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α subunits in $GABA_A$ receptors are dispensable for GABA and diazepam action

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The major isoform of the $GABA_A$ receptor is $\alpha_1\beta_2\gamma_2$. The binding sites for the agonist GABA are located at the β_2+/α_1- subunit interfaces and the modulatory site for benzodiazepines at α_1+/γ_2-. In the absence of α_1 subunits, a receptor was formed that was gated by GABA and modulated by diazepam similarly. This indicates that alternative subunits can take over the role of the α_1 subunits. Point mutations were introduced in β_2 or γ_2 subunits at positions homologous to α_1- benzodiazepine binding and GABA binding positions, respectively. From this mutation work we conclude that the site for GABA is located at a β_2+/β_2- subunit interface and that the diazepam site is located at the β_2+/γ_2- subunit interface. Computational docking leads to a structural hypothesis attributing this non-canonical interaction to a binding mode nearly identical with the one at the α_1+/γ_2- interface. Thus, the β_2 subunit can take over the role of the α_1 subunit for the formation of both sites, its minus side for the GABA binding site and its plus side for the diazepam binding site.

γ-Aminobutyric acid type A ($GABA_A$) receptors are the major inhibitory neurotransmitter receptors in the mammalian central nervous system. The $GABA_A$ receptor is a pentameric protein complex, whose subunits are drawn from the following different isoforms: α(1–6), β(1–4), γ(1–3), δ, ε, θ, π and ρ(1–3). The five subunits form a chloride selective ion channel[1–3]. The most common isoform of this receptor consists of two α_1, two β_2 and one γ_2 subunit(s)[4–6] arranged $\alpha_1\gamma_2\beta_2\alpha_1\beta_2$ counterclockwise when viewed from the extracellular space[7–9]. These receptors have two agonist GABA binding sites and one benzodiazepine binding site[10]. By using *in vitro* mutagenesis the binding sites for the agonist GABA were located to the β_2+/α_1- subunit interfaces[11,12], and the modulatory site for benzodiazepines was at the α_1+/γ_2- subunit interface[13]. Thus, the α_1 subunit is commonly accepted to contribute to the formation of both sites.

The $GABA_A$ receptors can be activated by the agonist GABA and modulated by many drugs[14]. Among these drugs are the benzodiazepines, such as diazepam, that have sedative, anxiolytic, anticonvulsant, hypnotic, and muscle relaxant properties[15]. Coexpression of different combinations of recombinant subunits has generated $GABA_A$ receptors with distinct pharmacological and electrophysiological properties.

As early as 1990, we observed that $\beta_2\gamma_2$ $GABA_A$ receptors, lacking the α_1 subunit, were activated by GABA and potentiated by diazepam[16]. Later this observation was confirmed by several groups for GABA and diazepam[17–19] or other modulators[20]. Expression was also documented for $\beta_1\gamma_2$[21,22] and $\beta_3\gamma_2$[22,23] $GABA_A$ receptors. In the present study, we tried to understand this apparent contradiction and decided to investigate whether alternative GABA and benzodiazepine-binding subunit interfaces exist. Site-directed mutagenesis was combined with two-electrode voltage clamp in *Xenopus* oocytes. Our findings suggest that the β_2 subunit may replace the α_1 subunit for the formation of either site. We have previously utilized experimentally guided computational docking that led to a diazepam bound structure model at the α_1+/γ_2- interface[24]. Computational docking at the β_2+/γ_2- interface yielded structural models which strongly suggest that diazepam can interact with this site in a binding mode nearly identical with the one observed at the canonical α_1+/γ_2- site, thus explaining the similar apparent potency.

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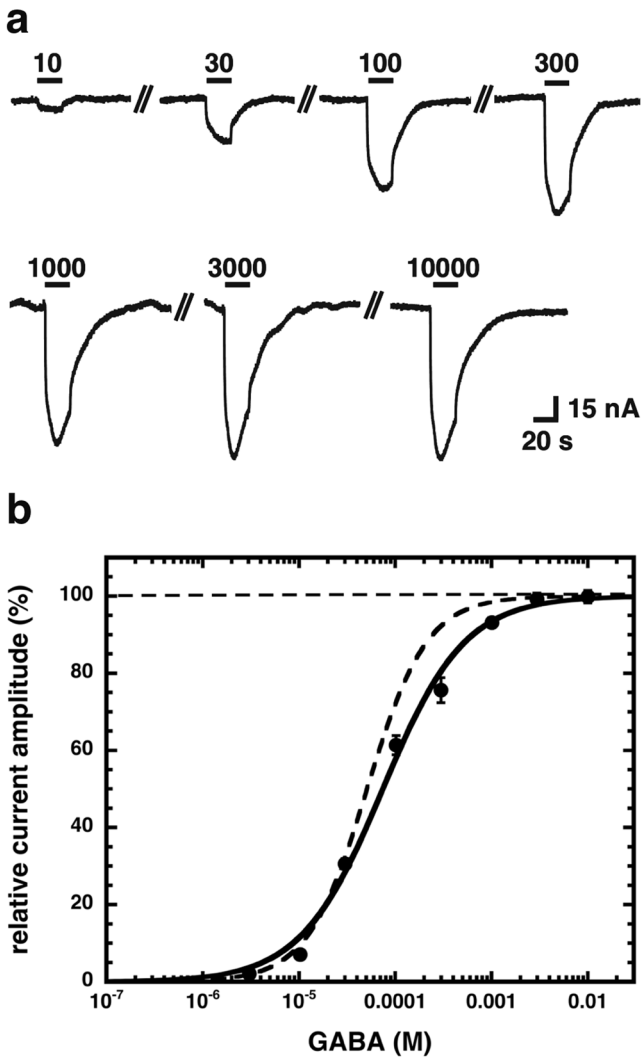

Figure 1. Concentration response curve for GABA at $\beta_2\gamma_{l2}$ $GABA_A$ receptors. Receptors were expressed in *Xenopus* oocytes and exposed to subsequently higher concentrations of GABA and the elicited current amplitude was determined. Individual curves were first normalized to the fitted maximal current amplitude and subsequently averaged. Data are expressed as mean ± S.E.M., n = 5 from two batches of oocytes. (**a**) Original current traces. GABA applications are indicated by a bar. The numbers indicate the concentration of GABA in μM. (**b**) Averaged concentration-response curve for $\beta_2\gamma_{l2}$ $GABA_A$ receptors. The dotted line shows for comparison corresponding data on $\alpha_1\beta_2\gamma_{l2}$ $GABA_A$ receptors.

