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Structural determinants for high affinity zolpidem binding to GABA-A receptors.

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Running Title: Determinants of zolpidem binding in the GABAR γ2 subunit

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ABBREVIATIONS:

GABAR, GABA-A receptor; BZD, benzodiazepine; χ, chimera; HEK, human embryonic kidney; AChBP, acetylcholine binding protein; nACh, nicotinic acetylcholine; SCAM, substituted cysteine accessibility method; EM, electron microscopy; MC, Monte Carlo; SA, simulated annealing; RMSD, root mean square deviation.

ABSTRACT

The imidazopyridine zolpidem (Ambien®) is one of the most commonly prescribed sleep aids in the nation (Rush, 1998). Similar to classical benzodiazepines (BZDs), zolpidem binds at the extracellular N-terminal α/γ subunit interface of the GABA-A receptor (GABAR). However, zolpidem differs significantly from classical BZDs in chemical structure and neuropharmacological properties. Thus, classical BZDs and zolpidem likely have different requirements for high affinity binding to GABARs. To date, three residues $\gamma2M57$, $\gamma2F77$ and $\gamma2M130$, have been identified as necessary for high affinity zolpidem binding (Wingrove et al, 1997; Buhr and Sigel, 1997). Here, we used radioligand binding techniques, $\gamma2/\alpha1$ chimeric subunits (χ), site-directed mutagenesis, and molecular modeling to identify additional $\gamma2$ subunit residues important for high affinity zolpidem binding. While $\alpha1\beta2\chi$ receptors containing only the first 161 amino-terminal residues of the $\gamma2$ subunit bind the classical BZD flunitrazepam with wild-type affinity, zolpidem affinity is decreased ~8 fold. By incrementally restoring $\gamma2$ subunit sequence, we identified a 7 amino acid stretch in the $\gamma2$ subunit Loop F region (a.a. 186-192) that is required to confer high affinity zolpidem binding to GABARs. When mapped to a homology model, these seven amino acids comprise part of loop F located at the α/γ interface. Based on *in silico* zolpidem docking, three residues within loop F, $\gamma2E189$, $\gamma2T193$, and $\gamma2R194$, emerge as being important for stabilizing zolpidem in the BZD binding pocket and likely interact with other loop F residues to maintain the structural integrity of the BZD binding site.

INTRODUCTION

Zolpidem (Ambien®), an imidazopyridine, is a popularly prescribed sedative-hypnotic in the treatment of insomnia. Similar to classical benzodiazepines (BZDs), such as flunitrazepam and diazepam, zolpidem binds to the extracellular N-terminal α/γ subunit interface of the GABA-A receptor (GABAR). Residues that are believed to form the BZD binding site are located in at least six non-contiguous regions of the α and γ subunits, which are by arbitrary convention designated 'loops' A-F (Galzi and Changeaux, 1995). Although zolpidem binds within the BZD binding site and induces many of the same behavioral effects as other positive modulators of the BZD site, it differs from classical BZDs in both chemical structure (Fig.1) and neuropharmacological profile (Rush, 1998). Zolpidem binds with high affinity to GABARs containing the $\alpha 1$ subunit isoform and low affinity for GABARs containing the $\alpha 2$ or $\alpha 3$ subunits while receptors containing the $\alpha 5$ subunit are virtually zolpidem insensitive (Pritchett and Seeburg, 1990). Furthermore, GABARs containing $\gamma 1$ or $\gamma 3$ subunits exhibit little to no zolpidem sensitivity, regardless of the co-assembled α subunit isoform (Sanna et al, 2002, Lüddens et al, 1994, Barnard et al, 1998). Thus, $\alpha 1\beta x\gamma 2$ GABARs display the highest affinity for zolpidem. In contrast, classical BZDs show a much broader binding profile, as all α subunit isoforms with the exception of $\alpha 4$ and $\alpha 6$, and all γ subunit isoforms bind the classical BZDs flunitrazepam and diazepam. Because classical BZDs and zolpidem show differences in subunit selectivity and possess divergent chemical structures, it is likely that they have different structural requirements for high affinity binding.

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Here, we used radioligand binding and $\gamma2/\alpha1$ chimeric subunits (χ) to identify $\gamma2$ subunit residues important for high affinity zolpidem binding. Although site-directed mutagenesis and photoaffinity labeling studies have made significant strides in uncovering amino acid residues that contribute to the binding domains of classical BZDs, the residues that preferentially contribute to the binding of non-classical BZD-site ligands, such as zolpidem, remain unresolved. A study exploiting the differences in zolpidem sensitivity between $\alpha1$ and $\alpha5$ subunit isoforms identified residues $\alpha1T162$, $\alpha1G200$, and $\alpha1S204$ as contributing to the high zolpidem sensitivity of the $\alpha1$ subunit isoform (Renard et al, 1999). Furthermore, replacing $\alpha1G200$ with residues of increasing side-chain volume correlates with decreasing zolpidem affinity (Wingrove et al, 2002). Hence, steric hindrance may dictate differences in zolpidem affinity across subunit isoforms since the $\alpha1$ subunit contains a glycine at position 200, whereas $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, and $\alpha6$ subunits possess a glutamate (larger in side-chain volume) at the aligned position.

