Four Amino Acid Exchanges Convert a Diazepam-Insensitive, Inverse Agonist-Preferring $GABA_A$ Receptor into a Diazepam-Preferring $GABA_A$ Receptor

Heike A. Wieland† and Hartmut Lüddens*

Laboratory for Molecular Neuroendocrinology, Center for Molecular Biology, Heidelberg, Germany

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Benzodiazepines (BZ) exert their effects through $GABA_A$ receptors, which belong to the superfamily of ligand-gated ion channels. Coexpression of recombinant α, β, and γ subunits in a cell culture system mimics the BZ binding sites. The α variants largely determine the nature of the BZ binding site in such $\alpha i\beta j\gamma k$ heteromultimers (i = 1–6; j = 1–3; k =1–3). Notably, the α1 and α6 variants confer high and low affinity for BZ agonists to the resulting receptor subtype, respectively. Glycine/glutamate and histidine/arginine positions in the α subunits of $\alpha x\beta 2\gamma 2$ receptors are involved in BZ I versus BZ II type selectivity. We now identify four amino acids in α6 which together increase the affinity of the mutant $\alpha x\beta 2\gamma 2$ receptor for classical BZ receptor agonists above the level seen for any wild-type $GABA_A$/BZ receptor. The most pronounced effect was due to an isoleucine to valine exchange. It simultaneously decreased the affinity for the BZ partial inverse agonist Ro 15-4513 20-fold and increased the affinity for diazepam 4-fold. The four amino acid residues stretch over most part of the N-terminal extracellular domain of the α subunit, suggesting that amino acids distant in the primary sequence form the BZ binding pocket.

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system.[1] It gates a chloride channel intrinsic to $GABA_A$ receptors. Several compounds allosterically modulate the activity of the channel opened by GABA,[1,2] the most prominent being the group of benzodiazepines (BZ). These and functionally similar but structurally diverse drugs are clinically important in the treatment of neuropsychiatric diseases, e.g., epilepsy, insomnia, and anxiety.[2]

Molecular cloning has revealed considerable diversity for the proteins assumed to participate in the formation of $GABA_A$ receptors. The mammalian subunits are grouped into the four classes α, β, γ, and δ according to their sequence identity, with six variants constituting the α and three each the β and γ classes.[3–6] Coexpression of subunits from the α, β, and γ classes leads to a large variety of $GABA_A$ receptors with functional, BZ-responsive ion channels. Indeed, the BZ receptor ligand Cl 218,872 (3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine) preferentially binds to a $GABA_A$ receptor subtype classically termed BZ I but recognizes with reduced affinity BZ type II receptors.[7–9] The two receptor types can be mimicked by $GABA_A$ receptors expressed in cultured mammalian cells from cDNAs encoding an α1, α2, α3, or α5 variant, any β variant (βx), and the γ2 subunit.[10,11] In these cells, $\alpha 1\beta x\gamma 2$ receptors display BZ type I pharmacology, whereas $\alpha 2\beta x\gamma 2$, $\alpha 3\beta x\gamma 2$, and $\alpha 5\beta x\gamma 2$ receptors are BZ type II receptors.[10,12] However, α4 and α6 subunits define $\alpha x\beta x\gamma 2$ receptors with virtually no affinity to classical BZ agonists like flunitrazepam (5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2*H*-1,4-benzodiazepin-2-one) and diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one)[13–15] but retained high affinity to the inverse agonist Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate).[15–17]

The pharmacology of $GABA_A$/BZ receptors of the $\alpha x\beta x\gamma 2$ group is largely determined by two amino acids at key positions in the α subunits. A glycine/glutamate exchange switches between BZ receptor type I and II,[18] and a histidine corresponding to position 100 in α6 is essential for the binding of BZ agonists to $GABA_A$ receptors.[15,19]

Here we provide evidence that a valine/isoleucine exchange in an α6-derived mutant increases the affinity for diazepam and flunitrazepam and drastically decreases the affinity for the partial inverse agonist Ro 15-4513. The mutated α6 variant, carrying four amino acid alterations, leads to $GABA_A$ receptors whose rank order of potency for benzodiazepine binding properties is reversed as compared to $\alpha 6\beta 2\gamma 2$ receptors. This $GABA_A$ receptor may prove helpful in modeling the $GABA_A$ receptor binding pocket for BZ agonists.

