containing $GABA_A$Rs. We found that residues in $\gamma_2$loop D and $\alpha_1$loops A and B are important for maintaining the overall structural

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[a] Abbreviations: BZD, benzodiazepine; $GABA_A$R, γ-aminobutyric acid type-A receptor; ZPM, zolpidem; ESZ, eszopiclone; WT, wild-type; i-BZD, imidazobenzodiazepine.

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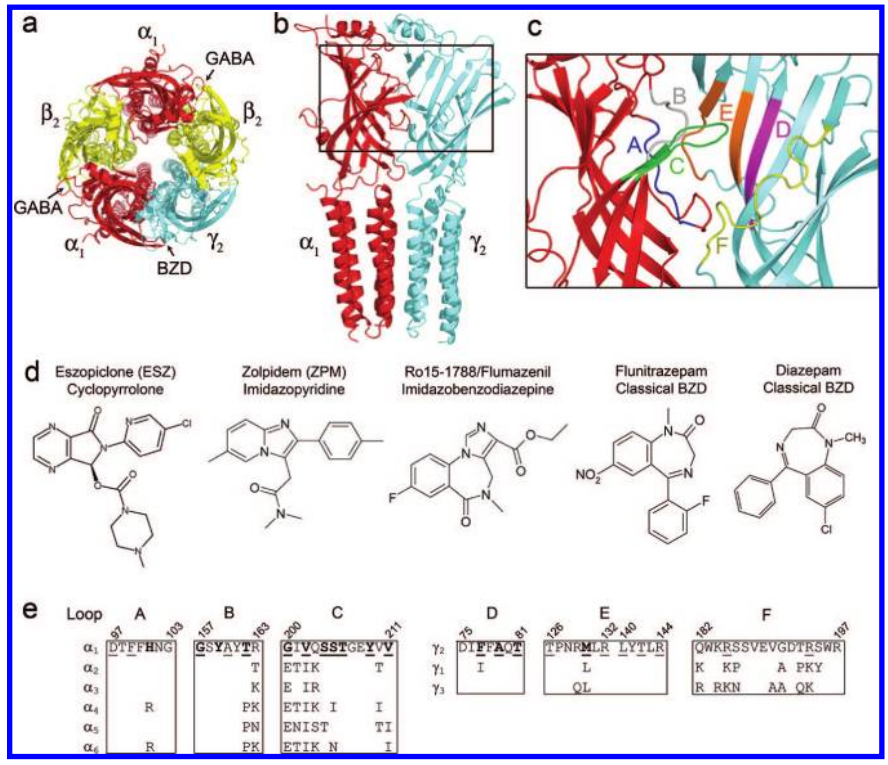

Figure 1. $GABA_A$ receptor α_1/γ_2 interface and structures of benzodiazepine binding site ligands. (a) Homology model of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor pentamer[36] as seen from the extracellular membrane surface. The α_1, β_2, and γ_2 subunits are highlighted in red, yellow, and blue, respectively. Arrows indicate that GABA binds at the β_2/α_1 interfaces whereas benzodiazepines (BZDs) bind at the α_1/γ_2 interface of the receptor. (b) Side view of the α_1 (red) and γ_2 (blue) subunits. The region of the interface encompassing the BZD binding site is boxed and highlighted in (c), where BZD binding site loops A−F are individually color-coded. (d) Structures of the BZD binding site ligands eszopiclone, zolpidem, Ro15-1788, flunitrazepam, and diazepam. (e) Rat $GABA_AR$ sequence alignment of loops A, B, C of α_{1-6} subunits and loops D, E, and F of γ_{1-3} subunits, where only differences from α_1 and γ_2 are shown. Amino acid residues shown previously to be important for BZD binding are bold, and residues examined in this study are underlined.

Table 1. Binding Affinities of Ro15-1788, Ro15-4513, Eszopiclone, and Zolpidem for $\alpha_x\beta_2\gamma_2$ Receptors[a]

	K_i (nM)			
receptor	Ro15-1788	eszopiclone	zolpidem	Ro15-4513
α1	3.3 ± 1.3	50.1 ± 10.1	61.9 ± 7.3	ND
α2	5.7 ± 0.1	114 ± 40.8	408 ± 35	ND
α3	8.1 ± 0.1	162 ± 29.5	975 ± 132	ND
α5	2.0 ± 0.1	102 ± 17.9	>15000	ND
α4	ND	>15000	>15000	3.1 ± 0.1
α6	ND	>15000	>15000	5.1 ± 0.1

[a] K_i values were determined by displacement of [^{3}H]ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (Ro15-1788)[52] binding (for α_1, α_2, α_3, and α_5) or [^{3}H]ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-1,4-benzodiazepine-3-carboxylate (Ro15-4513)[53] binding (for α_4 and α_6) and represent the equilibrium dissociation constant (apparent affinity) of the unlabeled ligand. Data represent mean ± SD from at least three separate experiments. ND: not determined.

integrity of the binding pocket whereas residues in $\gamma_2$loop E and $\alpha_1$loop C are important for ligand selectivity. Molecular docking is in good agreement with the binding data and suggests that unlike ESZ, ZPM binding relies more on the overall shape of the binding pocket than on specific residue interactions within the BZD site.

Results

Effects of BZD-Site Mutations on [^{3}H]Ro15-1788 Binding Affinity. Several residues that contribute to the binding of BZD ligands have previously been identified (Figure 1e). On the α subunit these residues include H101 (loop A);[8,14−19] G157, Y159, T162 (loop B);[8,15,20,21] and G200, V202, S204, S205, T206, Y209, and V211 (loop C).[8,15,20−28] On the γ subunit these include F77, A79, T81 (loop D)[16,22,29−33] and M130 (loop E).[22,34]

To help elucidate the unique structural requirements for ESZ and ZPM binding, 24 single cysteine mutations (12 each in the α_1 and γ_2 subunits) were made in or near the BZD binding site in the $GABA_AR$ (Figure 1e). These included all of the sites mentioned above with the exception of two residues and some new sites. We did not mutate α_1H101, as it has already been shown to contribute to the high affinity binding of both zopiclone and ZPM,[8,14,17] and α_1Y159, because serine and cysteine substitutions at this site abolish [^{3}H]Ro15-1788 and [^{3}H]flunitrazepam binding, thereby precluding further BZD affinity measurements.[15,20]

The mutant subunits were coexpressed with wild-type (WT) subunits in HEK293T cells to form $\alpha_1\beta_2\gamma_2$ $GABA_ARs$, and the binding of the BZD-site antagonist [^{3}H]Ro15-1788 (Figure 1d) was measured. The majority of mutant receptors bound Ro15-1788 with similar affinity as WT receptors (K_i = 3.3 ± 1.3 nM) (Table 2). Seven mutations caused small but significant decreases (2.0- to 4.5-fold) in Ro15-1788 affinity, whereas one mutation, α_1G157C, increased the affinity over 17-fold (Table 2). For three mutant receptors ($\alpha_1\beta_2\gamma_2$F77C, α_1D97C$\beta_2\gamma_2$, and α_1Y209C$\beta_2\gamma_2$) specific binding of [^{3}H]Ro15-1788 or the BZD-site agonist [^{3}H]flunitrazepam (Figure 1d) was not measurable (data not shown), precluding any further examination.