Similar site-directed mutagenesis studies have identified residues on the $\gamma2$ subunit that confer high zolpidem affinity, including F77 (Buhr et al, 1997a; Wingrove et al, 1997), M57 and M130 (Buhr and Sigel, 1997; Wingrove et al, 1997). However, $\gamma2M130$ is not solely selective for zolpidem binding, as mutations of $\gamma2M130$ have also been shown to disrupt high affinity binding of the classical BZDs flunitrazepam and triazolam (Wingrove et al, 1997). Interestingly, introducing both F77 and M130 into the homologous positions in the $\gamma1$ subunit was not sufficient to restore zolpidem affinity to that of $\gamma2$-containing receptors, suggesting that additional $\gamma2$ amino acids are necessary for high zolpidem affinity (Wingrove et al, 1997).

The specificity of binding to different subunit isoforms in part dictates the various behavioral endpoints of BZD ligands. For example, GABARs containing the α1 subunit are associated primarily with the sedative properties of classical BZDs, while α2/3 containing GABARs are responsible for the anxiolytic effects of classical BZDs (see Sieghart and Ernst, 2005 for review). These pharmacological differences may explain why zolpidem, an α1 selective BZD site ligand, is not a clinically efficacious anxiolytic but rather a sedative-hypnotic. Although classical BZDs such as diazepam are effective in treating anxiety, their use is accompanied by a myriad of side-effects including drowsiness, dependency and tolerance. These behaviorally ambiguous effects of classical BZDs are thought to result from a relative lack of selectivity in binding affinity and efficacy to GABARs containing different subunit isoforms. As such, identifying the structural determinants involved in the binding of a more selective BZD-site ligand, such as zolpidem, could help in the design and development of more pharmacologically and behaviorally selective BZD site ligands.

Here, we provide evidence that residues γ2S186-D192, comprising part of loop F of the BZD binding site, are necessary for conferring high affinity zolpidem binding to α1β2γ2 GABARs. In addition, by computational docking of zolpidem and flunitrazepam into a structural homology model of α/γ subunit extracellular interface, we describe possible orientations of these ligands in the BZD binding site of the GABAR.

MATERIALS AND METHODS

Molecular Cloning.

γ2/α1 chimeric subunits χ161 and χ167 were created by linearizing, then recircularizing

a dual plasmid construct in bacteria by random homologous crossover events as detailed in

Boileau et al, 1998. γ2/α1 chimeras χ185 and χ192 and χ198 were created using overlapping

α/γ complimentary oligonucleotides and standard overlap extension recombinant PCR

techniques. Chimeric subunits are numbered according to the amount of mature γ2 N-terminal

sequence contained in the subunit, where, for example, χ161 contains γ2 residues up to and

including amino acid 161 of the mature protein, and residues C-terminal of 161 are α1 subunit

sequence. γ2 subunit point mutations E189R, T193E and R194D were created using standard

recombinant PCR techniques. Wild-type (WT) rat γ2 cDNA in pCEP4 (Invitrogen) was used as

a template for site-directed mutagenesis protocols. Pfu *Ultra* polymerase (Strategene) was used

for PCR amplification at the recommended specifications. Resulting mutant and chimeric

products were subcloned into PCEP4 for transient expression in HEK 293 cells with β2 and γ2s

cDNAs in pCEP4. All mutant constructs were verified by double-stranded DNA sequencing.

Transient expression in HEK293 cells.

HEK293 cells were grown as described previously (Boileau et al, 1998). Cells were co-

transfected at ~70% confluency with WT rat α1, β2, γ2 or mutant subunit cDNAs and, when

necessary, the vector pAdvantage (Promega), at a ratio of 1:1:1:1.5-2 (3-4μg of DNA per subunit

and 6μg of pAdvantage per dish) using a standard $CaHPO_4$ precipitation method (Graham and

van der Eb, 1973).

Radioligand Binding.

Approximately 48hrs after transfection, membrane homogenates were prepared and homologous competition and saturation binding experiments were performed as described in Boileau et al, 1998. In brief, for competition binding, membrane homogenates (100µg) were incubated at room temperature for 1 hr with sub-Kd concentrations of radioligand ([^{3}H]Flunitrazepam, Perkins Elmer Life Sciences) in the absence or presence of 7 different concentrations of unlabeled ligand in a final volume of 250µl. Data were fit by non-linear regression analysis to a single site competition defined by the equation $y = B_{max}/(1+(x/IC_{50}))$, where y is bound ^{3}H ligand in dpm, 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, GraphPD software, San Diego, CA). K_I values were calculated using the Cheng-Prusoff/Chou equation: $K_I=IC_{50}/[1+(L/K_D)]$, where K_I refers to the equilibrium dissociation constant of the unlabled ligand, K_D refers to the equilibrium dissociation constant of the radioactive ligand and L refers to the concentration of radioactive ligand (Prism, GraphPD software, San Diego, CA).

For saturation binding, membrane homogenates were incubated at room temperature with 7-9 concentrations of [^{3}H]flunitrazepam. Data were fit by non-linear regression analysis to a single site using the equation $y=B_{max} \cdot x/(K_D+x)$, where y is specifically bound ^{3}H ligand in dpm, B_{max} is maximal binding, and x is concentration of ^{3}H ligand. In both cases, specific binding was defined as ^{3}H-drug bound in the absence of displacing ligand minus the amount bound in the presence of displacing ligand. Unlabeled flunitrazepam was obtained as a gift from Dr. Sepinwall (Hoffman-La Roche, Nutley, NJ). Unlabeled zolpidem was obtained from RBI/Sigma (Natick, MA).

Automated Ligand Docking.