Materials and Methods

Mutants and Chimera. The following antisense oligonucleotides were synthesized for use in site-directed mutagenesis (Amersham mutagenesis kit):

$\alpha 4^{his99}$ 5′-TCTTTCCATTGTGGAAGAAAGTATCCGGAGTCCAAACTT-3′

$\alpha 6^{his100}$ 5′-GACTTTTTCCCATTGTGGAAAAATGTGTCGGGAGTCC-3′

$\alpha 6^{his100,thr161}$ 5′-CGCTTTTCGTATAGGCGTAGCTCCC-3′

$\alpha 6^{his100,thr161,gly199}$ 5′-CGATTTAATGGTACCACTAGAAAC-3′

$\alpha 6^{his100,thr161,gly199,val210}$ 5′-GTGGAAGTACACTGTCATTACTACATATTC-3′

* Send correspondence to: Dr. Hartmut Lüddens, Laboratory for Molecular Neuroendocrinology, Center for Molecular Biology, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany. Tel.: *49-6221-566894. FAX: *49-6221-565894.

† Present address: Abt. Molekularpharmakologie, Thomae GmbH, Biberach/Riss, Germany.

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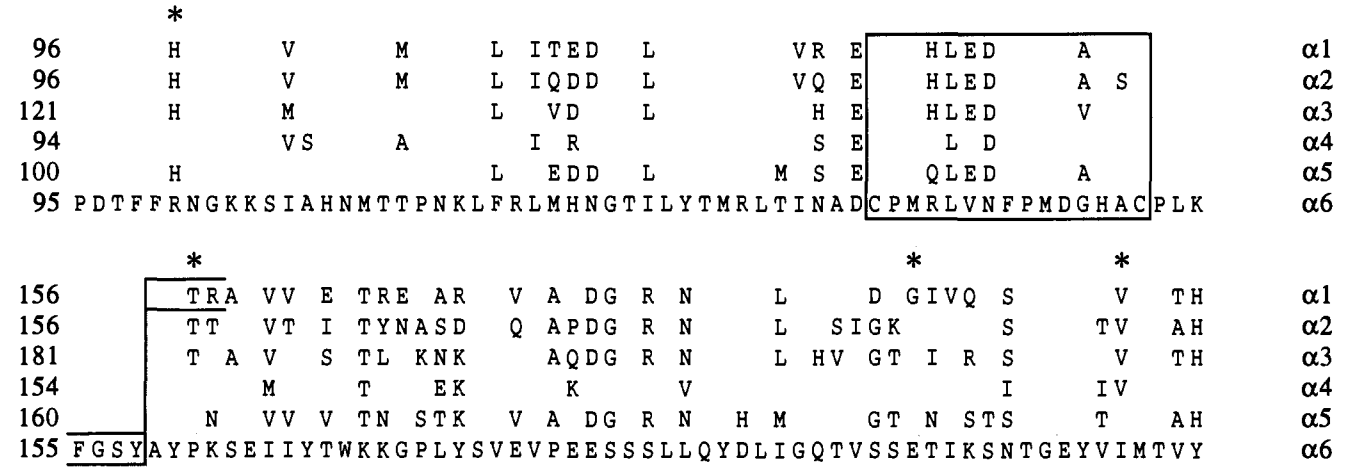

Figure 1. Wild-type, mutated, and chimeric $GABA_A$/BZ α variant N-terminal sequences. Shown is an alignment of the second half of the extracellular N-terminal domain of wild-type α1–α5 $GABA_A$/BZ receptor subunits to the α6 sequence. Numbers to the left indicate amino acid positions of individual proteins. Missing amino acids are identical to α6. The cysteine–cysteine loop is boxed. The transition of the α6 to α1 sequence for chimera C6 is indicated in the lower left of the figure. Exchanges in α6 to the corresponding amino acid of α1 are indicated by stars.