Effects of γ_2 Subunit Mutations on ESZ and ZPM Binding Affinity. ESZ and ZPM binding affinities were determined by their ability to competitively displace [^{3}H]Ro15-1788. In the γ_2 subunit, one mutation in loop D (β-strand 2), A79C,

Table 2. Binding Affinities of Ro15-1788, Eszopiclone, and Zolpidem for WT and Mutant $\alpha_1\beta_2\gamma_2$ Receptors[a]

		Ro15-1788		eszopiclone		zolpidem	
loop	receptor	K_i (nM)	mut/wt	K_i (nM)	mut/wt	K_i (nM)	mut/wt
	WT	3.3 ± 1.3	1.0	50.1 ± 10.1	1.0	61.9 ± 7.3	1.0
	αβγD56C	3.1 ± 0.2	0.9	82.4 ± 16.1	1.6	67.6 ± 8.0	1.1
D	αβγF77C	NB		ND		ND	
D	αβγA79C	9.2 ± 1.6*	2.8	393 ± 23.3**	7.9	577 ± 64.7**	9.3
D	αβγT81C	4.4 ± 1.4	1.3	75.2 ± 11.6	1.5	108 ± 9.2*	1.7
E	αβγT126C	4.4 ± 0.3	1.3	71.9 ± 3.5	1.4	72.7 ± 19.2	1.2
E	αβγM130C	13.8 ± 4.7*	4.1	102 ± 18.4*	2.0	15.5 ± 3.7**	0.3
E	αβγR132C	13.0 ± 4.7*	3.9	110 ± 23.7*	2.2	35.8 ± 4.8*	0.6
E	αβγL140C	3.8 ± 0.1	1.2	46.3 ± 1.4	0.9	63.1 ± 1.9	1.0
E	αβγT142C	9.2 ± 2.0*	2.8	503 ± 89.1**	10	1321 ± 300**	21
E	αβγR144C	3.7 ± 1.1	1.1	174 ± 50.6**	3.5	38.0 ± 11.5	0.6
F	αβχ161	9.6 ± 1.5*	2.9	22.4 ± 2.6*	0.4	427 ± 44.8**	6.9
F	αβγR185C	3.3 ± 0.0	1.0	70.9 ± 3.5	1.4	67.4 ± 6.8	1.1
F	αβγR194C	4.1 ± 0.0	1.2	67.0 ± 4.6	1.3	62.6 ± 0.7	1.0
A	αD97Cβγ	NB		ND		ND	
A	αF99Cβγ	15.0 ± 0.8**	4.5	407 ± 123**	8.1	180 ± 35.6*	2.9
B	αG157Cβγ	0.19 ± 0.06**	0.06	2103 ± 400**	42	1252 ± 367**	20
B	αA160Cβγ	4.0 ± 0.6	1.2	60.4 ± 32.2	1.2	81.4 ± 10.7	1.3
B	αT162Cβγ	2.2 ± 0.1	0.7	33.8 ± 5.6	0.7	109 ± 30.4	1.8
C	αG200Cβγ	3.0 ± 0.3	0.9	119 ± 13.8**	2.4	598 ± 104**	9.7
C	αV202Cβγ	1.5 ± 0.1	0.4	249 ± 52.9**	5.0	556 ± 153**	9.0
C	αS204Cβγ	8.8 ± 0.6**	2.7	57.9 ± 1.6	1.2	433 ± 56.4**	7.0
C	αS205Cβγ	6.5 ± 1.0*	2.0	34.9 ± 1.1	0.7	41.2 ± 7.6	0.7
C	αT206Cβγ	2.1 ± 0.5	0.6	0.83 ± 0.23**	0.02	76.4 ± 23.2	1.2
C	αY209Cβγ	NB		ND		ND	
C	αV211Cβγ	4.1 ± 1.4	1.2	62.4 ± 4.4	1.2	67.8 ± 22.2	1.1

[a] K_i values were determined by displacement of [^{3}H]Ro15-1788 binding and represent the equilibrium dissociation constant (apparent affinity) of the unlabeled ligand. The loop where each mutation is located inside the BZD binding pocket is indicated. The ratio of mutant to WT binding is shown and was calculated by dividing the K_i value for the mutant by the K_i value for WT. Data represent mean ± SD from at least three separate experiments. NB: no binding detected. ND: not determined. Values significantly different from WT are indicated (*, $p < 0.05$; **, $p < 0.01$).

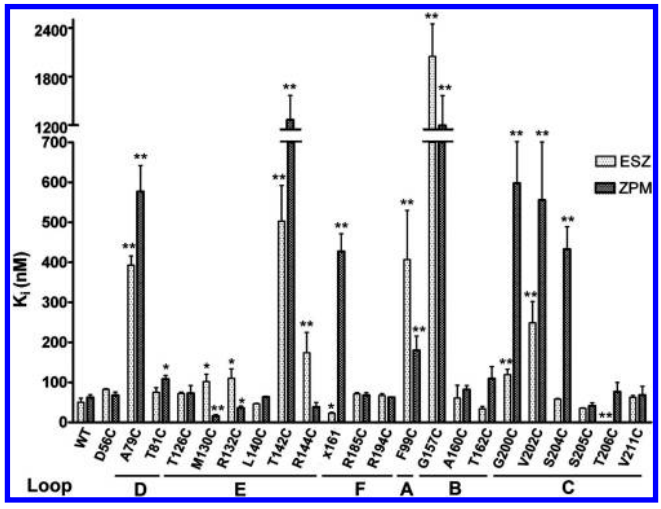

Figure 2. Cysteine mutations in the benzodiazepine binding site differentially affect eszopiclone and zolpidem affinity for the $GABA_A$ receptor. The apparent affinity (K_i) of WT, γ_2 mutant (loops D−F), and α_1 mutant (loops A−C) receptors for ESZ (light bars) and ZPM (dark bars) is graphed and was measured as described in the Experimental Section. x161 is the γ/α chimera where residues N-terminal to 161 are γ_2 sequence and residues C-terminal to 161 are α_1 sequence. Bars represent mean ± SD of at least three independent experiments. Values significantly different from WT are indicated (*, $p < 0.05$; **, $p < 0.01$).

significantly reduced both ESZ and ZPM affinity (~8- to 9-fold) compared to WT receptors (K_{iESZ} = 50.1 ± 10.1 nM; K_{iZPM} = 61.9 ± 7.3 nM), whereas γ_2T81C located adjacent to γ_2A79 had a small but significant effect on ZPM but not ESZ binding (Figures 2 and 3a, Table 2). γ_2D56C on the neighboring β-strand (Figure 3a) had no effect on the binding of either drug (Figure 2, Table 2).

