A point mutation in the γ2 subunit of γ-aminobutyric acid type A receptors results in altered benzodiazepine binding site specificity

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ABSTRACT Benzodiazepines allosterically modulate γ-aminobutyric acid (GABA) evoked chloride currents of γ-aminobutyric acid type A (GABA_A) receptors. Coexpression of either γ2 or γ3, in combination with α1 and β2 subunits, results both in receptors displaying high [3H]Ro 15-1788 affinity. However, receptors containing a γ2 subunit display a 178-fold reduced affinity to zolpidem as compared with γ2-containing receptors. Eight chimeras between γ2 and γ3 were constructed followed by nine different point mutations in γ2, each to the homologous amino acid residue found in γ3. Chimeric or mutant γ subunits were coexpressed with α1 and β2 in human embryonic kidney 293 cells to localize amino acid residues responsible for the reduced zolpidem affinity. Substitution of a methionine-to-leucine at position 130 of γ2 (γ2M130L) resulted in a 51-fold reduction in zolpidem affinity whereas the affinity to [3H]Ro 15-1788 remained unchanged. The affinity for diazepam was only decreased by about 2-fold. The same mutation resulted in a 9-fold increase in Cl-218872 affinity. A second mutation (γ2M57I) was found to reduce zolpidem affinity by about 4-fold. Wild-type and γ2M130L-containing receptors were functionally expressed in Xenopus oocytes. Upon mutation allosteric coupling between agonist and modulatory sites is preserved. Dose–response curves for zolpidem and for diazepam showed that the zolpidem affinity is drastically reduced. The apparent GABA_A affinity is not significantly affected by the γ2M130L mutation. The identified amino acid residues may define part of the benzodiazepine binding pocket of GABA_A receptors. As the modulatory site in the GABA_A receptor is homologous to the GABA site, and to all agonist sites of related receptors, γ2M130 may either point to a homologous region important for agonist binding in all receptors or define a new region not underlying this principle.

Neuronal inhibition of the mammalian brain is mainly effected by γ-aminobutyric acid type A (GABA_A) receptors. They are ligand gated chloride channels and belong to a larger channel family among nicotinic acetylcholine, glycine, and serotonin-3 receptors. Purification (1) and cDNA cloning (2) of two subunits belonging to the GABA_A receptor have initially been reported. Fourteen subunits or subunit isoforms have been described (ref. 3; for reviews, see refs. 4–8).

The GABA_A channel is functionally modulated by a large number of drugs (for review, see ref. 7). For example, it is subjected to positive allosteric modulation by clinically used drugs acting at the benzodiazepine binding site. Occupation of the binding site by these substances results in sedative, anxiolytic, myotonolytic, and anticonvulsant effects. In addition, substances are known to bind to this site, having no or a negative allosteric effect on the channel function. The location of this binding site on the receptor is of obvious interest.

A major site of photoaffinity labeling by the benzodiazepine [3H]flunitrazepam is a conserved histidine on α subunits (9). Several additional amino acid residues on various α subunit isoforms have been implicated in ligand binding (10–14). Expression of recombinant receptors indicates that a γ subunit is absolutely required for the formation of a benzodiazepine binding site (15, 16). Two point mutations in the γ2 subunit influence the benzodiazepine pharmacology (17–19). Upon deletion of γ2 from mice the [3H]Ro 15-1788 binding site nearly disappears, confirming the essential role of γ subunits (20). Thus, α and γ subunits are both thought to contribute to the benzodiazepine site ligand affinity, and its binding site is assumed to be located at the α/γ subunit interface.

Residues influencing the apparent γ-aminobutyric acid (GABA) affinity have been identified on α and β subunits assuming that the agonist site also is located at subunit interfaces. Residues important for GABA binding are at homologous positions to residues belonging to the agonist binding sites of the nicotinic acetylcholine and glycine receptors (for a review, see ref. 21). It appears that the localization of the neurotransmitter binding sites is conserved between these homologues (21). Remarkably, residues influencing benzodiazepine site ligand affinity at GABA_A receptors are located at homologous regions to regions contributing to these agonist sites (14, 18, 19, 21).

Zolpidem, an imidazopyridine, binds with high affinity to α1-containing GABA_A receptors and is able to displace the classical benzodiazepines (22). α2 and α3 apparently confer intermediate zolpidem affinity and the α subunit very low affinity to triple subunit combinations α2β2γ2 (23–25). Zolpidem displays lower affinity to γ1 than to γ2 containing receptors (26, 27), pointing to the importance of the α subunit for zolpidem binding.