Table 1. K_i Values of Wild-Type $GABA_A$ Receptors in the Form $\alpha x\beta 2\gamma 2$ (Shown are the means ± SEM of three experiments in nanomolar)

	diazepam	Cl 218,872	zolpidem	Ro 15-1788	Ro 15-4513	β-CCM
α1	16 ± 0.5[a]	108 ± 28[a]	19 ± 4[b]	0.5 ± 0.2[a]	15 ± 4[c]	0.8 ± 0.1[c]
a3	17 ± 2[a]	1620 ± 270[a]	400 ± 40[b]	0.6 ± 0.2[a]	nd	4 ± 1[a]
α4	>10 000[d]	>10 000[d]	>10 000	94 ± 27	5 ± 1[d]	nd
α5	15 ± 1	490 ± 120[b]	>15 000	0.5 ± 0.1	0.38 ± 0.03	27 ± 5[b]
α6	>10 000[c]	>10 000	>10 000[c]	90 ± 20[c]	5.4 ± 0.4[c]	2050 ± 20[c]

[a]Pritchett et al., 1989. [b]Pritchett and Seeburg, 1990. [c]Lüddens et al., 1990. [d]Wisden et al., 1991. nd = not determined.

Chimera C6 was constructed by exchange of fragment $\alpha 6^{his100}(1-158)$ with the corresponding fragment in α1 after introduction of appropriate restriction sites.[15] All mutants and chimeric receptor sequences were subcloned into an eukaryontic expression vector under the transcriptional control of a cytomegalovirus promoter.[12] For transfection, plasmids were purified by cesium chloride density gradient centrifugation.

Transfection and Membrane Preparation. Expression vectors coding for mutant α variants and the rat β2 and γ2 subunits were transfected in triple combination into human embryonic kidney 293 cells (ATCC CRL 1573) as described earlier.[15] Cells were harvested 48 h after transfection by washing with ice-cold 50 mM phosphate-buffered saline, pH 7.4, and centrifuged at 150*g*. Cell pellets were frozen at −80 °C. For immediate use, they were homogenized in an Ultraturrax homogenizer for 15 s in 50 mM potassium phosphate buffer (PPB), pH 7.0, centrifuged at 23000*g*, and resuspended in an appropriate volume of PPB.

Binding Assays. Resuspended cell membranes (final concentration 20–50 μg/tube) were incubated in a final volume of 0.5 mL of PPB with 6 nM [^{3}H]Ro 15-4513 or 2 nM [^{3}H]flunitrazepam (DuPont-New England Nuclear) and the competing ligand at varying concentrations. Ro 15-1788 (ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) at 10 μM determined nonspecific binding. After 1 h at 4 °C, the duplicate samples were rapidly filtered through glass fiber filters (Schleicher & Schuell, no. 34 or 52) presoaked in 0.3% poly(ethylenimine). After two washing steps with 5 mL of PPB, the filter-retained radioactivity was determined by liquid scintillation counting. The Inplot program (GraphPad Software, San Diego, CA) was used to calculate the best-fitting values for the parameters of the saturation (K_D) and displacement curves (IC_{50}). IC_{50} values were converted to K_i values according to the Cheng–Prusoff equation.[20]

Results

We constructed a chimeric α variant, C6, which obtained its N-terminus up to the cysteine loop from the $\alpha 6^{his100}$ sequence and the C-terminus from the α1 sequence (Figure 1). Cotransfection of C6 with vectors coding for the rat β2 and γ2 subunits resulted in the expression of [^{3}H]Ro 15-4513 receptors with K_i values for the non-benzodiazepines zolpidem and Cl 218,872 comparable to those for $\alpha 3\beta 2\gamma 2$ receptors, but $C6\beta 2\gamma 2$ receptors did not reach the high affinity of $\alpha 1\beta 2\gamma 2$ receptors for these two ligands (Tables 1 and 2).