γ_2Loop E of the BZD binding site is composed of two adjacent β-strands (5/6) that form the back and side of the BZD binding pocket (Figure 1c). Several mutations in the middle of these β-strands differentially affected ESZ and ZPM binding (Figures 2 and 3b, Table 2). Whereas cysteine substitution of γ_2M130 and γ_2R132 each *decreased* ESZ affinity by ~2-fold, these mutations *increased* ZPM affinity by ~2- to 4-fold. While γ_2T142C significantly reduced the affinity of both ligands, it had a larger effect on ZPM binding compared to ESZ (21-fold vs 10-fold change). Moreover, $\alpha_1\beta_2\gamma_2$R144C receptors showed a significant reduction in ESZ affinity (3.5-fold) but no change in ZPM binding. γ_2T126C and γ_2L140C, located at the periphery of the BZD-site (Figure 3b), did not affect the binding of ESZ or ZPM. Overall, the data suggest that loop E plays an important role in determining ligand selectivity of the BZD binding site.

Loop F of the γ_2 subunit (~residues 182−197) is a dynamic region of the receptor located between the BZD binding site and the transmembrane channel domain (Figure 1). Through the use of γ_2/α_1 chimeras, a portion of loop F ($\gamma_2$186−192) was shown to be important for high affinity ZPM binding.[35] A chimeric subunit, χ161 (containing γ_2 residues up to and including amino acid 161 and α_1 residues C-terminal to 161), when expressed with WT α_1 and β_2 subunits, retained WT binding affinity for flunitrazepam, but the binding affinity for ZPM decreased 8-fold. Here, we used the same chimera to test whether residues C-terminal to 161 were also important for ESZ binding. We found that χ161 slightly increased (~2-fold) the affinity of the receptor for ESZ (Figures 2 and 3c, Table 2).

We noticed that in our homology model of the $GABA_AR$[36] two arginine residues and a glutamate from loop F (γ_2R185, γ_2R194, and γ_2E189) point toward the ligand binding pocket (Figure 3c). We individually mutated the arginine residues to cysteine and found that the binding of Ro15-1788, ESZ, and ZPM to γ_2R185C- and γ_2R194C-containing receptors was indistinguishable from WT (Figure 2, Table 2). These data are

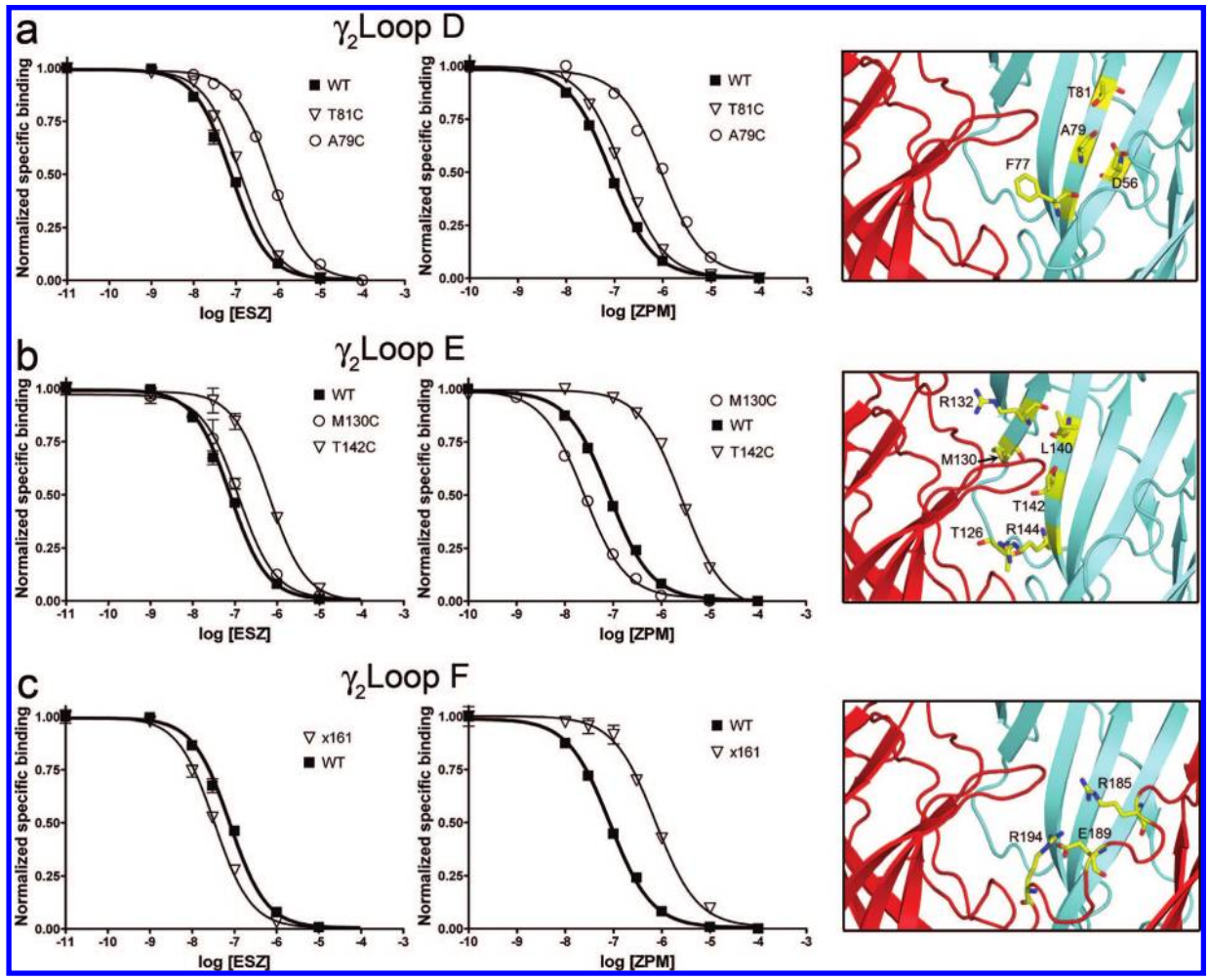

Figure 3. Mutations in the γ_2 subunit differentially affect eszopiclone and zolpidem binding to the $GABA_A$ receptor. (a−c) Representative radioligand binding curves depict the displacement of [^{3}H]Ro15-1788 binding by ESZ (left panels) and ZPM (middle panels) for WT $\alpha_1\beta_2\gamma_2$ receptors (filled squares) and the indicated γ_2 mutant receptors (open symbols) in loop D (a), loop E (b), and loop F (c), respectively. Representative binding curves are shown for a selected group of mutants. Each data point is the mean ± SEM of triplicate measurements. Data were fit by nonlinear regression as described in the Experiemental Section, and K_i values are reported in Table 2. A close-up view of the benzodiazepine binding site (right panels), with the α_1 subunit in red and the γ_2 subunit in blue, highlights all sites where individual cysteine substitutions were introduced (yellow). For x161, residues 1−161 are γ_2 sequence (blue), and residues C-terminal to 161 are α_1 sequence (red). The localization of residues γ_2R185, γ_2E189, and γ_2R194 is based on their positions in the WT γ_2 sequence.

consistent with our previous study of $\gamma_2$loop F where we demonstrated that mutations in this region (γ_2W183C, γ_2E189C, and γ_2R197C) affect modulation of GABA current by BZD agonists without affecting binding affinity of various BZD ligands including ZPM, Ro15-1788, the classical BZD, flurazepam, and the inverse agonist DMCM.[37] Overall, these results strongly suggest no one residue in $\gamma_2$loop F is critical for binding classical or non-BZDs.