Our GABAR model was constructed using SYBYL 7.1 (Tripos Inc., St. Louis, MO) as

described previously (Mercado and Czajkowski, 2006). In brief, the extracellular N-terminal

domain of the GABAR was built by residue replacement on the crystal structure of the

acetylcholine binding protein (AchBP) (Brejc et al, 2001). The N-terminal domain was then

merged with a model of the transmembrane domains created analogously from the refined 4 Å

resolution cryo-EM of the nicotinic acetylcholine receptor (Unwin, 2005). Atom potentials were

assigned from the Tripos force field and the entire structure was minimized by the Powell

conjugate gradient method. The resulting structure was then refined by manual adjustment to

correct gross distortions of side-chains and local electrostatic incompatibilities uncovered by

evaluation with the program PROCHECK (Laskowski et al., 1996).

BZD ligands were constructed in SYBYL 7.1 and docked at the $\alpha 1/\gamma 2$ interface using

Flo+ version of QXP (Quick eXPlore) (McMartin and Bohacek, 1997). The initial placement of

the ligand was determined ad-hoc, based on the extensive literature amassed on the requirements

for BZD binding at the α and γ subunit interface, implicating loops A, B and C on the α subunit,

and loops D, and E on the γ subunit. The automated docking strategy used a Monte Carlo search

followed by simulated annealing technique, assuming a vacuum (no solvent) environment for the

ligand-protein interaction. First, the ligands were docked by 1000 cycles of the default Monte

Carlo search, which included both ligand and residue side-chains within 4 Å of the initial ligand

coordinates. Poses were scored by default, according to energy evaluations using the AMBER

force field. The Monte Carlo search yielded twenty-five low energy receptor-ligand complexes

that deviated from one another by at least 0.4 Å root-mean-square-deviation (RMSD). Output

poses were binned into spatial clusters (RMSD less than 2 Å), where all members from a given

cluster represented a single common binding mode. Average cluster energies were then taken

from all members residing in the cluster.

To further optimize receptor-ligand interactions, the lowest energy representative pose

from each cluster was treated with simulated annealing molecular dynamics to further optimize

interactions in the receptor-ligand complex. The simulated annealing procedure consisted of 100

cycles of variable temperature molecular dynamics. In each cycle, the temperature was scaled

from 600 K to 50 K over an interval of 9 ps followed by quenching by the default Polak-Ribiere

conjugate gradients minimization to 0.1 kcal·mol^{-1} Å^{-1}. The lowest energy pose among the 100

generated was selected as the final representative pose. Ligands converged upon the same set of

modes regardless of starting coordinates for docking, suggesting a robust search procedure that is

not strongly influenced by the initial ligand placement. All images were produced using PyMOL

(DeLano Scientific, LLC, San Carlos, CA).

RESULTS

Flunitrazepam and zolpidem binding to αβχ receptors.

Four chimeric γ/α constructs, χ161, χ 167, χ 185, and χ198, containing varying amounts

of γ2 subunit N-terminal sequence (Fig 1) were expressed with WT α and β subunits in HEK 293

cells. Binding affinities (K_I) for flunitrazepam and zolpidem were measured by displacement of

[³H]flunitrazepam binding (Fig 2). Wild-type α1β2γ2 receptors bound flunitrazepam and

zolpidem with a K_I of 8.4 and 56.3 nM, respectively (Table 1). All chimeric receptors retained

WT binding affinity for flunitrazepam (Table 1, Fig 2A), indicating that unique γ2 subunit

residues C-terminal to 161 are not crucial for high affinity flunitrazepam binding. In contrast,

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only $\alpha1\beta2\chi198$ retained WT apparent binding affinity for zolpidem (K_I=54.3 nM), while

$\alpha1\beta2\chi161$, $\alpha1\beta2\chi167$, and $\alpha1\beta2\chi185$ displayed 8-9 fold decreases in zolpidem affinity ($p<0.01$;

Table 1, Fig 2B). Thus, $\gamma2$ subunit residues from the N-terminus to residue 185 are not sufficient

to confer high affinity zolpidem binding to $\alpha1\beta2\chi$ receptors. Because the addition of $\gamma2$ residues

S186-L198 restored zolpidem affinity in $\alpha1\beta2\chi198$ receptors to WT values, we hypothesized

that a subset of residues within this region are required for high affinity zolpidem binding. To

further delineate residues within this region that selectively contribute to high affinity zolpidem

binding, we constructed another γ/α chimera, $\chi192$. $\alpha1\beta2\chi192$ receptors bound zolpidem with

high affinity (K_I=16.5 nM, Table 1), indicating residues S186-D192, in loop F of the $\gamma2$ subunit,

are necessary for conferring high affinity zolpidem binding.

Molecular docking of flunitrazepam and zolpidem.

As depicted in a model of the N-terminal extracellular BZD binding domain of the

GABAR, the $\gamma2$S186-D192 region is part of a lengthy and flexible region of ambiguous

secondary structure known as Loop F/9 (Fig 3A&B). The model provides insight into the

structure of the BZD binding pocket, where loop F is nestled among various residues that have

previously been implicated in BZD binding (Fig 3B), highlighting the potential importance of

loop F in forming part of the binding pocket. We performed simulated molecular docking of

flunitrazepam and zolpidem within the α/γ BZD binding interface to help illuminate possible

residues within loop F that participate in zolpidem binding, and to examine the compatibility

between the predictions made by computational modeling and those conclusions drawn from

experimental observations.