Results

Functional expression of $\beta_2\gamma_{l2}$ $GABA_A$ receptors in *Xenopus* oocytes. We initially determined whether varying the subunit ratio led to a different extent of expression of $\beta_2\gamma_{l2}$ $GABA_A$ receptors. We injected β_2 and γ_{l2} cRNAs at the three ratios 1:1 (1 fMol each/oocyte), 2:1 (2 fMol and 1 fMol/oocyte) and 1:3 (1 fMol and 3 fMol/oocyte) into oocytes and measured the maximum current amplitudes elicited by 10 mM GABA. 5–7 days after microinjection of RNA, the $\beta_2\gamma_{l2}$ $GABA_A$ receptors formed by the 1:3 cRNA injection ratio gave the highest maximal current amplitude (131 ± 19 nA, n = 13). In contrast receptors formed from 1:1 and 2:1 cRNA ratios resulted in current amplitudes less than 100 nA. Thus, we used the 1:3 cRNA ratio coding for wild type or mutant β_2 or γ_{l2} subunits for all following experiments. Possibly the subunit arrangement is affected by the injection ratio as it has been documented in the case of $\beta_3\gamma_{l2}$ $GABA_A$ receptors[25]. Oocytes injected with 1 fMol coding for the β_2 subunit only, or with 3 fMol coding for the γ_{l2} subunit only, both did not result in current expression.

$\beta_2\gamma_{l2}$ receptors respond to GABA. 5–7 days after injection, *Xenopus* oocytes expressing $\beta_2\gamma_{l2}$ $GABA_A$ receptors were investigated for the presence of currents elicited by 10 mM GABA. Figure 1a shows original current traces obtained from oocytes clamped at −80 mV. Figure 1b shows an averaged concentration-response curve for $\beta_2\gamma_{l2}$ $GABA_A$ receptors. The curve was characterized by an EC_{50} of 75 ± 5 μM and a Hill coefficient of 1.0 ± 0.1 (n = 5). This EC_{50} is similar to that reported for $\alpha_1\beta_2\gamma_{l2}$, which amounts to 51 ± 15 μM[8].

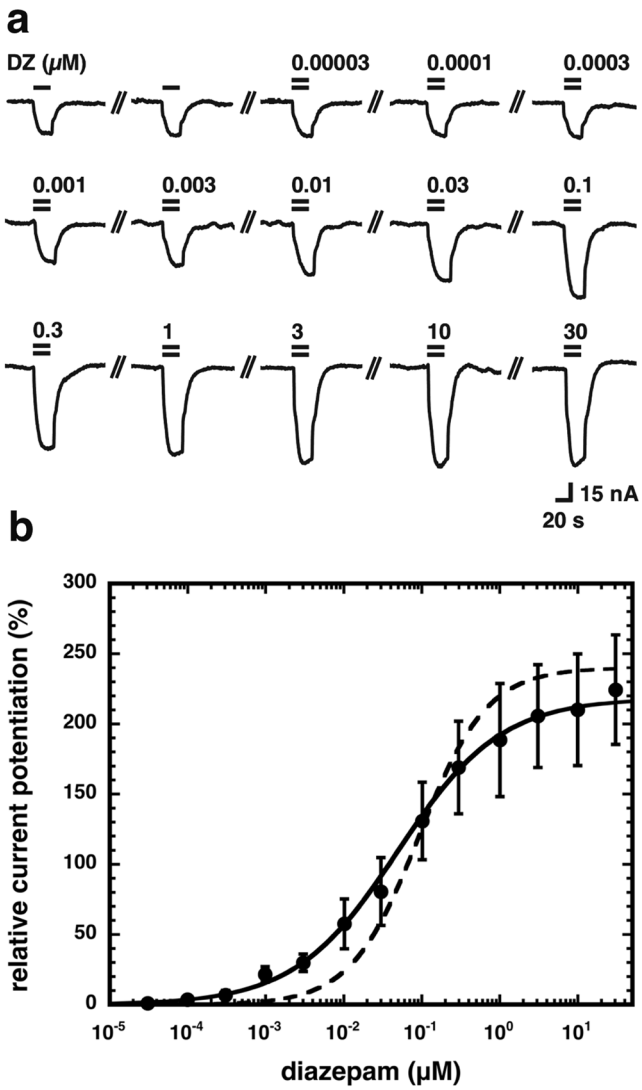

Figure 2. Concentration response curve for diazepam at $\beta_2\gamma_2$ $GABA_A$ receptors. Receptors were expressed in *Xenopus* oocytes and exposed to either GABA alone or GABA in the presence of subsequently higher concentrations of diazepam and the elicited current amplitude was determined. At each concentration of diazepam current potentiation was calculated. Individual curves for potentation were first normalized to the fitted maximal current amplitude and subsequently averaged. Data are expressed as mean ± S.E.M., n = 3 from two batches of oocytes. (**a**) Original current traces. (**b**) Averaged concentration-response curve for $\beta_2\gamma_2$ $GABA_A$ receptors. The dotted line shows for comparison corresponding data on $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors.