By expression of chimeric subunits and radioligand binding we show that the zolpidem affinity is influenced by two distinct regions within the N-terminal domain of the γ2 subunit. Site-directed mutagenesis of residues of the γ2 subunit differing between γ2 and γ3 identified two amino acid residues to be important for benzodiazepine site ligand affinity. Our work represents an important step leading to a rational drug design based on the identification of structural determinants on the receptor protein relevant for ligand binding.

MATERIALS AND METHODS

Construction of Receptor Subunits. The cDNAs coding for the α1, β2, γ2S, and γ3 subunits of the rat GABA_A receptor

Abbreviations: GABA, γ-aminobutyric acid; HEK 293 cells, human embryonic kidney 293 cells.
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channel have been described (26, 28–30). All subunits have been cloned in to the polylinker of pBC/CMV (31). This expression vector allows high level expression of a foreign gene under control of the cytomegalovirus (CMV) promoter and also allows in vitro transcription using SP6 RNA polymerase. Chimeras were constructed by PCR amplification of the desired sequences and three fragment ligation using standard molecular biology techniques. Site-directed mutagenesis was done using the QuikChange mutagenesis kit (Stratagene). In vitro-synthesized sequences have been verified by DNA sequencing.

**Binding Assays to Membrane Preparations of Transiently Transfected Cells.** Human embryonic kidney 293 cells (HEK 293 cells) (ATCC no. CRL 1573) were transfected with plasmids coding for GABA A receptor subunits by the calcium phosphate precipitation method (32), and membranes were prepared as described (19). Resuspended cell membranes (0.5 ml) were incubated for 90 min on ice in the presence of [3H]Ro 15-1788 (87 Ci/mmol; DuPont/NEN; 1 Ci = 37 GBq) and various concentrations of competing ligands. Membranes (20–50 μg protein/filter) were collected by rapid filtration on GF/C filters presoaked in 0.3% polyethyleneimine. After three washing steps with 4 ml of buffer, the filter-retained radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM Ro 15-1788. Data were fitted by using a nonlinear least-squares method to the equation $B(c) = B_{max}c/(K_c + c)$ for binding curves and $B(c) = B_{max}(1 + c/I_{C50})$ for displacement curves, where $c$ is the concentration of ligand; $B$, binding; $B_{max}$, maximal binding; and $K_c$, the dissociation constant. IC50 values obtained from displacement curves were converted to Ki values according to the Cheng–Prusoff equation (33). Protein concentration was determined with the Bio-Rad protein assay kit with BSA as standard. All wild-type, chimeric, and mutant receptor combinations expressed equally well ($B_{max}$, 0.5–2 pmol [3H]Ro 15-1788 binding sites/mg of protein).

**Functional Expression and Characterization.** Xenopus laevis oocytes were prepared, injected, and defolliculated, and currents were recorded as described (18, 34). Briefly, oocytes were injected with 50 nl of capped, polyadenylated cRNA dissolved in 5 mM K-Hepes (pH 6.8). This solution contained the transcripts coding for the different subunits at concentrations dissolved in 5 mM K-Hepes (pH 6.8). This solution contained the transcripts coding for the different subunits at concentrations: 100 ng/10 μl of each of the α1, β2, γ1, and γ2, respectively. Electrophysiological experiments were performed by the two-electrode voltage clamp method at a holding potential of −80 mV. Allosteric potencies via the benzodiazepine site was measured at a GABA concentration (1–3 μM) eliciting 2–10% of the maximal GABA current amplitude by coapplication of GABA and diazepam or of GABA and zolpidem. Oocytes were only exposed to a single drug in addition to GABA, to avoid contamination, and the perfusion system was cleaned by washing with dimethyl sulfoxide for the same reason. The apparent GABA affinity was measured at a holding potential of −60 mV. Agonist concentrations between 0.03 and 3.000 μM were applied for 20 s, and a washout period of 4–15 min was allowed to ensure full recovery from desensitization. Current responses have been fitted to the Hill equation: $I = I_{max}/(1 + (EC_{50}/I)^n)$ where $I$ is the peak current at a given concentration of GABA (A). $I_{max}$ is the maximum current, $EC_{50}$ is the concentration of agonist eliciting half maximal current, and $n$ is the Hill coefficient.