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Flunitrazepam docking yielded three different orientations of the ligand within the binding pocket (Fig 4A&B, Table 2). In contrast, zolpidem docking and subsequent energy minimization converged to a single pose of low energy (-43.5 kJ/mol; Fig 4C). Of the three flunitrazepam poses, the one with the lowest energy (-34 kJ/mol, 32% populated; Table 2) shown in Fig 4A most closely predicts the $\alpha 1$ and $\gamma 2$ residues involved in high affinity BZD binding, as previously determined experimentally. The most obvious difference between the lowest energy (Fig 4A) and most populated (Fig 4B) flunitrazepam poses is in the π-π stacking interactions of the pendant phenyl group of flunitrazepam, which in the lowest energy pose is sandwiched between the aromatic rings of $\alpha 1$Y159 and $\alpha 1$H101 whereas in the most populated pose it stacks exclusively with $\alpha 1$Y209. The variability in flunitrazepam orientation may result from a greater conformational freedom of flunitrazepam relative to zolpidem within the BZD binding site.

Given the technical and methodological constraints of current molecular simulation techniques, it is difficult to fully recapitulate the true biological environment within which protein-protein, and protein-ligand interactions occur. Nevertheless, to gain insight into the potential interactions between zolpidem or flunitrazepam and the GABAR in our docked receptor-ligand complexes, we measured the distances between residue atoms of the protein and atoms of each ligand (Fig5). We assigned proximity ratings to each interaction, where the higher the proximity rating number, the closer the ligand is to a given subunit residue, ranging from 6 (2.5 Å) to 1 (5 Å). Functional groups less than 4 Å of one another possess potential for forming salt bridges and hydrogen bonds (Kumar and Nossinov, 1999), while functional groups greater than 7Å in distance from one another likely do not participate meaningful electrostatic interactions (Schreiber and Ferscht, 1995). Hydrophobic interactions, on the other hand, can occur at much longer distances (Israelachvili&Pashely, 1982).

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Residues α1H101, α1Y159, α1T206, α1Y209, γ2F77, and γ2T142, all previously

implicated in both flunitrazepam and zolpidem binding and/or efficacy (Wingrove et al, 1997;

Mihic et al, 1994; Sigel et al, 1998; Amin et al, 1997; Buhr et al, 1997a; Buhr et al, 1997b), are

located within 4 Å of both flunitrazepam and zolpidem (Fig5A&B). Thus, these residues could

potentially participate in a myriad of non-covalent intermolecular interactions with the ligand,

including van der Waals, salt bridges, and hydrogen bonds. In addition, our docks predict that

α1T162, α1S204, and α1E208 come within 5 Å of zolpidem, but not flunitrazepam (Fig5A), and

potentially participate in longer-range (4-7 Å) molecular interactions with zolpidem.

Interestingly, α1T162 and α1S204 have previously been shown to confer binding selectivity for

zolpidem to the α1 subunit (Renard et al, 1999). That our simulated molecular docking results

are consistent with past experimental findings lends credence to the accuracy of our homology

model and the prediction that flunitrazepam and zolpidem are situated differently within the

BZD binding pocket.

Within Loop F of the γ2 subunit, only R194 was found to come within at least 3 Å of

flunitrazepam (Fig 5B). In the most energetically favorable (lowest energy) pose, R194 comes

within 2.5Å of flunitrazepam (Fig 4A&5B), where the guanidine group of the arginine is

pointing towards the nitro group of flunitrazepam. However, the nitro group of flunitrazepam

(Fig 1A) may also participate in hydrogen bonding with the side-chain functional group of

α1T206, which also comes within 2.5 Å of the substrate (Fig 4A). In the most highly populated

binding mode for flunitrazepam (Fig 4B), R194 is pointing towards the pendant phenyl of

flunitrazepam, coming within 2.5 Å of the substrate. None of the residues in the γ2S186-D192

region are in close proximity to flunitrazepam, falling greater than 5Å away from the substrate

(Fig 4A&B; Fig 5B), which may explain why mutations in this region do not disrupt high affinity flunitrazepam binding.

Our model of the zolpidem pharmacophore predicts that γ2 loop F residue R194 comes within 2.5 Å of zolpidem (Fig 5B). What distinguishes the interaction observed between R194 and zolpidem from that of R194 and flunitrazepam is the potential for strong hydrogen bonding exclusively between the guanidine group of the arginine and the carbonyl oxygen (Fig 1B) on zolpidem (Fig 4C). Additionally, γ2T193 could potentially participate in longer-range molecular interactions with zolpidem, even possibly hydrogen bonding, positioned within 5 Å of the substrate (Fig 4C&5B). Surprisingly, the loop F residues γ2S186-D192 that we have experimentally shown to be important in zolpidem binding do not seem to participate in any direct interactions with zolpidem based on our docking results. However, there are some notable intra-subunit interactions within loop F, including potential electrostatic interactions between R185, E189, and R194 (Fig 4C). It is important to note that a residue can influence ligand binding in multiple ways. Some residues may directly contact the ligand, some may be important for maintaining the structural integrity of the binding site, while others may mediate local conformational movements near the binding site. Therefore, although residues γ2S186-D192 may not directly contact zolpidem, they likely contribute to zolpidem binding by maintaining the structural integrity of this loop region and strategically orienting R194 within the binding pocket. In order to test the importance of residues γ2E189, γ2T193, and γ2R194 in zolpidem binding, we mutated each to their aligned α1 subunit residue. Given our sequence alignment, these residues show the most dramatic divergence in the chemical properties of their side-chains (Fig 6A).