Effects of α_1 Subunit Mutations on ESZ and ZPM Binding Affinity. In α_1 loops A and B, cysteine substitution of two residues, α_1A160 and α_1T162, had no significant effect on either ESZ or ZPM binding affinity (Figure 2, Table 2). In contrast, cysteine substitution of α_1F99 and α_1G157 caused significant changes in the affinity of both ligands. α_1F99C reduced ESZ and ZPM affinity by ∼8- and 3-fold, respectively, compared to WT receptors. α_1G157C had a larger effect, with 42- and 20-fold changes in ESZ and ZPM binding affinity, respectively (Figures 2 and 4a, Table 2).

Cysteine substitution of several residues in $\alpha_1$loop C also significantly altered the binding affinity of both ligands. α_1G200C and α_1V202C reduced ESZ affinity by 2.4- and 5.0-fold, respectively, and reduced ZPM affinity 9.7- and 9.0-fold, respectively (Figures 2 and 4b, Table 2). Other mutations differentially affected ZPM and ESZ affinity. α_1S204C reduced ZPM affinity by 7-fold but had no effect on ESZ binding, whereas α_1T206C increased ESZ affinity over 60-fold while having no effect on ZPM binding (Figures 2 and 4b, Table 2). Receptors containing α_1S205C and α_1V211C bound both ligands with WT affinity. These results suggest that together with $\gamma_2$loop E, $\alpha_1$loop C is an important determinant for BZD-site ligand selectivity.

Molecular Docking of Eszopiclone and Zolpidem. Independent of the radioligand binding experiments described above, we used our homology model of the $GABA_AR$ to dock ESZ and ZPM into the BZD binding site. ESZ docking yielded a single most populated pose with low energy (−6.61 kcal/mol). In this pose (termed ESZ-dock), the free carbonyl of ESZ is pointed up toward loop C and the ring carbonyl is near γ_2R144 (loop E) and α_1H101 (loop A) near the base of the pocket (Figures 5a and 6a). In contrast, ZPM docking yielded three equally populated poses with similar energies (between −7.0 and −6.7 kcal/mol): one with the imidazopyridine ring under $\alpha_1$loop C, the carbonyl pointed up toward the tip of $\alpha_1$loop C and the dimethyl amide pointed toward $\gamma_2$loop D (ZPM-up-

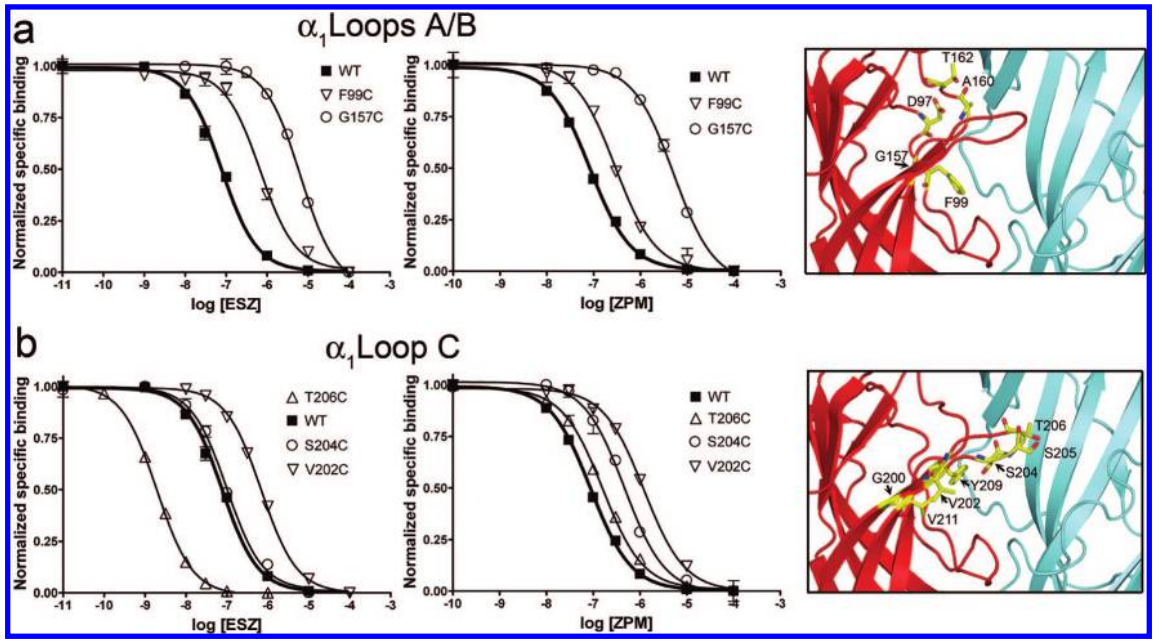

Figure 4. Mutations in the α_1 subunit differentially affect eszopiclone and zolpidem binding to the $GABA_A$ receptor. (a, b) Representative radioligand binding curves depict the displacement of [^{3}H]Ro15-1788 binding by ESZ (left panels) and ZPM (middle panels) for WT $\alpha_1\beta_2\gamma_2$ receptors (filled squares) and the indicated α_1 cysteine mutant receptors (open symbols) in loops A and B (a) and loop C (b), respectively. Representative binding curves are shown for a selected group of mutants. Each data point is the mean ± SEM of triplicate measurements. Data were fit by nonlinear regression as described in the Experiemental Section, and K_i values are reported in Table 2. A close-up view of the benzodiazepine binding site (right panels), with the α_1 subunit in red and the γ_2 subunit in blue, highlights all sites where individual cysteine substitutions were introduced (yellow).

dock) (Figure 5b); one with the imidazopyridine ring under $\alpha_1$loop C and the dimethyl amide pointed down in the pocket toward α_1H101 (ZPM-down-dock) (Figures 5c and 6b); and one with the imidazopyridine ring pointed toward the back wall of the pocket, the carbonyl pointed down away from $\alpha_1$loop C and the dimethyl amide positioned under the tip of $\alpha_1$loop C (ZPM-out-dock) (Figure 5d).

To gain insight into the potential interactions between ESZ or ZPM and the $GABA_AR$ in our docked ligand−receptor complexes, we measured the distance between atoms in each ligand and atoms in the protein, with the idea that functional groups separated by less than 7 Å have the potential to interact[38] and those within 4 Å may form salt bridges or hydrogen bonds.[39] In ESZ-dock, residues γ_2F77, γ_2A79, γ_2T142, γ_2R144, α_1H101, α_1Y159, α_1T206, and α_1Y209 all come within 4 Å of the ligand (Figures 5a and 6a) and, when mutated, alter ESZ binding (Table 2) and/or BZD binding in general. In this docking, potential polar contacts exist between ESZ and γ_2R144, α_1Y159, α_1S204, α_1Y209, and the backbone of $\alpha_1$loop C (shown as dashed lines in Figures 5a and 6a). In addition, residues α_1F99, α_1V202, γ_2M130, and γ_2R132, which when mutated all adversely affect ESZ binding (Table 2), are all within 7 Å of ESZ in our model.