$\beta_2\gamma_2$ receptors respond to diazepam. Diazepam is a positive allosteric modulator of certain $GABA_A$ receptors enhancing the GABA-induced chloride ion influx. We examined the current potentiation by 1 μM diazepam using a GABA concentration that elicited about 5% of the respective maximal current amplitude. Figure 2a shows original current traces from an experiment were oocytes were exposed to either GABA alone or in combination with increasing concentrations of diazepam. Figure 2b shows an averaged concentration-response curve. The curve was characterized by an EC_{50} of 69 ± 14 nM and a Hill coefficient of 0.6 ± 0.1 (n = 3). The EC_{50} is similar to that reported earlier for $\alpha_1\beta_2\gamma_2$ with 92 ± 6 nM[8], while the Hill coefficient is, for reasons we do not understand, significantly lower than 1. No evidence for a possible receptor heterogeneity that could explain this finding was found (see below). Potentiation by 1 μM diazepam amounted to 216 ± 30% (n = 15).

Selection of point mutations. Obviously the α_1 subunit is dispensable for the formation of a $GABA_A$ receptor responsive to both channel agonist GABA and diazepam as shown above. We aimed to localize both binding sites in $\beta_2\gamma_2$ receptors. For this purpose we selected some point mutations that have been described to affect either the response to GABA or that to diazepam in $\alpha_1\beta_2\gamma_2$[11,12,26–29] (Table 1). A total of nine different point mutations were introduced into either the β_2 subunit or γ_2 subunit. In the β_2 subunit we generated one mutation at the minus side (β_2Y62L) and four mutations at the plus side (β_2T202A, β_2T202S, β_2Y205S, β_2Y205Q) and in the γ_2 subunit one mutation on the minus side (γ_2F77Y) and three mutations on the plus side (γ_2S217A, γ_2Y220S and

mutation	effect in $\alpha_1\beta_2\gamma_2$	homologous residues in α_1 or β_2	effect of homologous mutations in $\alpha_1\beta_2\gamma_2$	Lit.
β_2Y62L	30-fold decrease in EC_{50} GABA	α_1F64L	200-fold decrease in EC_{50} GABA	11
	no shift in antagonist apparent affinity		200-fold decrease in antagonist apparent affinity	
β_2T202S	20-fold decrease in EC_{50} GABA	(α_1T206C)	(5-fold increase in EC_{50} GABA)	12,27
			(no effect on DZ affinity)	
		(α_1T206V)	(7-fold decrease in DZ affinity)	28
γ_2F77Y	230-fold decrease in DZ affinity			29
	no effect on EC_{50} GABA			
γ_2S217A	Not available	(β_2T202A)	(drastic loss of EC_{50} GABA)	12
		(α_1T206A)	(3-fold decrease in DZ affinity)	26
γ_2Y220Q	Not available	α_1Y209Q	see above	26

Table 1. Point mutations in β_2 and γ_2 used in this study. Point mutations were selected on the basis of previous findings in $\alpha_1\beta_2\gamma_2$ $GABA_A$. The receptors β_2T202Aγ_2, β_2Y205Sγ_2, $\beta_2\gamma_2$Y220S and β_2Y205Qγ_2 were additionally studied, but were not activated by GABA or etomidate.

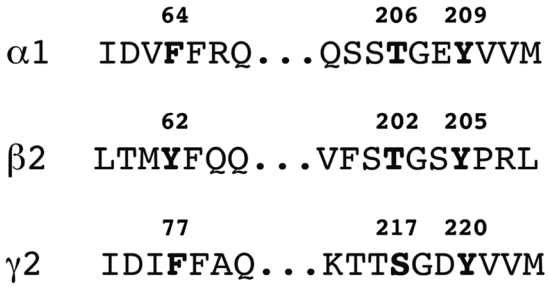

Figure 3. Sequence alignment of α_1, β_2 and γ_2 subunits of the rat $GABA_A$ receptor. Mutated residues of β_2 and γ_2 subunits are indicated with numbers. Homologous positions of the α_1 subunit are also highlighted.

γ_2Y220Q). A sequence alignment of the corresponding regions in α_1, β_2 and γ_2 is shown in Fig. 3. Table 1 summarizes the consequences of the mutations in $\alpha_1\beta_2\gamma_2$ receptors. In addition the effect of homologous mutations in other subunits is listed.

All mutated subunits were expressed in *Xenopus* oocytes in combination with wild-type subunits to result in $\beta_2\gamma_2$ $GABA_A$ receptors. Functional properties were determined by using two-electrode voltage clamp.

Functional expression of receptors was verified using 50 µM etomidate as agonist since mutations affecting the response to GABA are not very likely to influence the response to this agent. Amino acid residues affecting the latter property have been described to be located within the membrane embedded part of the receptor[30]. The mutation β_2Y62L interfered very strongly with channel activation by etomidate. As the mutation is located far away from the suspected etomidate site[31], this indicates that this mutation also affects gating by etomidate (see below). Unfortunately, β_2T202Aγ_2, β_2Y205Sγ_2 and β_2Y205Qγ_2 all resulted in no or very little expression (Table 2), so that these receptors could not be further investigated.

Effect of a mutation at the plus side of β_2. β_2T202S was the only investigated point mutation at the plus side of β_2 that did not disrupt $\beta_2\gamma_2$ receptor assembly as evidenced by the large response to etomidate (Table 2). In $\alpha_1\beta_2\gamma_2$ receptors this mutation led to a 20-fold decrease in GABA sensitivity[12]. In $\beta_2\gamma_2$ receptors the same mutation led to a 42-fold decrease in GABA sensitivity (Fig. 4, Table 2). The EC_{50} for GABA dependent channel gating was 3130 ± 260 µM and a Hill coeffient of 0.8 ± 0.1 ($n = 4$). Potentiation by 1 µM diazepam was not significantly affected by the mutation (Fig. 5, Table 2).