The effects of point mutations in loop F of the γ2 subunit on zolpidem and flunitrazepam binding.

Of the three mutations tested, γ2E189R, γ2T193E, and γ2R194D, none disrupted high affinity zolpidem binding (Fig 6B). Both α1β2γ2T193E and α1β2γ2E189R receptors had affinities for zolpidem comparable to WT (K_I=40 nM and 85.6 nM, respectively), while α1β2γ2R194D receptors displayed a 3.5 fold increase in affinity for zolpidem (K_I=16.6 nM, $p<0.01$; Fig. 6B; Table 3). Therefore, the results suggest that residues γ2E189, γ2T193, and γ2R194 are not individually responsible for conferring high affinity zolpidem binding. Interestingly, γ2E189R resulted in 5.5-fold increase in flunitrazepam affinity (Table 3). Introducing an arginine at position γ2E189 would disrupt the predicted salt-bridge triad with γ2R185 and γ2R194, suggesting that these electrostatic interactions are not necessary for high affinity flunitrazepam or zolpidem binding. Given the purported structural flexibility of loop F, and the presence of other charged residues within the region, it is possible that alternate electrostatic interactions compensate for the introduction of a positive charge at γ2E189.

To compliment our experimental findings, molecular docking of zolpidem was carried out on γ2E189R and γ2R194D mutant receptors. While the binding energy and the position of zolpidem in the binding pocket of the γ2E189R mutant were comparable to wild-type, the amino acid side-chains adopted slightly different orientations within the site, which may explain why the γ2E189R mutation had little effect on zolpidem binding affinity (Table 2). In the γ2R194D mutant background, the binding energy for zolpidem was comparable to that of wild-type receptors and the amino acid side chains maintained similar positions to those in the WT dock. Interestingly, zolpidem adopted a flipped orientation within the site, with the amide

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group on zolpidem coming within 4.5 Å of the carboxyl group of the aspartic acid at position 194. The re-orientation of zolpidem within the binding pocket may account for the inability of the γ2R194D mutation to reduce the apparent affinity of zolpidem (Table 2). When considering our mutagenesis findings in the context of our docking data, it becomes clear that residues S186-D192 are likely not contact residues, since the docking results suggest that these residues are not located near zolpidem and our mutagenesis results indicate that individual point mutations fail to disrupt high affinity zolpidem binding. Taken collectively, these findings indicate that residues within loop F of the γ2 subunit likely work in a synergistic fashion to maintain the proper configuration of the BZD binding site.

DISCUSSION

Multiple methods have been used to identify residues within loops A-E that are involved in the efficacy and/or binding of BZD site ligands. In this study, we demonstrate that γ2S186-D192 in loop F is necessary for high affinity binding of the imidazopyridine zolpidem. In contrast, unique γ2 subunit loop F residues are not required for high affinity flunitrazepam binding, as affinity for flunitrazepam is preserved in α1β2χ167 receptors (Table 1). To our knowledge, this study is the first to identify residues in loop F of the γ subunit that are important for the action of a BZD-site ligand.

Molecular docking reveals that zolpidem and flunitrazepam likely inhabit overlapping, yet partially distinct, locations within the BZD binding pocket. The dockings accurately identify residues in loops A-C of the α1 subunit, and loops D and E of the γ2 subunit, that previous experiments have shown to contribute to the binding of both classical BZDs as well as zolpidem.

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On the α1 subunit, several aromatic residues stabilize zolpidem and flunitrazepam in the binding

site. For example, the aromatic ring α1Y159 (loop B) stacks with the pendant phenyl group of

both flunitrazepam (lowest energy pose, Fig. 4A), and zolpidem (Fig 4C). Removing the

aromatic ring at α1Y159 completely abolishes diazepam-mediated potentiation of GABA current

in α1β2γ2 receptors (Amin et al, 1997), and based on our modeling we predict this residue may

similarly abolish the actions of zolpidem. In addition, the aromatic ring of α1Y209 (loop C)

stacks with the fused phenyl ring on flunitrazepam (Fig 4A) and zolpidem (Fig 4C), potentially

forming π-π interactions. In the most populated flunitrazepam dock (Fig 4B), the pendant

phenyl of flunitrazepam also participates in π-π stacking with α1Y209, underscoring the

importance of an aromatic residue at this position. Studies have shown that an aromatic ring at

position 209 is crucial for maximal diazepam mediated modulation of GABA current as well as

high affinity zolpidem, flunitrazepam and diazepam binding in α1β2γ2 GABARs (Amin et al,

1997; Buhr et al, 1997b). In our dock, a potential hydrogen bond between α1T206 (loop C), and

the nitro group (Fig 1A) of flunitrazepam (lowest energy pose, Fig 4A), or the fluorine of the

pendant phenyl (most populated pose, Fig 4B) is also observed. Previous findings have shown

that the mutation α1T206A significantly disrupts high affinity binding of classical BZDs, as well

as zolpidem (Sigel et al, 1998; Buhr et al, 1997b). Interestingly, our model predicts that

α1T206C and the carbonyl oxygen of zolpidem interact via hydrogen bonding, suggesting that

α1T206C may also influence zolpidem affinity (Fig 4C).