For ZPM, the three poses are similar in that residues including γ_2F77, γ_2M130, γ_2T142, α_1H101, α_1Y159, α_1S204, and α_1Y209 all come within 5 Å of the ligand in each case (Figures 5 and 6b). Likewise, residues α_1F99, α_1V202, γ_2A79, and γ_2R132 are within ~7 Å or less from ZPM in each model. Thus, even though the free carbonyl of ZPM is oriented differently in each pose, the space occupied by ZPM within the binding pocket is similar. The major difference between the three models lies in the potential for hydrogen bonding. In ZPM-up-dock, potential hydrogen bonds exist between ZPM and the backbone of loop C near α_1T206/G207; in ZPM-down-dock, the free carbonyl may form a hydrogen bond with α_1S204 in loop C; and in ZPM-out-dock the carbonyl may interact with γ_2R194 in loop F (dashed lines, Figures 5 and 6b).

Discussion

Although several studies have revealed important information on the amino acid side chains that contribute to classical BZD binding in the $GABA_AR$, a complete description of the residues that participate in non-BZD binding has been lacking. Residues previously shown to participate in ZPM binding include γ_2F77, γ_2M130, α_1H101, α_1T162, α_1G200, α_1S204, α_1T206, α_1Y209, and α_1V211.[8,14,21−25,28,32−34] To our knowledge, only one site, α_1H101, has been identified that is important for zopiclone (the racemate of ESZ) binding.[17] Here, we define receptor models for how ESZ and ZPM are oriented in the binding site, evaluate how specific residues in the binding site interact with ESZ and ZPM, and provide new insight into the pharmacophores for these drugs.

Residues in Loops A, B, and D Are Critical for the Overall Structure of the BZD Site. Several lines of evidence suggest that residues in $\gamma_2$loop D and $\alpha_1$loops A and B are critical to the binding of BZDs in general and thus are important for the overall structure of the BZD binding site. Mutations in these areas affect the binding affinities of a variety of structurally diverse BZD-site ligands. In $\gamma_2$loop D, we were unable to detect specific binding of BZD antagonist [^{3}H]Ro15-1788 or the BZD agonist [^{3}H] flunitrazepam to F77C-containing receptors, suggesting that the native phenylalanine at this position is a key structural element in the BZD binding pocket. This is consistent with previous findings that showed that a variety of substitutions at γ_2F77 dramatically alter the affinity of various BZDs including Ro15-1788, diazepam, flunitrazepam, and ZPM,[22,32,33] and where γ_2F77C was shown to completely abolish flurazepam potentiation.[29]

Moreover, we found that mutation γ_2A79C reduced the binding of ESZ and ZPM to a similar extent (~8- to 9-fold)

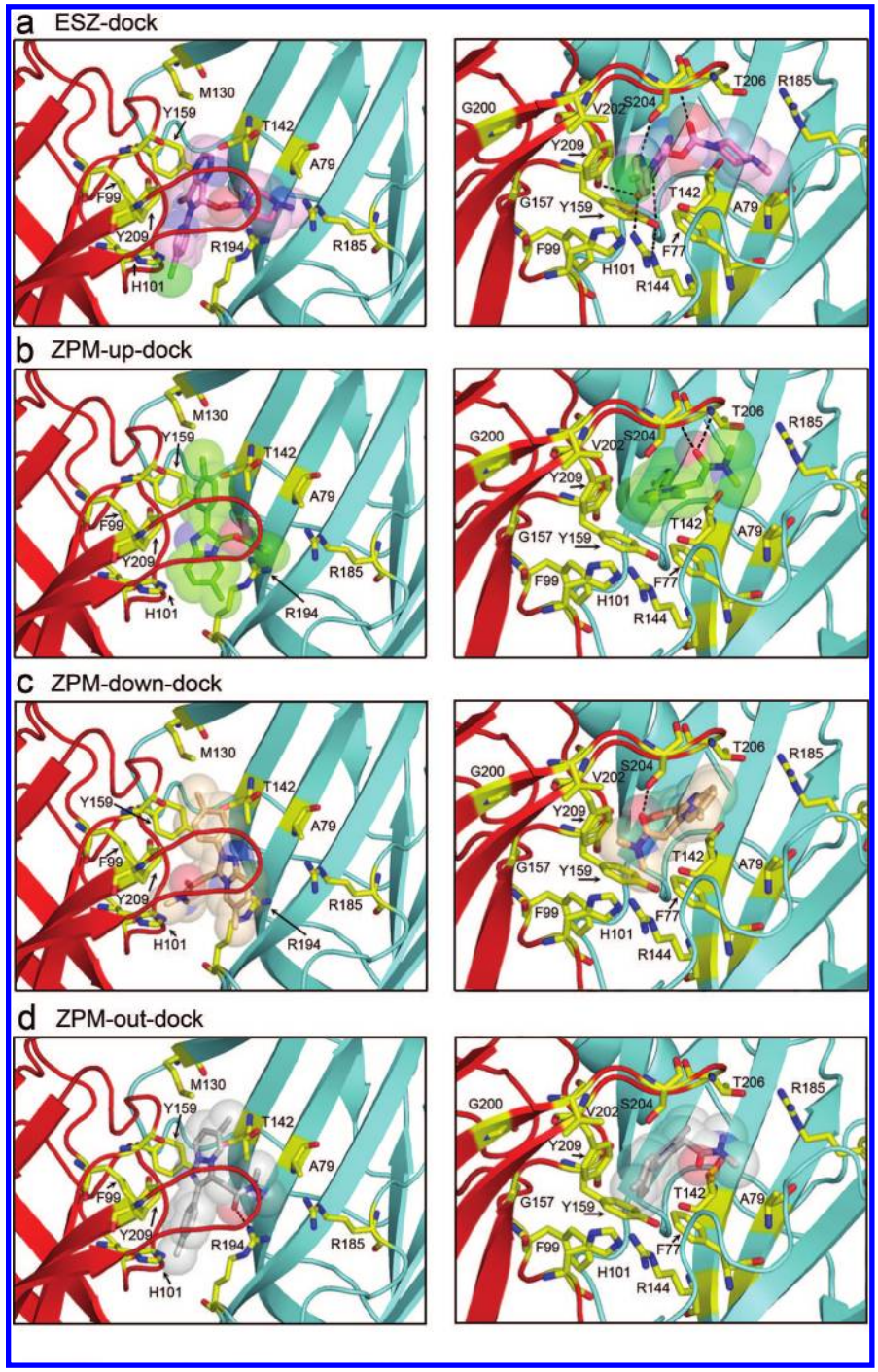

Figure 5. Molecular docking of eszopiclone and zolpidem. View looking down on $\alpha_1$loop C (left panels) and underneath $\alpha_1$loop C (right panels) of (a) ESZ and (b−d) ZPM docked into the benzodiazepine binding site of the $GABA_AR$ using AutoDock 4.0 software as described in the Experiemental Section. The α_1 subunit is red, the γ_2 subunit is blue, and residues of interest are highlighted in yellow. Docked ligands are represented as sticks with transparent space-fill. Atoms are color-coded as follows: oxygen, red; nitrogen, blue; sulfur, yellow; chloride (ESZ), green. Potential hydrogen bonds are represented by dashed lines. The pdb files containing eszopiclone and zolpidem docked at the BZD site of the $GABA_AR$ are provided in the Supporting Information.