Effect of a mutation at the minus side of β_2. In $\alpha_1\beta_2\gamma_2$ receptors the mutation β_2Y62L leads to a 30-fold decrease in GABA sensitivity with no effect on the antagonist affinity as compared to wild-type $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors[11]. The homologous mutation in the α_1 subunit, α_1F64L caused a 200-fold drop in both properties[11]. Here, we found that combination of β_2Y62L with the γ_2 subunit showed 2-fold smaller current amplitude (Table 2) as compared to wild-type $\beta_2\gamma_2$ receptors, indicating that this mutation might somehow disturb efficient assembly of functional channels and/or that gating is affected. In addition the mutation β_2Y62L led to a 3-fold decrease in the sensitivity for GABA with an EC_{50} of 228 ± 50 µM and a Hill coefficient of 1.0 ± 0.1 ($n = 7$) (Fig. 4, Table 2). Again, potentiation by 1 µM diazepam was not significantly affected by the mutation (Fig. 5, Table 2).

Subunit combination	Etomidate (nA; 50 μM)	GABA (nA; 10 mM)	EC_{50} (μM)	DZ potentiation (%)	Homology to mutated residues
β_2	4 ± 1 (4)	−4 ± 1 (4)	—	—	—
γ_{l2}	0 ± 0 (5)	−4 ± 1 (5)	—	—	—
$\beta_2\gamma_{l2}$	946 ± 8 (4)	131 ± 19 (13)	75 ± 5 (5)	216 ± 30 (15)	—
β_2Y62Lγ_{l2}	13 ± 3 (11)	53 ± 8 (17)	228 ± 50 (7)	121 ± 12 (5)	α_1F64, γ_{l2}F77
β_2T202Aγ_{l2}	8 ± 2 (5)	5 ± 0 (5)	—	—	α_1T206, γ_{l2}S217
β_2T202Sγ_{l2}	380 ± 77 (5)	203 ± 18 (5)	3130 ± 260 (4)	100 ± 7 (4)	α_1T206, γ_{l2}S217
β_2Y205Sγ_{l2}	0 ± 0 (5)	0 ± 0 (16)	—	—	α_1Y209, γ_{l2}Y220
β_2Y205Qγ_{l2}	6 ± 2 (5)	1 ± 0 (4)	—	—	α_1Y209, γ_{l2}Y220
γ_{l2}F77Yβ_2	193 ± 25 (4)	148 ± 23 (11)	72 ± 15 (5)	5 ± 2 (5)	α_1F64, β_2Y62
γ_{l2}S217Aβ_2	141 ± 31 (5)	100 ± 25 (13)	133 ± 20 (3)	185 ± 23 (4)	α_1T206, β_2T202
γ_{l2}Y220Sβ_2	76 ± 17 (5)	10 ± 1 (12)	—	—	α_1Y209, β_2Y205
γ_{l2}Y220Qβ_2	777 ± 113 (5)	127 ± 40 (6)	367 ± 139 (3)	127 ± 11 (6)	α_1Y209, β_2Y205

Table 2. Functional properties of expressed wild type and mutated GAB_{AA} receptor subunits. Individual subunits or subunit combinations were expressed in *Xenopus* oocytes. Assembly of receptors was verified by determining amplitudes of current responses to 50 μM etomidate. Responses to 10 mM GABA and potentiation by 1 μM diazepam were determined.

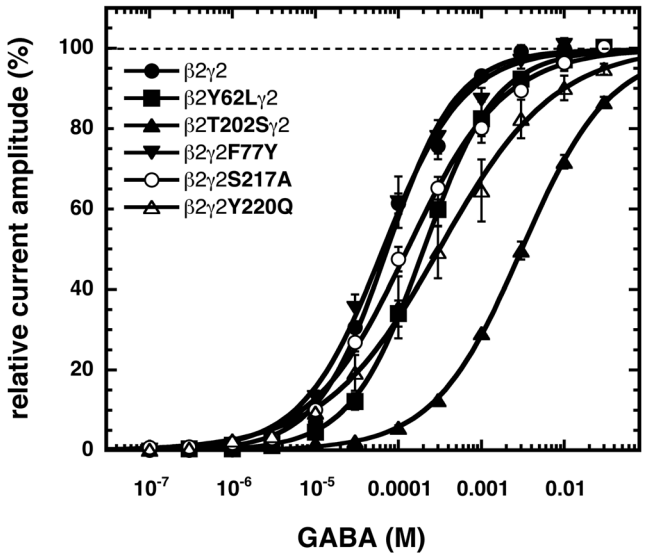

Figure 4. Influence of different point mutations on the concentration dependence of GABA. Wild type or mutant $\beta_2\gamma_{l2}$ $GABA_A$ receptors were expressed in *Xenopus* oocytes and exposed to subsequently higher concentrations of GABA and the elicited current amplitude was determined. Individual curves for each subunit combination were first normalized to the fitted maximal current amplitude and subsequently averaged. Averaged concentration-response curve are shown. Data are expressed as mean ± S.E.M., n = 3–7 from two batches of oocytes.

The notion that gating is affected by the mutation is strongly supported by the fact that 50 μM etomidate elicits about 4-fold smaller currents than saturating concentrations of GABA (Table 2). In wild type $\beta_2\gamma_{l2}$ receptors this current was about 7-fold larger than the one elicited by GABA. Similarly, in mutated $\alpha_1\beta_2$Y62Lγ_{l2} receptors this current was about 30-fold smaller than the one elicited by GABA. As the site for etomidate is located far away from the mutated residue, this is a strong indication that β_2Y62 is not only involved in binding of GABA, but also in gating.