We considered three amino acid substitutions between the α1 and $\alpha 6^{his}$ sequences in the region between the cysteine loop and the first transmembrane region (Figure 1) as most likely to be involved in the switch to medium affinity for zolpidem and high affinity for diazepam and Cl 218,872, respectively. A threonine is present in $\alpha 1\beta 2\gamma 2$, $\alpha 2\beta 2\gamma 2$, and $\alpha 3\beta 2\gamma 2$ receptors at positions corresponding to 162 in α1. In the zolpidem-insensitive receptor subtypes $\alpha 5\beta 2\gamma 2$, and $\alpha 6^{his100}\beta 2\gamma 2$, this amino acid is replaced by a proline (Figure 1). We introduced the threonine at position 161 into the $\alpha 6^{his100}$ sequence yielding the $\alpha 6^{his100thr161}$ variant. Cotransfecting this mutant with the β2 and γ2 subunit vectors in 293 cells, we obtained receptors with the high affinity for diazepam seen in $\alpha 1\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$, and $\alpha 5\beta 2\gamma 2$ receptors, but the affinity of the resulting $GABA_A$ receptor for the partial agonists zolpidem and Cl 218,-872 did not change with respect to $\alpha 6^{his100}\beta 2\gamma 2$ (Tables 1 and 2). Additionally, the affinities of the antagonist Ro 15-1788, the partial inverse agonist Ro 15-4513, and the full inverse agonist β-CCM (3-methyl-β-carboline) were unaffected by this amino acid substitution (Table 2).

We further exchanged a glutamate for a glycine at position 199 of the $\alpha 6^{his100thr161}$ variant. The analogous position in the α3 sequence had been shown to confer

Table 2. K_i Values of Mutant and Chimeric $GABA_A$ Receptors in the Form $\alpha x\beta 2\gamma 2$ (Shown are the means ± SEM of three experiments in nanomolar)

	diazepam	Cl 218,872	zolpidem	Ro 15-1788	Ro 15-4513	β-CCM
$\alpha 1^{arg}$	>10 000[a]	>10 000[a]	>10 000[a]	106 ± 31[a]	10 ± 4[a]	nd
$\alpha 4^{his}$	23 ± 6	720 ± 220	76 ± 2	0.6 ± 0.1	1.9 ± 0.5	9 ± 3
$\alpha 6^{his}$	98 ± 3[a]	6000 ± 1,300[a]	>10 000	17 ± 2[a]	7 ± 1[a]	45 ± 2
$\alpha 6^{his,thr}$	20 ± 3	4,300 ± 930	>10 000	17 ± 4	6 ± 1	39 ± 1
$\alpha 6^{his,thr,gly}$	9 ± 1	620 ± 100	980 ± 90	15 ± 3	8 ± 1	8 ± 4
$\alpha 6^{his,thr,gly,val}$	2.1 ± 0.1	780 ± 80	1400 ± 200	33 ± 13	190 ± 33	11 ± 2
chimera C6	20 ± 6	160 ± 30	125 ± 50	0.6 ± 0.2	2.0 ± 0.5	1.1 ± 0.3

[a]Wieland et al., 1992. nd = not determined.

high-affinity binding of BZ I-selective agonists, i.e., Cl 218,872 and zolpidem, to $\alpha 3^{gly}\beta 2\gamma 2$ receptors.[18] Similarly, $\alpha 6^{his100thr161gly199}\beta 2\gamma 2$ receptors exhibited an increased affinity for the BZ I-selective compounds β-CCM, Cl 218,872, and zolpidem (Table 1). None of these compounds bound to $\alpha 6^{his100thr161gly199}\beta 2\gamma 2$ receptors with the high affinity seen for $\alpha 1\beta 2\gamma 2$ or $C6\beta 2\gamma 2$ receptors. However, the affinities for diazepam and β-CCM increased significantly, with diazepam reaching a level 2-fold higher than for any wild-type receptor, but the affinities of $\alpha 6^{his100}\beta 2\gamma 2$, $\alpha 6^{his100thr161}\beta 2\gamma 2$, and $\alpha 6^{his100thr161gly199}\beta 2\gamma 2$ receptors for the antagonist Ro 15-1788 and the inverse agonist Ro 15-4513 were identical (Table 2).