(Figure 2) and the affinity of the receptor for Ro15-1788 was also significantly decreased (Table 2). Previous studies found mutation of γ_2A79 was also detrimental to flunitrazepam, Ro15-4513, Ro15-1788, and diazepam binding.[16,30,31] These results are in good agreement with cysteine accessibility studies that showed γ_2A79 is part of the BZD binding pocket.[29,30]

Cysteine substitution of γ_2T81 had no effect on ESZ or Ro15-1788 and only a minor effect on ZPM affinity (<2-fold) (Table 2). However, larger volume BZD-site ligands such as Ro15-4513, Ro40-6129, and Ro41-3380 are affected by mutations at this site.[30] Thus, even though γ_2T81 may not contribute significantly to ESZ or ZPM binding, it likely forms part of the binding site for other BZDs.

In $\alpha_1$loop A, we observed no specific binding of [^{3}H]Ro15-1788 or [^{3}H]flunitrazepam to α_1D97C$\beta_2\gamma_2$ receptors. Functional α_1D97C$\beta_2\gamma_2$ receptors that respond to GABA can be expressed in *Xenopus* oocytes.[40] Thus, it is unlikely that cysteine substitution at this location impairs proper folding or expression of the receptor. Molecular docking indicates that α_1D97 is within 8 Å of ESZ and ZPM. In our model, α_1D97 appears part of an

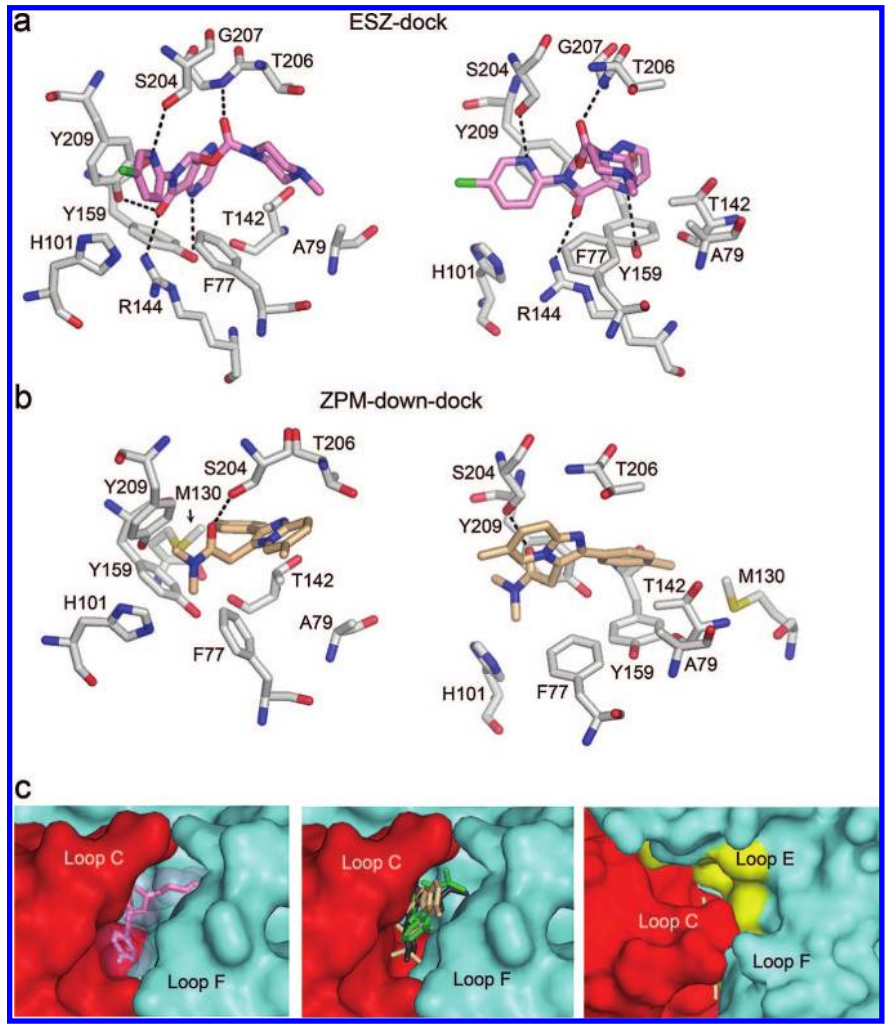

Figure 6. Eszopiclone and zolpidem in the BZD binding pocket. Two views of ESZ-dock (a) and ZPM-down-dock (b) showing the relative orientation of ligand and residues of interest in the BZD-site. Potential hydrogen bonds are represented by dashed lines. Atoms are color-coded as follows: oxygen, red; nitrogen, blue; sulfur, yellow; chloride (ESZ), green. (c) The surface of the α_1 (red) and γ_2 (blue) subunits near the BZD-site is shown to highlight the size of the binding pocket. Left panel: ESZ (pink) is represented as sticks with transparent space-fill. Middle panel: The three orientations of ZPM (green, tan, gray) are represented as sticks. Right panel: Surface view of ZPM-down-dock (tan, sticks) as seen looking down on $\alpha_1$loop C. (Note: most of the molecule is occluded by loop C). The surface of loop E residues examined in this study is highlighted in yellow. Observe the large unfilled volume of space bordered by loop E residues.

electrostatic network of residues that bridges the α_1/γ_2 subunit interface. We speculate that the lack of specific binding for this mutant is because α_1D97 is important for maintaining the structural integrity of the BZD pocket.

Mutation of α_1F99 in loop A significantly reduces $GABA_AR$ affinity for ESZ, ZPM, and Ro15-1788. This may be because α_1F99 participates in hydrophobic interactions with bound drug or because mutation of α_1F99 to cysteine alters the positions of α_1H101 (loop A) and α_1Y159 (loop B), which lie on either side of it in the binding pocket (Figure 5). Indeed, the necessity of α_1H101 in binding ZPM,[8,14] zopiclone,[17] and several other BZDs[16,18,19] has been established. The importance of α_1Y159 is underscored by its potential to hydrogen-bond directly with ESZ in the binding pocket (Figure 5) and the inability of [^{3}H]Ro15-1788 or [^{3}H]flunitrazepam to bind α_1Y159C- or α_1Y159S-containing receptors.[15,20]

One of the most dramatic shifts in BZD binding affinity was measured for the $\alpha_1$loop B mutant G157C. This residue appears to be at the side wall of the ESZ and ZPM binding pocket (Figure 5). In our homology model of the $GABA_AR$, a larger cysteine side chain would decrease the volume of the binding site. This may hinder occupation of the site by ZPM and ESZ and/or affect the positioning of nearby residues including α_1H101 (loop A) and α_1Y209 (loop C) (Figure 5). The fact that G157C drastically reduces ZPM and ESZ binding (Figure 2, Table 2) but increases Ro15-1788 affinity 17-fold supports the idea that G157C alters the shape of the BZD binding pocket and that imidazobenzodiazepines (i-BZDs) such as Ro15-1788 have different structural requirements than the non-BZDs. Indeed, a recent study showed that a sulfhydryl-reactive derivative of the i-BZD, Ro15-4513, was able to covalently attach to a cysteine at α_1G157.[15]

Since mutation of residues in $\gamma_2$loopD and $\alpha_1$loops A and B alter the binding affinity of a variety of structurally diverse BZD-site ligands (our work and others), we envision that these regions define the core of the binding site for BZD-site ligands. Thus, a question remains as to what defines ligand specificity at the BZD binding site.