Effect of mutations at the plus side of γ_2. Among the investigated mutations, γ_{l2}S217A and γ_{l2}Y220Q led to sizeable expression in combination with β_2 and were further studied. To our knowledge nothing is known about both mutations. The homologous mutation to γ_2S217A in the β_2 subunit, β_2T202A, led to a drastic loss in GABA sensitivity[12]. The homologous mutation to γ_2Y220Q in the α_1 subunit, α_1Y209Q, disrupted the site for diazepam, while leaving GABA sensitivity unaffected[26]. This residue was also identified with photoaffinity labeling by the benzodiazepine binding site ligand Ro15–4513[32]. In $\beta_2\gamma_{l2}$S217A and $\beta_2\gamma_{l2}$Y220Q the sensitivity to GABA was decreased 2-fold and 5-fold, respectively with EC_{50s} of 133 ± 20 μM (n = 3) and 367 ± 139 μM (n = 3),

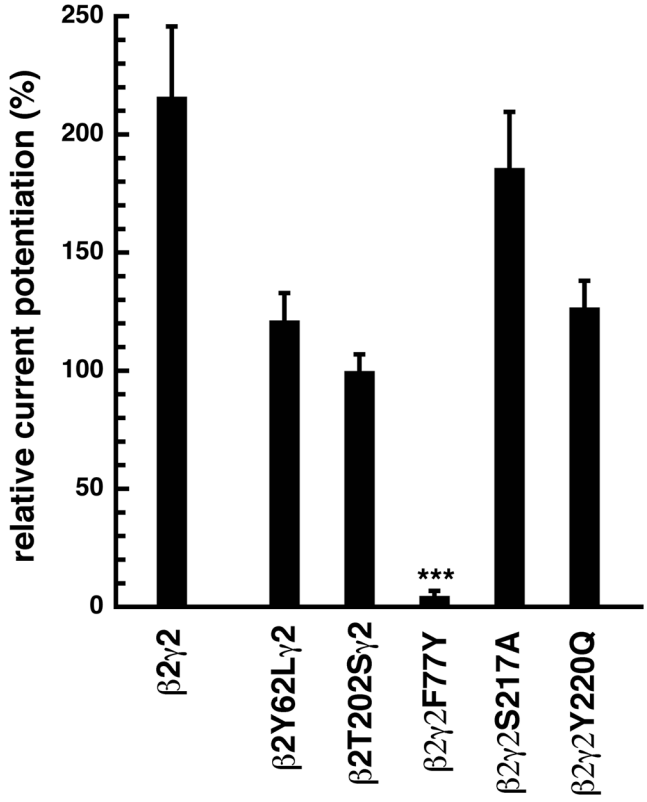

Figure 5. Influence of different point mutations on the potentiation by diazepam. Receptors were expressed in *Xenopus* oocytes and first exposed to 7 μM GABA alone or the same concentration of GABA in the presence of 1 μM diazepam and the elicited current amplitude was determined. Current potentiation by diazepam was calculated and averaged for each subunit combination. Data are expressed as mean ± S.E.M., n = 4–15 from two batches of oocytes.

and Hill coefficients of 0.8 ± 0.1 and 0.7 ± 0.1, respectively (Fig. 4, Table 2). Potentiation by 1 μM diazepam was not significantly affected by both mutations (Fig. 5, Table 2).

Effect of a mutation at the minus side of γ_2. We studied the mutation γ_2F77Y. In $\alpha_1\beta_2\gamma_2$ receptors this mutation abolishes the binding site for diazepam, while leaving GABA sensitivity unaffected[29]. Similar findings were made in $\beta_2\gamma_2$ receptors. The EC_{50} for GABA was 72 ± 15 μM (n = 5) and the Hill coefficient 0.9 ± 0.1 (Fig. 4, Table 2). Modulation by 1 μM diazepam was nearly lost (Fig. 5, Table 2).

Summary of the findings. No appreciable currents could be elicited upon expression of β_2 or γ_2 subunits alone and the receptors β_2T202Aγ_2, β_2Y205Sγ_2, $\beta_2\gamma_2$Y220S and β_2Y205Qγ_2. Modulation by diazepam was nearly lost in $\beta_2\gamma_2$F77Y receptors, whereas the reponse to GABA remained unaffected. Activation by GABA was strongly affected in β_2T202Sγ_2 receptors and weakly affected in β_2Y62Lγ_2, $\beta_2\gamma_2$S217A and $\beta_2\gamma_2$Y220Q receptors.

Computational Docking. We performed computational docking of diazepam utilizing a homology model of the β_2+/γ_2- interface based on the β_3 crystal structure 4COF[33] as specified in the Methods section. The overal sequence similarity between β_2+ and α_1+ is high, especially in loops B and C where several aromatic and polar amino acids are conserved. We have shown previously that loop C residues are engaged in key interactions with diazepam[24,26]. The docking as specified in the Methods section provided for sidechain flexibility (loops D, G, E, B and C,) as well as a limited degree of backbone flexibility in the loop C tip, very similar to the approach used in our previous docking studies at the canonical high affinity α_1+/γ_2- site[24]. Computational docking usually generates correct binding poses, but they are not always correctly ranked by the different scoring functions[34]. We therefore analyzed the top 100 poses of the docking run based on multiple different criteria: Only poses that display interactions with γ_2F77 were considered, to limit poses to those that reflect experimental findings. Poses were then filtered by similarity to the binding mode in the high affinity α_1+/γ_2- site[24], where similarity was judged on ligand binding mode and major interactions with the pocket. Lastly, two different scoring functions were employed to identify the best candidate poses based on consensus scoring. Overall, six poses were identified that show high similarity with the high affinity binding mode at α_1+/γ_2-. Among these, two were found in rank one and two positions in the ChemScore ranking, and five were among top 30 ChemScored. Similarly, one of the six candidates was found in the rank two position of the GoldScore Fitness ranking, and a total of three were among