$\alpha 4\beta 2\gamma 2$ receptors are insensitive to diazepam but recognize [^{3}H]Ro 15-4513 with high affinity.[14] Exchange of arginine[99] to histidine resulted in $\alpha 4^{his99}\beta 2\gamma 2$ receptors with affinities for the agonist diazepam, the antagonist Ro 15-1788, and the inverse agonist Ro 15-4513 that were in the same range as those for $\alpha 1\beta 2\gamma 2$, $\alpha 3\beta 2\gamma 2$, and $C6\beta 2\gamma 2$ receptors but higher than those for $\alpha 6^{his100}\beta 2\gamma 2$ receptors (Tables 1 and 2). Furthermore, the affinities of $\alpha 4^{his99}\beta 2\gamma 2$ for the non-benzodiazepines zolpidem and Cl 218,872 put this receptor closer to $\alpha 1\beta 2\gamma 2$ than to $\alpha 3\beta 2\gamma 2$ receptors, i.e., they resembled more BZ type I than type II receptors. Sequence comparison of α variants between the cysteine loop and the first transmembrane region led us to isoleucine[211] of α6 which is replaced by valine in α1, α2, α3, and α4. We therefore modified the α6 variant to yield $\alpha 6^{his100thr161gly199val211}$. When coexpressed with β2 and γ2, it recognized [^{3}H]flunitrazepam with a K_D = 0.67 ± 0.07 nM (n = 4) and diazepam with a K_i = 2.1 ± 0.1 nM (Table 2). However, the K_i value for the partial inverse agonist Ro 15-4513 rose to 190 ± 50 nM. The affinities of the mutant receptor for the non-BZs zolpidem, Cl 218,872, and β-CCM were not significantly affected (Tables 1 and 2). Thus, $\alpha 6^{his100thr161gly199val211}\beta 2\gamma 2$ receptors recognized the clinically important BZ receptor ligands flunitrazepam and diazepam with a high affinity, whereas wild-type $\alpha 6\beta 2\gamma 2$ receptors are prototypic diazepam-insensitive and flunitrazepam-insensitive $GABA_A$/BZ receptors.

Discussion

Most clinically important benzodiazepine receptor ligands act as anxiolytics, sedatives, muscle relaxants, and anticonvulsants. Two different BZ receptor subtypes have been defined by their affinities for selective ligands, e.g., BZ type I receptors have a high affinity for 2-oxoquazepam, Cl 218,872, and zolpidem, whereas BZ type II receptors have a lower affinity for these ligands.[21-23] The partial inverse agonist Ro 15-4513 is one of the few BZ receptor ligands which recognize all known BZ receptors with similar affinity, including the diazepam-insensitive $GABA_A$ receptor of cerebellar granule cells.[11,13,24]

Previously it was shown that a glutamate to glycine exchange in the α3 variant switches between BZ receptor types II and I. Furthermore, arginine[100] in α6 hinders the binding of BZ agonists like diazepam to $\alpha 6\beta 2\gamma 2$ receptors.[15,18,19] We extended the search for amino acids in the α6 variant of $GABA_A$/BZ receptors to identify residues which enhance agonist binding in order to gain further insight into the structural requirements of the BZ agonist pharmacophore.

$\alpha 4\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ contain arginine in place of histidine at homologous positions, and both are BZ agonist insensitive.[14,15] Mutant $\alpha 4^{his99}\beta 2\gamma 2$ receptors were diazepam-sensitive as are their $\alpha 6^{his100}\beta 2\gamma 2$ counterparts (Table 2), underlining the importance of the histidine position. Otherwise, the two mutants differed strikingly. $\alpha 6^{his100}\beta 2\gamma 2$ receptors displayed micromolar affinity for Cl 218,872 but did not recognize zolpidem, by this resembling $\alpha 5\beta 2\gamma 2$ receptors,[12] whereas $\alpha 4^{his99}\beta 2\gamma 2$ receptors were Cl 218,872 and zolpidem sensitive. The latter showed high affinity for the antagonist Ro 15-1788 and the agonist diazepam as well seen in $\alpha 1/\alpha 2/\alpha 3/\alpha 5\beta 2\gamma 2$ but reduced in $\alpha 6^{his100}\beta 2\gamma 2$ receptors (Tables 1 and 2). Therefore, the histidine is essential for BZ receptor agonist binding, e.g., Cl 218,-872 and diazepam, but not sufficient for the capability to bind zolpidem or to reach an affinity for diazepam in the 10 nM range.