Although there is a significant amount of overlap in residues forming the recognition site

for flunitrazepam and zolpidem, our findings suggest that there are differences in their binding

requirements. On the alpha subunit, α1H101 (loop A), α1T162 (loop B), and α1S204 (loop C)

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lie within the core of the zolpidem binding pocket. Our docking results reveal the pendant

phenyl of both zolpidem and flunitrazepam participate in π-π stacking with the aromatic ring of

α1H101 (loop A) (Fig 4A&C). α1H101 has long been noted as a crucial residue in high affinity

classical BZD binding. Zolpidem is also sensitive to mutations at this position. For example,

mutating α1H101 to arginine abolishes flunitrazepam and zolpidem binding, as well as the *in*

vivo behavioral effects of zolpidem (Crestani et al, 2000; Wieland et al, 1992). However, the

converse mutation at the aligned position in the α6 subunit (α6R100H), which restores

sensitivity of α6β2γ2 receptors to classical BZDs, does not restore high affinity zolpidem

binding (Wieland et al, 1992). Additionally, GABARs photoincorporated with flunitrazepam

have drastically reduced affinities for classical BZDs, whereas the binding affinity of zolpidem

remains intact (McKernan et al, 1998). These data suggest that zolpidem may have more

tolerance for substitutions at α1H101, and requires additional residues in the α1 for high affinity

binding. We predict that two residues in particular, α1T162 and α1S204, are selectively

involved in zolpidem binding because they reside within at least 4.5 Å of zolpidem, but not

flunitrazepam (Fig 5). α1S204 in particular comes within 4 Å of zolpidem, making this residue a

candidate for hydrogen bonding with the substrate. Replacing the corresponding residues in the

zolpidem-insensitive α5 subunit with the aligned residues from the α1 subunit (i.e.,

α5P166T& α5T208S) significantly increased zolpidem affinity in α5β2γ2 mutant receptors

(Renard et al, 1999), further supporting our predictions.

On the γ2 subunit, F77 (loop D) is positioned within 4 Å of zolpidem as well as

flunitrazepam, and is likely involved in hydrophobic and/or electrostatic interactions with both

ligands (Fig 4&5). Mutagenesis, radioligand binding, and studies on γ2F77I transgenic knock-in

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mice indicate that γ2F77 is critical for the high affinity binding of classical BZDs and zolpidem, as well as the *in vivo* behavioral effects of zolpidem (Cope et al, 2004; Ogris et al, 2004; Sigel et al, 1998; Wingrove et al, 1997). In addition, γ2R144 (loop E) is positioned within 4 Å of both flunitrazepam and zolpidem and potentially H-bonds with the carbonyl oxygen of flunitrazepam (Fig. 4A). Interestingly, γ2R144 appears to be involved in a potential intersubunit salt-bridge with α1E137 and may be important for stabilizing the structure of the BZD binding site (Fig 4A-C). A study by Harrison and Lummis (2006) suggests that the aligned residue in □he GABA-C receptor ρ1 subunit (ρ1R170, i.e. GABAR γ2R144) is important for proper receptor function and is likely involved in intersubunit salt-bridge interactions. Finally, γ2T142 (loop E) is positioned within 4.5 Å of both flunitrazepam and zolpidem (Fig 5), and has previously been shown to influence the efficacy of classical BZDs as well as zolpidem (Mihic et al, 1994).

Our docking and experimental findings indicate that the binding requirements for zolpidem and flunitrazepam also differ in the loop F region of the γ2 subunit. γ2T193 and γ2R194 are positioned within 5 Å of zolpidem (Fig 5B). Although γ2R194 is also located near flunitrazepam, we speculate that it plays a more critical role in orienting zolpidem, as the guanidine group of the arginine likely participates in a hydrogen bond with the carbonyl oxygen (Fig 1B) of zolpidem (Fig 4C). Furthermore, α1β2γ2R194D receptors bound zolpidem with even higher apparent affinity than WT, while flunitrazepam apparent affinity was unaltered, supporting the conclusion that this residue may play a more critical role in dictating zolpidem affinity. It is possible that a negatively charged aspartate at position 194 preferentially or more favorably interacts with the partial positive charge of the amide moiety on zolpidem (Fig 1B), instead of the carbonyl oxygen, since the smaller side chain volume of the aspartate would be

able to better accommodate the bulkier amide group. Binding studies using a variety of

phenylimidazopyridine derivatives have shown that shortening the methylene linker or

lengthening the carbon chain before the amide group (Fig 1B) of the ligand results in a

significant decrease in apparent affinity (Trapani et al, 1997). The spacer length between the

imidazopyridine nucleus and the amide group is likely important for correctly positioning the

hydrogen bond accepting site, the carbonyl oxygen of zolpidem, within the BZD binding pocket.

Consistent with this hypothesis, when we performed molecular docking of zolpidem in the

α1β2γ2R194D receptor background, zolpidem assumed a different orientation within the binding

pocket such that the amide group of zolpidem moved within 4.5 Å of the carboxyl side-chain of

the aspartate at position 194.