Residues in Loops C and E Determine Ligand Selectivity at the BZD Site. Unlike residues in loops A, B, and D, $\gamma_2$loop E residues are located at the back of the binding pocket where extra space exists for ligand placement/movement (Figure 6c, right panel). The large unfilled volume bordered by loop E is likely ideal for accommodating ligands of different size and

chemical composition. Indeed, we found that mutations M130C, R132C, and R144C in $\gamma_2$loop E differentially affect ESZ, ZPM, and Ro15-1788 affinity (Table 2, Figure 2). In addition, the magnitude of the effect of the T142C mutation was different for all three ligands tested. Interestingly, previous studies have shown that mutation of γ_2M130 to leucine reduces ZPM affinity while having very small or no effects on the binding of several other BZDs.[22,34] Thus, substitution of native loop E residues may cause a change in the volume of the binding site that results in altered positioning of the ligand in the pocket, thereby affecting affinity. For example, molecular docking shows that the native arginine at position 144 stabilizes the ring carbonyl of ESZ in the binding pocket via a hydrogen bond (Figure 5); thus, removal of this H-bond via cysteine substitution likely causes the observed reduction in ESZ affinity. In contrast, ZPM dockings show no interaction with γ_2R144, explaining the lack of effect of γ_2R144C on ZPM binding.

Mutations in $\alpha_1$loop C also differentially affect ligand binding to the BZD-site of the $GABA_AR$ (Figure 2), suggesting that these residues also play a role in determining ligand selectivity. We found that three mutations, G200C, V202C, and S204C, had a much greater effect on ZPM affinity than ESZ or Ro15-1788, whereas T206C dramatically increased ESZ affinity without affecting ZPM or Ro15-1788 (Figure 2, Table 2). In addition, previous studies have shown that mutations T206V and T206A selectively alter the affinity of diazepam, flunitrazepam, and ZPM but not that of several other BZDs.[22,25]

On the basis of the low sequence homology of $\alpha_1$loop C to other α subunit isoforms (Figure 1e), loop C is likely a significant determinant in the α_1-subunit selectivity of ZPM. This is supported by previous studies. First, replacement of α_1G200 with the aligned glutamate residue present in all other α subunit isoforms reduces ZPM binding,[23] whereas replacement of the glutamate in α_3, α_5, and α_6 with glycine increases the affinity of these receptors for ZPM.[8,21,24] Second, replacement of the threonine in α_5 with the aligned serine at 204 in α_1 increases the affinity of α_5 for ZPM.[21] Lastly, mutation of α_1V211 to the aligned isoleucine in α_5 and α_6 decreases flunitrazepam and ZPM affinity while increasing the affinity of α_5-selective ligands.[28] All of these substitutions show that $\alpha_1$loop C residues promote ZPM binding whereas loop C residues from other α subunit isoforms reduce ZPM affinity, supporting the idea that $\alpha_1$loop C contributes to ZPM selectivity.

Loop C residues α_1Val211, α_1Val202, and α_1Ser205 also appear to be especially important for i-BZD binding. A sulfhydryl-reactive derivative of Ro15-4513 has been shown to covalently attach to α_1V202C and α_1V211C,[15] whereas α_1S205C reduced the affinity for Ro15-1788 (Table 2), and replacement of α_1S205 with the aligned asparagine in α_6 was shown to decrease the affinity of i-BZDs and β-carbolines.[27] Of the cysteine substitutions at these sites, only α_1V202C had an effect on ESZ and ZPM binding (Figure 2, Table 2). Overall, mutations in $\gamma_2$loop E and $\alpha_1$loop C differentially affect binding of structurally diverse classes of BZD-site ligands, supporting the idea that these regions define specificity.

Zolpidem Interaction with the BZD Site Is Less Specific Than Eszopiclone. The orientation of ESZ in the BZD binding pocket as presented in Figures 5 and 6 is supported by our mutagenesis data. This docking was the lowest energy, most highly populated pose obtained using AutoDock 4.0. A similar orientation of ESZ was observed using AutoDock 3.0 and SureFlex Dock (data not shown), and the docking is consistent with the recently described unified pharmacophore/receptor model of the BZD site.[41] Essential functional groups defining the ESZ pharmacophore and its orientation in the site are its two carbonyls, which hydrogen-bond with the backbone of loop C and γ_2R144 (loop E), respectively, and two ring nitrogens, which hydrogen-bond with α_1S204 (loop C) and α_1Y159 (loop B), respectively (Figure 6).

In contrast, ZPM may bind in multiple orientations in the BZD site. Our molecular docking revealed three equally populated poses with equivalent energies that occupy a similar space within the BZD site (Figures 5 and 6) but with few potential polar contacts in any orientation. Thus, the essential descriptor of the ZPM pharmacophore is likely its size and shape. ZPM has only a single carbonyl, which appears capable of interacting with several different residues in the BZD binding site (Figure 5). We found that this carbonyl could hydrogen-bond with $\alpha_1$loop C (at α_1S204 or the backbone near α_1T206/G207) or with $\gamma_2$loop F (Arg194) (Figure 5). Similar orientations were observed with Autodock 3.0 and SureFlex Dock software (data not shown). Our ZPM-up-dock pose is similar to that reported by Sancar et al., which allowed flexible movement of the side chains during docking.[35] While our mutagenesis data would favor hydrogen bonding with α_1S204, we cannot exclude any of the poses presented here. Overall, we believe that unlike ESZ, ZPM binding relies more on shape recognition of the binding pocket than on specific interactions in the BZD site. This would explain several observations:

First, this would account for the α_1 subunit selectivity of ZPM (Table 1).[8−10,42,43] The included volume of the BZD binding pocket for α_1-containing receptors has a slightly different shape, polarity, and lipophilicity compared to α_2- and α_3-containing receptors,[41] explaining the reduced but still measurable affinity of ZPM for α_2 and α_3 (Table 1). Furthermore, the included volume of α_1-containing receptors is much larger than α_4-, α_5-, and α_6-containing receptors,[41] thus explaining the lack of ZPM binding at these α subtypes (Table 1). It follows that differences in the included volumes of the BZD-site for γ_2- versus γ_1- and γ_3-containing receptors also likely play a role in ZPM selectivity.