For the C6 chimera, we replaced the region between the N-terminus and amino acid 160 of the α1 variant with that of the $\alpha 6^{his100}$ mutant. $C6\beta 2\gamma 2$ receptors were zolpidem sensitive, and the affinity for Cl 218,872 and diazepam reached the levels of $\alpha 1\beta 2\gamma 2$ receptors (Table 1). From previous studies we could exclude the C-terminal transmembrane region of the α variants as containing amino acids important for the BZ affinity of $\alpha x\beta 2\gamma 2$ receptors.[15,18] Therefore, the region between the cysteine loop and the first transmembrane region (Figure 1) seems to determine the high-affinity binding for diazepam and Cl 218,872[9,13,23] and the medium range affinity for zolpidem.[22] Our data further indicate that unidentified amino acids in the N-terminal 160 amino acids of the α variants, not derived from α1 in chimera C6, are involved in the low nanomolar affinity of zolpidem.

To pinpoint the origin of the diazepam sensitivity of $C6^{his100}\beta 2\gamma 2$ receptors, we could concentrate on the region located between the cysteine loop and the first transmembrane region. We identified three potential amino acid residues by sequence comparison of the α subunits showing high (α1, α2, α3, $\alpha 4^{his99}$, α5, and C6) and low ($\alpha 6^{his100}$) affinity for diazepam. A stepwise

exchange of the these amino acids in $\alpha 6^{his}$ (Figure 1) increased the affinity for the BZ agonist diazepam, yielding an overall improvement of >40-fold and resulting in an affinity for diazepam 8-fold higher than for any described wild-type BZ receptor (Table 2). All amino acid substitutions interfered with only a limited number of BZ ligand affinities, leaving others unchanged. These data rule out that the modifications of the BZ binding site are due to gross distortions of the whole $GABA_A$/BZ receptor molecule. In addition to their effect on potency, the mutations may influence the efficacy of diazepam and other BZ receptor ligands,[25-28] as the efficacy depends on the intricate interaction of α, β, and γ subunits.[11]

The most prominent amino acid substitution (isoleucine to valine) is generally considered a conserved exchange. The affinity for Ro 15-1788 was only marginally affected by the isoleucine[211] to valine exchange in $\alpha 6^{his100thr161gly199}$, but it decreased the affinity for the partial inverse agonist Ro15-4513 20-fold. Direct steric hindrance, as postulated for the arginine to histidine or glutamate to glycine exchanges,[15,18] may only play a minor role as both diazepam, i.e., its additional 5-phenyl group, and isoleucine are bulkier than Ro 15-4513 and valine, respectively. One explanation could be a direct and specific hydrophobic interaction of the isoleucine side chain with a hydrophobic portion of the Ro 15-4513 molecule, absent in the valine-substituted mutant. This hypothesis could only be tested by a range of inverse agonists and additional mutant receptors. The valine site is most likely different from the hydrophobic narrow cleft postulated as part of the inverse agonist pharmacophore,[29-32] since the affinity of the β-carboline methyl ester is not affected by the mutation (Table 2). As the diazepam and Ro 15-4513 affinities are differentially affected in $\alpha 6^{his100thr161gly199val211}\beta 2\gamma 2$ receptors, this mutant is well suited to explore the structural differences of the agonist and the different inverse agonist pharmacophores of $GABA_A$/BZ receptors. However, recent reports indicate that only the $\gamma 2$ variant is required together with either an α or a β subunit to result in receptors responsive to BZs.[33,34] Furthermore, $\alpha 1\beta 3\gamma 3$ and $\alpha 5\beta 3\gamma 3$ receptors display a BZ pharmacology different from the corresponding $\gamma 2$-containing receptors.[11] Therefore, the BZ pharmacophore cannot be solely determined by the nature of the α variant, but its formation depends on the proper interaction of α and γ subunits.

Our studies indicate that single amino acids determine features of the BZ binding pocket, i.e., the accessibility of the agonist site or BZ I type pharmacology. However, 'fine tuning' of the binding pocket involves the interplay of large numbers of amino acid residues, as evidenced by the lack of single amino acids as switches for zolpidem recognition and the stepwise increase in affinity for BZ agonists with single amino acid exchanges. Furthermore, our data indicate that individual point mutations can change the affinity of $GABA_A$/BZ receptors for subsets of BZ receptor ligands without affecting others, indicating that naturally occurring point mutations could be responsible for certain types of BZ ligand hypo- or hypersensitivity.

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