## RESULTS

**Binding Properties of Receptors Containing γ1 or γ2.** Coexpression of either γ2 or γ1 subunits together with α1 and β2 results in receptors displaying high affinity to the benzodiazepine site antagonist [3H]Ro 15-1788 (Table 1). Receptors containing a γ1 subunit instead of a γ2 subunit differ significantly in their zolpidem binding properties. They exhibit an approximately 178-fold reduced affinity for zolpidem. To locate amino acid residues responsible for reduced zolpidem affinity, eight different γ subunit chimeras were constructed (Fig. 1A).

### Binding Properties of Chimeric Receptors.

Chimeric γ subunits were coexpressed with α1 and β2 subunits in cultured

![Figure 1](image_url)  
**Fig. 1.** Two distinct regions within the amino acid sequence of γ subunits determine zolpidem affinity. (A) Chimeric subunits were constructed by exchanging various parts of the N-terminal extracellular domains between the γ2 (open bars) and γ1 (hatched bars) subunits. Numbers indicate residues of the mature form of the γ2 subunit present in the chimera. Filled boxes denote the four presumed transmembrane segments (M1–M4). (B) Specific binding of 2 nM [3H]Ro 15-1788 in the presence of 1 μM zolpidem to GABA A receptors expressed as triple subunit combinations consisting of γ2, γ3, or chimeric γ subunits each in combination with α1 and β2 subunits. Error bars indicate SD of two independent determinations performed in duplicate.

<table>
<thead>
<tr>
<th>αβγxγ receptor</th>
<th>[3H]Ro 15-1788</th>
<th>Diazepam</th>
<th>Zolpidem</th>
<th>CI 218872</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ1</td>
<td>0.94 ± 0.16</td>
<td>180 ± 30</td>
<td>2,670 ± 350</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>γ1*</td>
<td>0.61 ± 0.24</td>
<td>12 ± 8</td>
<td>15 ± 3</td>
<td>46 ± 1</td>
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<tr>
<td>γ1M130L</td>
<td>0.50 ± 0.01</td>
<td>30 ± 1</td>
<td>770 ± 70</td>
<td>5.3 ± 0.6</td>
</tr>
<tr>
<td>γ1M57I</td>
<td>0.77 ± 0.05</td>
<td>22 ± 4</td>
<td>60 ± 10</td>
<td>62 ± 1</td>
</tr>
<tr>
<td>γ1T65V</td>
<td>0.47 ± 0.03</td>
<td>ND</td>
<td>24 ± 4</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Ki* values were determined by binding of [3H]Ro 15-1788 to washed membranes of transiently transfected HEK 293 cells. *Ki* values for each compound were determined by displacement of [3H]Ro 15-1788 binding, and were calculated according to the equation of Cheng and Prusoff (33). IC50 values for each compound were determined by nonlinear least-squares regression assuming a Hill coefficient of 1. Data indicate mean ± SD of two experiments performed in duplicate. ND, not determined.

*Data are from Buhr et al. (18).
HEK 293 cells and assayed for their ability to bind the antagonist \(^{[3]}\)H\text{Ro 15-1788} in the presence or absence of zolpidem (1 \(\mu M\)). All wild-type and chimeric receptors displayed comparable high affinity binding of \(^{[3]}\)H\text{Ro 15-1788}. This was verified by determining specific binding of \(^{[3]}\)H\text{Ro 15-1788} at different concentrations. Binding was increased between 10% and 25% upon increasing the radioligand concentration from 2 nM to 6 nM (data not shown), indicating that the \(K_d\) for \(^{[3]}\)H\text{Ro 15-1788} was between 0.4 nM and 1 nM for all receptors. From these results it was obvious that the antagonist binding site did not undergo remodeling in receptors containing chimeric \(\gamma\) subunits.

Receptors containing a chimeric subunit consisting of the entire N-terminal extracellular domain of the \(\gamma_2\) subunit combined with the transmembrane domain of the \(\gamma_3\) subunit displayed the same zolpidem sensitivity as wild-type \(\gamma_2\) containing receptors. Furthermore, chimeric receptors containing the entire extracellular domain of the \(\gamma_2\) subunit display identical binding properties as receptors containing a wild-type \(\gamma_3\) subunit. Therefore, it could be concluded that the first 235 amino acids of the mature \(\gamma_2\) subunit determine the difference in zolpidem affinity. A total of 68 amino acid residues are different within the putative N-terminal extracellular domain of both isoforms. Further chimeras were constructed and analyzed. Chimeric receptors displayed high, low, and intermediate sensitivity toward zolpidem (Fig. 1B), indicating that two distinct amino acid regions influence zolpidem affinity. One region drastically influences zolpidem sensitivity and is located between amino acid residues 115 and 133 (termed region I) of the \(\gamma_2\) subunit. The concentration of zolpidem to affect affinity to a lesser extent is located between residues 44 and 59 (termed region II).