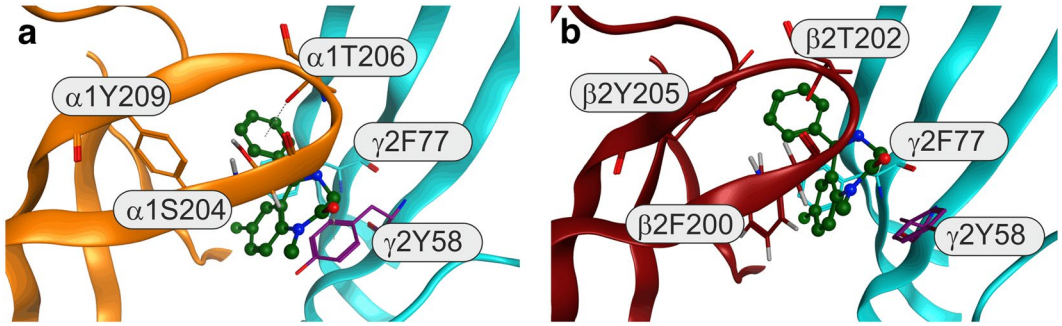

Figure 6. Structural hypothesis for diazepam binding at the extracellular β_2+/γ_2 interface. Panel (a) shows the reference binding pose from our previous studies[17] at the α_1+ (orange)/ γ_2- (cyan) interface. Panel (b) shows the most closely corresponding binding pose from the computational docking at the β_2+ (red)/γ_2- (cyan) interface. The homologous key amino acids in the binding pockets, as well as diazepam, are rendered in stick representation. While sidechain rotamers show some differences, ligand position, binding mode and key interations are very similar.

the GoldScore top 30. Thus, consensus scoring leads to a binding mode model that features a binding mode very similar to the one that is observed at the canonical high affinity site.

Due to the sterically demanding sidechain β_2F200, we find that sidechain rotamers adjust differently to ligand binding compared to the high affinity pocket, but gross binding mode and key interactions are highly similar (Fig. 6) where one of the representative poses is shown in comparison with the pose depicted in our previous study[24].

In the proposed binding mode the pendant phenyl ring is in close contact to loop A, which bears in the diazepam sensitive α isoforms a histidine, and in α_4 and α_6 an arginine which is known to interfere with diazepam binding[35]. Several H101X mutations were investigated in the past, and it was demonstrated that F (Phe), Y (Tyr) or Q (Gln) have only a small impact on flunitrazepam modulation[36]. The homologous position in β_2 is the hydrophobic Leu99, which is sterically similar to Gln, and as hydrophobic as Phe. Thus, the proposed binding mode is compatible with previous mutagenesis studies and with the present observations.

Discussion

Several early studies reported responsiveness of $\beta_2\gamma_2$ $GABA_A$ receptors expressed in heterologous systems to both GABA and diazepam[16–19] or to GABA in $\beta_x\gamma_2$ $GABA_A$ receptors[20–23]. Later, the two binding sites for GABA in $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were localized to the two β_2+/α_1- subunit interfaces[11,12] and the diazepam binding site to α_1+/γ_2- subunit interface[13]. Thus, the α_1 subunit seemed to be required for the formation of both sites. The major point of this study was to localize the subunit interfaces that harbor the alternative GABA and benzodiazepine binding sites in $\beta_2\gamma_2$ $GABA_A$ receptors lacking the α_1 subunit.

After having shown that $\beta_2\gamma_2$ $GABA_A$ receptors responded similarly to GABA and diazepam as $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, we used point mutations abrogating one of the two in $\alpha_1\beta_2\gamma_2$. Unfortunately, some of the chosen mutations interfered with receptor expression or gating, presumably by negatively affecting assembly or by leading to mainly inactive channels.

The γ_2+/β_2- subunit interface may be excluded for both sites. There are four possible subunit interfaces in $\beta_2\gamma_2$ $GABA_A$ receptors: β_2+/β_2-, β_2+/γ_2-, γ_2+/β_2- and γ_2+/γ_2-. Of these, the γ_2+/β_2- subunit interface also occurs in the $\alpha_1\beta_2\gamma_2$ receptors. Mutation β_2T202A dramatically impaired GABA activation in these receptors[12] and mutation α_1Y209Q led to loss of flumazenil sensitivity[26]. Obviously the γ_2+/β_2- subunit interface can not take over the formation of both sites. In addition three mutations located at this interface in $\beta_2\gamma_2$ receptors, β_2Y62Lγ_2, γ_2S217Aβ_2 and γ_2Y220Qβ_2, had no strong impact on the responses to GABA or diazepam (Table 2). Taken together, we can exclude that the γ_2+/β_2- subunit interface is the location of GABA and benzodiazepine binding sites.

Localization of the diazepam binding subunit interface. Mutations β_2T202S[12] and γ_2F77Y[29] have been described to disrupt GABA and diazepam binding sites in $\alpha_1\beta_2\gamma_2$ receptors, respectively. Mutations β_2T202S and γ_2F77Y similarly strongly affect the response of $\beta_2\gamma_2$ receptors to GABA and diazepam. Mutations β_2T202S and γ_2F77Y had little impact on diazepam and GABA sites, respectively. This implies a role of β_2+ and γ_2- in the formation of GABA site and diazepam site, respectively. Thus, the GABA binding site must be located at β_2+/β_2- or β_2+/γ_2- subunit interfaces and that for diazepam at β_2+/γ_2- or γ_2+/γ_2-.