 We demonstrate experimentally that γ2S186-D192 is important for high affinity zolpidem

binding. Based on molecular docking, these residues do not come within 5 Å of zolpidem (Fig

5), suggesting that residues S186-D192 contribute to zolpidem binding not by forming direct

contacts with the ligand, but by maintaining the secondary structure of the binding site. It is

important to note, however, that the positioning of loop F is open to interpretation as the crystal

structure of AChBP, upon which our model is based, is poorly resolved within loop F (Brejc et

al, 2001). Furthermore, loop F protein sequence is poorly conserved among GABAR subunit

isoforms, and other ligand gated ion channels, making sequence alignment difficult. It is

possible that γ2S186-D192 plays a role in zolpidem binding by a complex series of interactions,

perhaps serving to properly position γ2R194 in the binding pocket. Loop F is likely a highly

mobile and adaptable structure, thus garnering the ability to compensate for conservative

alterations in sequence identity. Our finding that point mutations in the γ2 loop F region do not

disrupt high affinity zolpidem binding supports our hypothesis, suggesting that multiple loop F

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residues act together to preserve the structure of the BZD site and stabilize zolpidem within the binding pocket.

Our finding that Loop F of the γ2 subunit is involved in BZD binding compliments previous reports that residues in the homologous regions of the GABA binding site interface, as well as the related nicotinic acetylcholine (nACh) and 5-HT3 receptors, participate in orthosteric agonist binding (Newell and Czajkowksi, 2003; Lyford et al, 2003; Thompson et al, 2006). Studies using molecular dynamics simulation, hydrophobic photolabeling, and SCAM have shown that Loop F of AChBP, as well as the nACh, GABA-A, and 5-HT3 receptors, undergoes conformational changes during channel activation and/or agonist binding (Gao et al, 2005; Lyford et al, 2003; Leite et al, 2003; Newell and Czajkowski, 2003; Thompson et al, 2006). Given these findings, it is reasonable to hypothesize that loop F of the γ subunit undergoes structural rearrangements upon binding of BZDs. It is possible that loop F is important for regulating not only BZD binding, but also BZD efficacy. These are intriguing possibilities that need to be explored in future experimental endeavors.

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FOOTNOTES

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LEDGENDS FOR FIGURES

Figure 1. **Chemical structures of flunitrazepam (A), zolpidem (B), and schematic representations of chimeric (χ) $\gamma 2/\alpha 1$ subunits (C) used in this study.** In chimeric subunits (C), N-terminal $\gamma 2$ sequences are highlighted in grey, and the remaining C-terminal $\alpha 1$ sequences are highlighted in white, with the transmembrane domains lableled (T1, T2, T3, T4). Chimeras are numbered according to the amount of mature $\gamma 2$ sequence contained in the subunit, where $\chi 161$ contains $\gamma 2$ residues up to and including amino acid 161 of the mature protein sequence and residues C-terminal of 161 are $\alpha 1$ sequence.

Figure 2. **$\gamma 2/\alpha 1$ chimeric subunit constructs differentially affect zolpidem binding in $\alpha 1\beta 2\chi$ receptors.** Representative radioligand binding curves depict the displacement of [3H]flunitrazepam binding by flunitrazepam (A) and zolpidem (B) in $\alpha 1\beta 2\gamma 2$ WT ($\blacklozenge$), $\alpha 1\beta 2\chi 167$ (Δ), $\alpha 1\beta 2\gamma 2\chi 185$ ($\square$), and $\alpha 1\beta 2\gamma 2\chi 198$ ($\bigcirc$) receptors, where each point is the mean+/- SEM of triplicate measurements. Data were fit by non-linear regression analysis as described in *Materials and Methods*. K_I values are reported in TABLE 1.

Figure 3. **Homology model of the extracellular $\alpha 1/\gamma 2$ subunit interface of the GABAR.** The $\alpha 1$ subunit is pink, the $\gamma 2$ subunit is purple, and Loop F is highlighted in yellow (A&B). A close-up view of the BZD binding pocket is illustrated in (B), where several residues previously implicated in benzodiazepine binding are highlighted in teal. Data from this study suggest that

residues within loop F of the γ2 subunit, γ2S186-D192A shown in yellow, are involved in conferring high affinity zolpidem binding to α1β2γ2 GABARs.

Figure 4. **Molecular docking of flunitrazepam (A&B), and zolpidem (C).** (A) The lowest energy orientation of flunitrazepam within the binding site, after Monte Carlo (MC) and simulated annealing (SA) analysis. (B) An alternative, most highly populated (based on the MC), binding mode for flunitrazepam after MC and SA (see *Materials and Methods*). (C) The orientation of zolpidem after MC and SA. Panels on the right side are zoom-in views of the docked ligands an A, B and C. The α1 subunit is pink, and the γ2 subunit is purple. The substrate is colored such that carbon is white, nitrogen is blue, oxygen is red, and fluorine is light blue. Loop F residues γ2R185-R197 are highlighted in yellow. Residues within 3 Å of the docked ligand are highlighted in teal. Results from the molecular docking procedure are summarized in TABLE 2.

Figure 5. **Proximity of BZD binding site residues to flunitrazepam and zolpidem.** The distances between various amino acid side-chains and flunitrazepam (lowest energy pose in white, and most populated mode in grey) or zolpidem (black) on the α1 subunit (A) and the γ2subunit (B) were measured. The proximity ratings of these residues to the docked ligands are plotted, where 1=5Å, 2=4.5Å, 3=4Å, 4=3.5Å, 5=3Å, and 6=2.5Å. Distances were measured from the atom on the residue that is closest to the docked ligand. Note that within Loop F of the γ2 subunit, two residues come within 5Å of zolpidem (γ2T193 and γ2R194).