Receptors Mutagenized in Region I. A total of 4 amino acid residues are different between \(\gamma_2\) and \(\gamma_3\) subunits in region I. Each of these four residues of the \(\gamma_2\) subunit has been substituted by the corresponding amino acid residue of the \(\gamma_3\) subunit. Mutants were coexpressed together with the \(\alpha_1\) and \(\beta_2\) subunits, and receptors were assayed for \(^{[3]}\)H\text{Ro 15-1788} affinity and zolpidem sensitivity. Receptors containing a methionine-to-leucine substitution at position 130 of the \(\gamma_2\) subunit (\(\gamma_2\text{M130L}\)) displayed a drastically reduced zolpidem sensitivity of \(^{[3]}\)H\text{Ro 15-1788} binding (Fig. 2A). Detailed analysis showed that there was little effect on Ro 15-1788 affinity (Fig. 3), whereas the zolpidem affinity was drastically affected (Fig. 4), showing a 51-fold decrease in affinity for zolpidem (Table 1). The diazepam affinity is decreased by 2.5-fold in receptors containing \(\gamma_2\text{M130L}\) (Table 1). Interestingly, the same mutation resulted in a 9-fold increase in \(\text{Cl}^{-}\) affinity (Table 1). Receptors containing the \(\gamma_3\) subunit display almost the same high affinity to this ligand as receptors containing \(\gamma_2\text{M130L}\) (Table 1).

Receptors Mutagenized in Region II. The zolpidem affinity of receptors containing \(\gamma_2\text{M130L}\) was still about 3.5-fold higher than the affinity of receptors containing the \(\gamma_3\) subunit. Results obtained by chimeric receptors also indicated that at least one additional residue contributes to the zolpidem affinity and that it must be located in region II of the \(\gamma_2\) subunit. Within this region a total of five residues are different within the putative N-terminal extracellular domain of both isoforms. Further chimeras were constructed and analyzed. Chimeric receptors displayed high, low, and intermediate sensitivity toward zolpidem (Fig. 1B), indicating that two distinct amino acid regions influence zolpidem affinity. One region drastically influences zolpidem sensitivity and is located between amino acid residues 115 and 133 (termed region I) of the \(\gamma_2\) subunit. The concentration of zolpidem to affect affinity to a lesser extent is located between residues 44 and 59 (termed region II).

Functional Effects of \(\gamma_2\text{M130L}\). Wild-type and mutant \(\gamma\) subunits were coexpressed with \(\alpha_1\) and \(\beta_2\) subunits in \(\text{Xenopus}\) oocytes to study functional consequences of the \(\gamma_2\text{M130L}\) mutation. Mutant ion channels expressed GABA-activated chloride currents of comparable maximal current amplitude as wild-type channels (3–4 days after injection, measured at 60 mV; \(\alpha_1\beta_2\gamma_2\), 10.4 ± 0.9 \(\mu A\), \(n = 3\); \(\alpha_1\beta_2\gamma_2\text{M130L}\), 12.1 ± 1.7 \(\mu A\), \(n = 3\)), 3.6 ± 0.8 \(\mu A\), \(n = 3\)). The apparent GABA affinities were 9.6 ± 6.9, 12.5 ± 0.8, and 9.7 ± 1.6 \(\mu M\) (mean ± SD, \(n = 3\)) for channels containing \(\gamma_2\), \(\gamma_2\text{M130L}\), or \(\gamma_3\).
respectively. Therefore, expression and agonist affinity are not affected by the mutation. Allosteric stimulation by zolpidem and diazepam of GABA-activated currents was measured in cumulative dose–response curves (Fig. 5). Stimulation of mutant channels requires much higher concentrations of zolpidem as compared with wild-type $\gamma_2$-containing channels (Fig. 5A). In contrast to zolpidem, diazepam stimulation is similar in wild-type and mutant $\gamma_2$ containing channels (Fig. 5B). These results indicate that mutant channels retain allosteric coupling to the agonist site and confirm the importance of $\gamma_2$M130 for zolpidem binding.