Second, this clarifies why χ161, but not specific mutations in loop F, specifically reduces ZPM binding (Table 2). The replacement of the entire $\gamma_2$loop F, which is poorly conserved, with the $\alpha_1$loop F that is also two amino acids longer, likely changes the size of the binding pocket to the detriment of ZPM binding.

Third, shape recognition by ZPM would explain why minor mutations in loop C, like α_1G200C, α_1V202C, and α_1S204C, affect ZPM binding to a much greater extent than ESZ (Figure 2). Loop C, which comprises the entire lid over of the BZD site (Figure 6c), is highly flexible and is believed to move upon ligand binding.[44−46] Thus, it stands to reason that mutation of α_1G200, located at the hinge of loop C (Figure 5), would change the overall flexibility of the loop, perhaps preventing its full closure on the binding site, resulting in an altered shape of the binding pocket, which in turn precludes high affinity ZPM binding. In contrast, ESZ, which is anchored to the BZD site by multiple interactions (e.g., Arg144, Tyr209, Tyr159, etc.) (Figure 6a), is less affected by any single mutation.

Overall, this evidence suggests that ZPM binding depends largely on the size and shape of the BZD binding pocket rather than on specific polar interactions.

Summary and Conclusions

In summary, our data provide new insights toward defining the pharmacophore for non-BZD hypnotics as well as the structure of the BZD binding site (Figure 6). We provide a comprehensive description of the amino acid residues that

contribute to the binding of these ligands and present molecular models for their orientation in the BZD binding site. We show that residues in $\gamma_2$loop D and $\alpha_1$loops A and B provide the necessary framework for ligand binding in the pocket, while specific residues in $\gamma_2$loop E and $\alpha_1$loop C play a key role in determining ligand selectivity. We conclude that $\gamma_2$loop F does not directly contribute to BZD binding but may serve indirectly to maintain the structural integrity of the region. We also provide evidence that the subunit selectivity of ZPM results mainly from the overall shape of the binding pocket and is based largely on its interaction with loop C.

Thus, their footprint within the BZD binding pocket of the $GABA_AR$ may in part determine the pharmacological properties of the non-BZDs. Further experiments will be necessary to determine if specific residues in loops C and E differentiate the efficacies of the classical and non-BZD ligands. The identification of the structural elements important for the high affinity binding and efficacy of these drugs provides insight into the unique neuropharmacological profile of ESZ and ZPM in the central nervous system and will be beneficial in the design and development of more pharmacologically and behaviorally selective BZD site ligands.

Experimental Section

Site-Directed Mutagenesis. Cysteine mutants of γ_{2L} and α_1 receptor subunits were made by recombinant PCR in the pUNIV vector[47] and verified by double-stranded DNA sequencing.

Radioligand Binding. HEK293T cells were grown in Minimum Essential Medium with Earle's salts (Invitrogen, Grand Island, NY) containing 10% fetal bovine serum in a 37 °C incubator under 5% CO_2 atmosphere. Cells were plated on 100 mm dishes at ~40% confluency[47] for transient transfection using a standard $CaHPO_4$ precipitation method.[48] Cells were transfected with equal ratios of α, β, and γ subunit DNA in the same vector (4 μg/subunit). For experiments involving cysteine mutants and WT $\alpha_1\beta_2\gamma_2$ receptors, cells were co-transfected with WT pUNIV-α_1, pUNIV-β_2, pUNIV-$\gamma_{2\,L}$, or mutant subunit cDNA. For expression of $\alpha_2\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$, and $\alpha_6\beta_2\gamma_2$ receptors, cells were co-transfected with β_2-pRK5 and γ_2-pRK5 and either α_2-pRK5, α_3-pRK5, or α_6-pRK5 (constructs kindly provided by S. Dunn (Department of Pharmacology, University of Alberta, Edmonton, Canada)). For expression of $\alpha_4\beta_2\gamma_2$ and $\alpha_5\beta_2\gamma_2$ receptors, cells were co-transfected with α_4-pUNIV, β_2-pUNIV, and γ_2-pUNIV or α_5-pCEP4, β_2-pCEP4, and γ_2-pCEP4 cDNA, respectively. Cells were harvested and membrane homogenates prepared 48 h post-transfection as described.[49] Briefly, membrane homogenates (50 μg) were incubated at room temperature for 40 min with a sub-K_d concentration of radioligand ([^{3}H]Ro15-1788, 70.7Ci/mmol; [^{3}H]flunitrazepam, 85.2 Ci/mmol; [^{3}H]Ro15-4513, 35.7 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) in the absence or presence of seven different concentrations of unlabeled ligand in a final volume of 250 μL. Data were fit by nonlinear regression to a one-site competition curve defined by the equation $y = B_{max}/[1 + (x/IC_{50})]$, where y is bound [^{3}H]ligand in disintegrations per minute, B_{max} is maximal binding, x is the concentration of displacing ligand, and IC_{50} is the concentration of unlabeled ligand that inhibits 50% of [^{3}H]ligand binding (Prism, GraphPad Software). Equilibrium dissociation constant values for the unlabeled ligand (K_i) were calculated using the Cheng−Prusoff−Chou equation: $K_i = IC_{50}/[1 + L/K_d]$, where K_d is the equilibrium dissociation constant of the radioligand and L is the concentration of the radioligand.

Statistical Analysis. Binding data represent the mean ± SD from three experiments performed in triplicate. The data were analyzed by one-way ANOVA with Dunnett's post-test for significance of differences (StatView, version 5.0.1, SAS Institute, Inc., Cary, NC).

Automated Ligand Docking. The homology model of the $GABA_AR$ used in this study was constructed as described.[36] ESZ and ZPM were built using Sybyl Modeling software (Tripos Corp., St. Louis, MO). Each of the drug structures were energy-minimized using the Tripos force field, and then a random search was performed for the lowest energy conformations. The single lowest energy form was placed in the $GABA_AR$ α_1/γ_2 interface using Sybyl, and a 15 Å sphere of residues around the ligand was chosen as the starting active site. The active site was set up for docking using AutoDock 4.0 Tools, placing Gatseiger charges and desolvation parameters on the chosen 15 Å receptor sphere. Autodock 4[50,51] parameters were chosen for the genetic algorithm (GA) to examine 150 individuals in a population with a maximum of 5 million energy evaluations, followed by 300 iterations of Solis & Wets local search (Lakmarkian algorithm). A total of 10 to 30 of these hybrid dockings were performed on each drug. The binding results were clustered on the basis of lowest energy, visual similarities, and the orientation in the active site. The reported binding energies in kcal/mol is the sum of the final intermolecular energy, the internal energy of the ligand, and the torsional free energy minus the unbound systems energy. The orientation with stronger binding has the lower total energy, and the cluster of highest number of bindings represents a higher probability of binding. The drug was allowed to flexibly dock, but the receptor's backbone and side chains remained rigid during docking. Each docking gave an ensemble of docking modes, with many orientations nearly identical and only differing by less than 0.1 kcal/mol.

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Supporting Information Available: pdb files containing eszopiclone and zolpidem docked at the BZD site of the $GABA_AR$. This material is available free of charge via the Internet at http://pubs.acs.org.

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