Figure 6. **Point mutations in Loop F of the γ2 subunit do not disrupt high affinity zolpidem**

binding. In (A), sequence alignments of the loop F region of WT α1 and γ2 subunits and the

chimeric γ2χ192 subunit are shown. γ2 sequence is depicted in black and α1 sequence is

depicted in grey, where each star represents a residue in α1 that does not have a corresponding

residue in γ2. Residues in loop F of the γ2 subunit implicated in zolpidem binding by molecular

docking (circled) were mutated to their corresponding α1 residue. Of these residues, one

(γ2E189) is contained within the region defined by our study (γ2χ192), as being selectively

involved in zolpidem binding. In (B), representative radioligand binding experiments depict

displacement of [^{3}H]flunitrazpam binding by zolpidem in α1β2γ2 WT (◆) and α1β2γ2E189R

(□), α1β2γ2T193E (○), and α1β2γ2R194D (Δ) mutant receptors, where each point is the mean

+/-SEM of triplicate measurements. Data were fit by non-linear regression analysis as described

in *Materials and Methods*. K_I values for zolpidem and flunitrazepam are summarized in

TABLE 3.

TABLES

TABLE 1. Binding affinities of flunitrazepam and zolpidem for α1β2γ2□ WT and

α1β2χ receptors.

Values shown are the mean±SEM for N number of independent experiments. K_I is the

equilibrium dissociation constant (apparent affinity) of the unlabeled ligand. K_I values for

flunitrazepam were determined by [^{3}H]flunitrazepam saturation and competition binding

experiments. K_I values for zolpidem were determined by the displacement of [^{3}H]flunitrazepam.

Statistical differences between WT and chimera $\log K_I$ values were determined using one-way

ANOVA with Dunnett's post-test.

GABAR	Flunitrazepam		Zolpidem	
	K_I (nM)	N	K_I (nM)	N
α1β2γ2s	8.4±1.2	35	56.3±3.8	7
α1β2χ161	10.9±2.0	4	478±13**	4
α1β2χ167	12.8±2.3	7	486±18**	3
α1β2χ185	14.1±22	3	518±73**	3
α1β2χ192	3.7±0.6	3	16.5±1.5**	3
α1β2χ198	7.8±10	4	54.3±18	4

** Significantly different from α1β2γ2 WT ($p<0.01$)

TABLE 2. Zolpidem and flunitrazepam docking energies.

Docking simulations were carried out using the Flo+ version of QXP (Quick eXPlore), as detailed in *Materials and Methods*. Docking yielded one orientation of zolpidem and three orientations of flunitrazepam (1-3). The lowest and population average energies after the Monte Carlo (MC) and after simulated annealing (SA) procedures are shown (see *Materials and Methods*). Values are the mean±SEM, where error is reported.

Pose (Binding Mode)	MC population (%)	MC Energy (kJ/mol)	MC Avg. Energy (kJ/mol)	SA population (%)	SA Energy (kJ/mol)	SA Avg. Energy (kJ/mol)
Zolpidem1	100	-43.5	-38.4±0.6	100	-60.2	-56.5±2.1
Flunitrazepam1	32	-34.1	-32.0±0.4	100	-52.8	-45.7±3.2
Flunitrazepam2	60	-33.1	-31.0±0.2	100	-44.3	-40.4±1.8
Flunitrazepam3	8	-31.4	-31.6±0.1	----	----	----

TABLE 3. Binding affinities of flunitrazepam and zolpidem for α1β2γ2s WT and

α1β2γ2s mutant receptors.

Values shown are the mean±SEM for N number of independent experiments. K_I is the equilibrium dissociation constant (apparent affinity) of the unlabeled ligand. K_I values were determined by the displacement of [^{3}H]flunitrazepam. Statistical differences between WT and chimera $logK_I$ values were determined using one-way ANOVA with Dunnett's post-test.

GABAR	Flunitrazepam		Zolpidem	
	K_I (nM)	N	K_I (nM)	N
α1β2γ2s	8.4±1.2	35	56.3±3.8	7
α1β2γ2E189R	1.5±0.6**	3	61.8±16	3
α1β2γ2T193E	6.4±2.0	3	40.0±6.5	4
α1β2γ2R194D	3.9±1.0	3	16.6±4.2**	4

** Significantly different from α1β2γ2 WT ($p<0.01$)

FIGURE 1.

A.
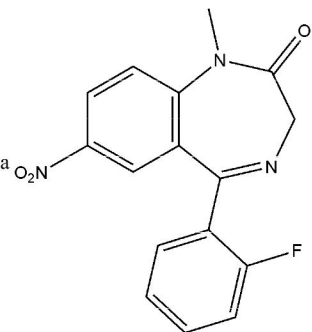
B.
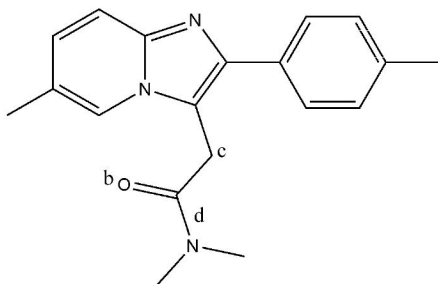
a. Nitro group b. Carbonyl Oxygen c. Methylene linker d. C-C bond before amide

C.

FIGURE 2.

A.

B.

FIGURE 3.

A.

α1   γ2

B.

M130

T206

F77

G203   S204

H101

S186

D192

FIGURE 4.

FIGURE 5.

A.

B.

FNZM: lowest energy dock
FNZM: highest frequency dock
Zolpidem dock

FIGURE 6.

A.

B.