The mutation α_1Y209Q abrogates the diazepam site in $\alpha_1\beta_2\gamma_2$ receptors[26]. Therefore, it may be expected that the homologous mutation in the γ_2 subunit, γ_2Y220Q, affects the apparent affinity for diazepam. Similarly, the mutation α_1T206A decreases the affinity for diazepam in $\alpha_1\beta_2\gamma_2$ receptors[26]. Thus, it may be expected that the homologous mutation in the γ_2 subunit, γ_2S217A, affects the apparent affinity for diazepam. Both mutations failed to affect the response to diazepam, arguing strongly against involvement of the γ_2+ subunit interface in

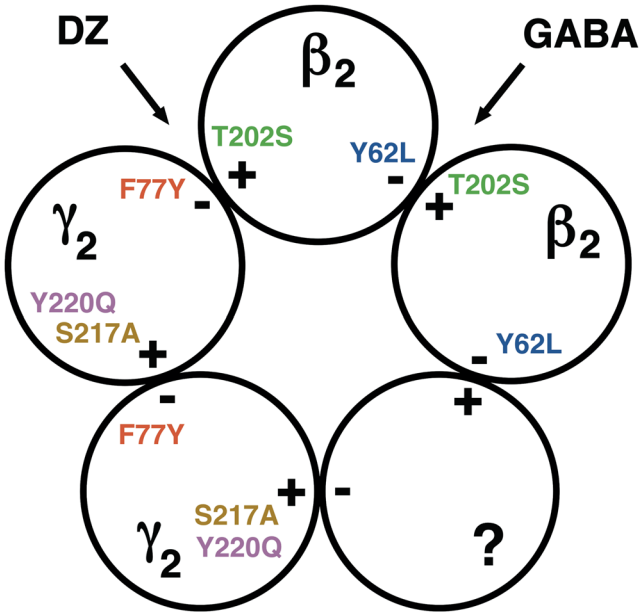

Figure 7. Schematic representation of $\beta_2\gamma_2$ $GABA_A$ receptors. The location of amino acid residues of interest is indicated. Point mutations resulting in disrupting assembly are not shown. The binding site for GABA is concluded to locate at the β_2+/β_2- interface, that for diazepam at the β_2+/γ_2- interface. Please note that the subunit arrangement was not addressed in this study.

the diazepam site. For these reasons, we locate this site to the β_2+/γ_2- subunit interface (Fig. 7). The failure of the mutation β_2T202S to affect the response to diazepam may be explained by the fact that a homologous, similar mutation α_1T206C did not affect the response to diazepam in $\alpha_1\beta_2\gamma_2$ receptors[27]. The high affinity interaction of diazepam with the β_2+/γ_2- subunit interface is also supported by the docking experiments (Fig. 6), which strongly suggest that diazepam binds in this non-canonical site in a fashion very similar to the one that is observed in the high affinity site.

Putative localization of the GABA binding subunit interface. Thus, we are left with the β_2+/β_2- and the γ_2+/γ_2- subunit interfaces. The mutation β_2T202S at the β_2+ has very strong effect on the EC_{50} for GABA shifting the concentration response curve 42-fold, while effects of mutations at the γ_2+ side are much smaller. Therefore we localize the GABA binding site at the β_2+/β_2- subunit interface (Fig. 7). However, we can not fully exclude an additional site at a γ_2+ side. As β_2+/γ_2- has been excluded, this additional GABA site would have to be at the γ_2+/γ_2- interface. It should be noted that a binding site for GABA has also been described at the $\beta_3+/\delta-$ subunit interface[37].

Functional expression. We observed that individual β_2 or γ_2 subunits did not form functional receptors on the surface of oocytes. Within 1 day after injection, the $\alpha_1\beta_2\gamma_2$ receptors expressed currents in μA range[38]. In contrast, we observed in this work that the $\beta_2\gamma_2$ receptors need longer period for channel expession (5–7 days) and the maximal current amplitudes elicited by 10 mM GABA amount to only 100–200 nA. If silent receptors and different single channel open frequency between receptors are ignored, $\beta_2\gamma_2$ receptors form less efficiently than $\alpha_1\beta_2\gamma_2$ receptors. A previous study described a important role of α_1 subunits for receptor trafficking and assembly in $\alpha_1\beta_2\gamma_2$ receptors[39]. Thus, in the presence of large amounts of α_1, $\beta_2\gamma_2$ may not be formed. Under special circumstances where α subunit expression is low, the formation of a limited amount of $\beta_2\gamma_2$ receptors may occur. Recent single cell RT-PCR data indicate that cells devoid of mRNA coding for α subunits are not present in the hypothalamus, where diversity of neurons is huge. However, the endocrine system may have receptors without α subunits. Chromaffine cells (at least in certain developmental stages) have only mRNA coding for β_3 and ε subunits (personal communication, I. Adameyko).

While so far it is not considered a candidate receptor to exist in the adult mammalian nervous system, the possible existence of such receptors has also not been specifically excluded. Given that fact that in the developing mammalian brain expression of all three γ isoforms is higher than in the postnatal brain, non-canonical receptor arrangements should be considered. In this vein, it is very important to realize that a high affinity benzodiazepine binding site at the β_2+/γ_2- interface implies that such receptors cannot be distinguished from $\alpha+/\gamma_2-$ "canonical" receptors in radioligand and PET studies where benzodiazepine ligands are used as presumably selective probes for $\alpha+/\gamma_2-$ canonical benzodiazepine-sites.

What may be the biological relevance of our observations? Ralvenius *et al.*[40] studied mice carrying a point mutation in all those four alpha subunits that can form diazepam sensitive $GABA_A$ receptors. At 10 mg/kg diazepam these mice were completely protected from diazepam-induced muscle relaxation and motor impairment. However, they showed a trend towards reduced locomotor activity that was quite prominent at higher doses. At

least part of this response could be due to $\beta_2\gamma_2$ $GABA_A$ receptors. We have not tested whether β_1 or β_3 (that may form $\beta_1\gamma_2$ and $\beta_3\gamma_2$) behave as β_2. As their loop C differs (see Supplementary Fig. S1), it is conceivable that the diazepam site described here does not exist or has different properties in these receptors.