DISCUSSION

This study demonstrates that one particular amino acid residue at position 130 of the mature $\gamma_2$ subunit of $\alpha_1\beta_2\gamma_2$ GABA\(_A\) receptors drastically affects benzodiazepine site ligand affinities. Both $\alpha$ and $\gamma$ subunits contribute to the benzodiazepine pharmacology of GABA\(_A\) receptors. Therefore, it is assumed that the benzodiazepine binding site is located at the $\alpha$/$\gamma$ subunit interface. Until now, only one amino acid residue on $\gamma$ subunits influencing benzodiazepine binding affinity (for functional data, see below) has been identified (18, 19). A $\gamma_2$ or $\gamma_3$ subunit is required for high affinity binding of the benzodiazepine site antagonist $[^3H]$Ro 15-1788. Receptors containing a $\gamma_3$ instead of a $\gamma_2$ subunit display a 178-fold reduced affinity for the ligand zolpidem. The $\gamma_2$ subunit shows 64.6% sequence identity to the $\gamma_3$ subunit. About 150 amino acid residues are different between the mature subunits and therefore could be responsible for reduced zolpidem affinity. Chimeric $\gamma$ subunits were constructed to identify amino acid regions containing the responsible residues. Receptors were expressed and analyzed for the ability of zolpidem to displace $[^3H]$Ro 15-1788 binding.
Coexpression of the γ2 or the γ3 subunit together with α1 and β2 in cultured HEK 293 cells results in receptors displaying high affinity to [3H]Ro 15-1788. All receptors containing chimeric γ subunits expressed equally well and retained high affinity to [3H]Ro 15-1788. These data rule out that this drastic type of mutagenesis had an influence on the overall structure of the receptor complex or a disruptive effect on the benzodiazepine binding pocket. Two regions important for the interaction with zolpidem were identified. One region had a strong effect (termed region I) and the other region had an intermediate effect (termed region II) on zolpidem sensitivity of [3H]Ro 15-1788 binding. In region I and region II a total of four and five amino acid residues are different between γ2 and γ3. Each of these nine residues of the γ2 subunit was individually mutagenized to the residue present in the γ3 subunit, and mutant receptors were expressed and analyzed.

The methionine-to-leucine substitution at position 130 of the γ2 subunit had a dramatic influence on zolpidem sensitivity of [3H]Ro 15-1788 binding. Whereas the affinity to [3H]Ro 15-1788 is not affected the affinity to zolpidem is drastically reduced amounting to a 51-fold change. The affinity for diazepam is reduced by about 2.5-fold. Interestingly, the affinity to Cl 218872 is increased by about 9-fold. Wild-type and mutant subunit combinations were also expressed in Xenopus oocytes and analyzed. These functional data confirmed the importance of γM130 for zolpidem stimulation of GABA-activated currents. The apparent zolpidem affinity is drastically reduced whereas diazepam potentiation is much less affected. GABA dose–response curves indicate that the apparent agonist affinity is not affected by the γM130L mutation. These results and also the fact that the binding affinity to one benzodiazepine site ligand is not affected at all, whereas other ligands are affected in different ways strongly suggest that γM130 plays an important role in the specificity of the binding pocket and that it is most likely part of it.

The analysis of receptors mutagenized in region II identified methionine at position 57 to be important for zolpidem sensitivity of [3H]Ro 15-1788 binding. Whereas the affinity to [3H]Ro 15-1788 is not affected the affinity to zolpidem is drastically reduced amounting to a 51-fold change. The affinity for diazepam is reduced by about 2.5-fold. Interestingly, the affinity to Cl 218872 is increased by about 9-fold. Wild-type and mutant subunit combinations were also expressed in Xenopus oocytes and analyzed. These functional data confirmed the importance of γM130 for zolpidem stimulation of GABA-activated currents. The apparent zolpidem affinity is drastically reduced whereas diazepam potentiation is much less affected. GABA dose–response curves indicate that the apparent agonist affinity is not affected by the γM130L mutation. These results and also the fact that the binding affinity to one benzodiazepine site ligand is not affected at all, whereas other ligands are affected in different ways strongly suggest that γM130 plays an important role in the specificity of the binding pocket and that it is most likely part of it.