Summary. While in $\alpha_1\beta_2\gamma_2$ receptors diazepam binds to the α_1+/γ_2- subunit interface and GABA to β_2+/α_1-, in $\beta_2\gamma_2$ receptors diazepam binds to the β_2+/γ_2- subunit interface and GABA to β_2+/β_2- (Fig. 7). Thus, the β_2 subunit can take over the role of the α_1 subunit for the formation of both sites, its minus side for the GABA binding site and its plus side for the diazepam binding site.

Methods

Construction of mutated receptor subunits. The point mutations β_2Y62Lγ_2, β_2T202Aγ_2, β_2T202Sγ_2, β_2Y205Sγ_2, β_2Y205Qγ_2, $\beta_2\gamma_2$F77Y, $\beta_2\gamma_2$S217A, $\beta_2\gamma_2$Y220S and $\beta_2\gamma_2$Y220Q were prepared using the QuickChange™ mutagenesis kit (Stratagene, Agilent Technologies, Basel, Switzerland).

Expression in *Xenopus* oocytes. Animal experiments were carried out in strict accordance to the Swiss ethical guidelines, and have been approved by the local committee of the Canton Bern Kantonstierarzt, Kantonaler Veterinärdienst Bern (BE85/15). Surgery of *Xenopus laevis* to obtain the oocytes was done under anesthesia, and all efforts were made to diminish animal suffering. Oocytes were prepared, injected and defolliculated as described previously[41,42]. Polyadenylated cRNA coding for the subunits of $GABA_A$ receptors were prepared *in vitro* with the mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA). Oocytes were injected with 50 nl of solution containing cRNA coding for wild type or mutants β_2 (1 fMol) or γ_2 (3 fMol) subunits and then incubated in modified Barth's solution (10 mM HEPES, pH 7.5, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.34 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 100 units/ml penicillin, 100 µg/ml streptomycin) at 18 °C for 5–7 days before measurements.

Functional characterization in *Xenopus* oocytes. Electrophysiological experiments were performed using an Oocyte Clamp OC-725 (Warner Instrument Corp., Hamden, USA) two-electrode voltage clamp amplifier. Currents were digitized at 5 kHz with MacLab/200 (AD Instruments, Spechbach, Germany).

The holding potential was −80 mV. The perfusion medium contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM Na-HEPES (pH 7.4). The perfusion solution (6 ml/min) was applied through a glass capillary with an inner diameter of 1.35 mm, the mouth of which was placed about 0.5 mm from the surface of the oocyte. Individual concentration response curves for GABA were fitted with the equation $I(c) = I_{max}/[1 + (EC_{50}/c)^n]$, where c is the concentration of GABA, EC_{50} the concentration of GABA eliciting half-maximal current amplitude, I_{max} is the maximal current amplitude, I is the current amplitude, and n is the Hill coefficient. Maximal current amplitudes (I_{max}) were obtained from the fits of the concentration-response curves. The individual curves were fitted and standardized to I_{max} and subsequently averaged. For all receptors studied, potentiation was measured at a GABA concentration eliciting 1–5% of the maximal GABA current amplitude. GABA was applied twice alone for 20 s, and then in combination with diazepam for 20 s. The duration of washout periods was 4 min in between agonist or agonist/drug applications to prevent receptor desensitization. At the beginning of the experiments, GABA applications were repeated when the elicited current amplitude altered by >5%. Potentiation was calculated by the following equation: $(I_{Modulator+GABA}/I_{GABA} - 1) * 100\%$. Concentration dependent potentiation was fitted with the equation $I(c) = I_{max}/[1 + (EC_{50}/c)^n]$, where c is the concentration of diazepam, EC_{50} the concentration of diazepam eliciting half-maximal current amplitude, I_{max} is the maximal current amplitude, I is the current amplitude, and n is the Hill coefficient. Maximal current amplitudes (I_{max}) were obtained from the fits of the concentration-response curves. The individual curves were fitted and standardized to I_{max} and subsequently averaged.

All data are from at least two different batches of oocytes. Data represent mean ± S.E.M as indicated in each case. An unpaired *t-test* was used to compare two means. ***$p < 0.001$.

Computational Modelling and Docking. Homology models of the β_2+/γ_2- interface were generated based on the 4COF $GABA_A$ receptors human β_3 homopentamer structure[24]. Due to the high homology of the β_2 and β_3 subunits, no insertions or deletions requiring gaps occur in the extracellular domain, while an alignment of the γ_2 subunit as described previously[43] was used to account for the lower homology in the loop F region. Computational docking was subsequently performed using the GOLD software v1.6.2[44], where the binding site was defined to be at the interface between loops A – G of the subunits. Sidechains β_2Y157, β_2T160, β_2Y159, β_2T161, β_2F200, β_2T202, and β_2Y205 as well as γ_2Y58, γ_2F77 and γ_2T142 were kept flexible, soft potential were applied to the tip of loop C (β_2V198 - β_2G203) to allow some degree of backbone flexibility, default settings for the docking run were used and the top 100 ranked poses were retained for subsequent analysis. Ligand interactions of poses were computed using the MOE program (MOE (The Molecular Operating Environment), Version 2011.10, Chemical Computing Group Inc., Montreal), and poses featuring interactions with γ_2F77 were inspected using two scoring functions (ChemScore Fitness as implemented in GOLD, GoldScore Fitness). "Hits" were defined as poses among top 30 ranked in both scoring functions (consensus score hits) and featuring interactions with γ_2F77 and the resulting hit poses were subsequently compared to our poses from previous work at the canonical binding site[24].

Data availability. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

N.W., M.C.M., X.S. and R.B. performed molecular biology and electrophysiological experiments, X.S. and M.E. performed computational studies, N.W. and E.S. designed the experiments, analysed the data and wrote the manuscript.

Additional Information

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