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Several amino acid residues on α subunit isoforms have also been identified to influence benzodiazepine site ligands affinities. If α2E225 is mutated to a glycine (which is the homologous residue to α2G200), there is at least a 10-fold increase in binding affinities for zolpidem and Cl 218872 (10). Replacement of histidine 101 by arginine in the α1 subunit leads to a complete loss in detectable diazepam, zolpidem, and Cl 218872 binding, while the binding affinity for [3H]Ro 15-1788 is reduced by more than 100-fold (11, 12). Similarly, two additional amino acid residues (proline 161 and isoleucine 210) have been identified in an α6-derived mutant that together alter affinities to benzodiazepine site ligands (13). Residues that influence benzodiazepine pharmacology have been identified on the α1 subunit after functional expression in Xenopus oocytes. α1Y161A and α1T206A both increase stimulation by diazepam and by zolpidem (18) and at least α1T206A also affects benzodiazepine ligand binding affinities upon expression in HEK 293 cells (unpublished results). The same study showed that receptors containing the α1Y209A substitution displayed a reduced zolpidem stimulation. Preliminary binding experiments indicate that this residue also plays an important role in benzodiazepine site ligand binding (unpublished results). After submission of this work, Amin et al. (14) published that two tyrosine residues (α1Y159 and α1Y209) are crucial for benzodiazepine binding and allosteric modulation of GABA-activated currents, pointing again at the importance of α1Y209.

It is not clear if all of the above-mentioned amino acid residues that affect binding of benzodiazepines are in direct contact with benzodiazepines or if the substitutions have an more indirect effect on the benzodiazepine binding site. However, observations that make it likely that at least some of these residues are in direct contact with the ligands comes from an independent approach. Using bovine brain membranes and photoaffinity labeling by [3H]flunitrazepam, Duncalfe et al. (9) showed that the conserved histidine (residue no. 102 of bovine α subunits, corresponding to 101 in rat α1) is the major site of photoincorporation. Furthermore, residues C-terminal of residue 104 can be photolabeled by [3H]Ro 15-4513 (35). Most of the residues on α subunits that have been identified by site-directed mutagenesis are downstream of the conserved histidine. It is likely that further analysis of [PH]Ro 15-4513 photoincorporation will point to one of these residues. Similarly, photoincorporation of the agonist [3H]muscimol into α6F64 (36) identified a residue that is important for apparent GABA affinity of channel gating as shown by functional expression of mutant channels (37). Therefore, site-directed mutagenesis and the study of properties of mutant receptors are important strategies for the identification of amino acid residues interacting with ligands.

Interestingly, it appears that the amino acid residues known to be important for benzodiazepine site ligand affinity are located in regions homologous to regions that affect neurotransmitter binding of GABA, and related receptors. For example, α1F64, which is involved in GABA binding, is homologous to γF77, which is important for benzodiazepine site specificity. A four loop model of neurotransmitter binding has been suggested by Galzi and Changeux (21) for the members of the acetylcholine receptor subfamily (including GABA_{A}, glycine, and 5HT_{3} receptors). As for the benzodiazepine binding site, peptide loops of two neighboring subunits are
important for neurotransmitter affinity indicating that both binding sites are located at subunit boundaries. \( \gamma \)\textsubscript{M130} is not located in a region homologous to any region involved in agonist binding. As the \( \gamma \)\textsubscript{M130L} mutation drastically affects ligand affinity and because of the homology of the benzodiazepine and GABA binding sites it is likely that new residues of the GABA binding site can be identified. Presently, we are testing this hypothesis by systematically mutagenizing the homologous region of the \( \alpha \) subunit and determining the apparent GABA affinity of mutant ion channels. As the location of the neurotransmitter binding site is strongly conserved in different receptors it is likely that the regions homologous to the region were \( \gamma \)\textsubscript{M130} is located are of general importance. Alternatively, we have described here a region important for the modulatory site that has no related location in the agonist site.

We identify here new amino acid residues on the \( \gamma \) subunit differentially affecting binding of ligands of the benzodiazepine binding site. There is good chance that at least one of these residues is part of the binding pocket and directly interacts with some ligands of this site. The analysis of various substitutions at key positions might help to understand how precisely ligands interact with the binding pocket. Ultimately, such an analysis may provide a rational basis for drug design. However, final verification of such structural predictions has to await crystallization and structural resolution of the GABA\textsubscript{A} receptor.

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