Mechanism of benzodiazepine effects on $\text{GABA}_A$ receptors

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ABSTRACT

Benzodiazepines are positive allosteric modulators of the $\text{GABA}_A$ receptor ($\text{GABA}_A$R), acting at the $\alpha$$-\gamma$ subunit interface to enhance $\text{GABA}_A$R function. $\text{GABA}$ or benzodiazepine binding induces distinct conformational changes in the $\text{GABA}_A$R. The molecular rearrangements in the $\text{GABA}_A$R following benzodiazepine binding remain to be fully elucidated. Using two molecular models of the $\text{GABA}_A$R, we identified electrostatic interactions between specific amino acids at the $\alpha$$-\gamma$ subunit interface which were broken by, or formed after, benzodiazepine binding. Using two-electrode voltage clamp electrophysiology in $\text{Xenopus laevis}$ oocytes, we investigated these interactions by substituting one or both amino acids of each potential pair. We found that Lys-104 in the $\alpha_1$ subunit forms an electrostatic bond with Asp-75 of the $\gamma_2$ subunit after benzodiazepine binding, and that this bond stabilizes the positively-modified state of the receptor. Substitution of these two residues to cysteine and subsequent covalent linkage between them increased the receptor’s sensitivity to low $\text{GABA}$ concentrations and increased the receptor’s response to benzodiazepines. The $\alpha_1$ Lys-104 and $\gamma_2$ Asp-75 interaction did not play a role in ethanol or neurosteroid modulation of $\text{GABA}_A$R, suggesting that different modulators induce different conformational changes in the receptor. These findings may help explain the additive or synergistic effects of modulators acting at the $\text{GABA}_A$R.

The ionotropic $\text{GABA}_A$ receptor ($\text{GABA}_A$R) is a pentameric protein belonging to the cys-loop superfamily family of ligand-gated ion channels. Various subunits ($\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$ and $\delta$, $\epsilon$, $\theta$ and $\pi$) combine in multiple combinations to form $\text{GABA}_A$Rs. $\text{GABA}$ is the predominant inhibitory neurotransmitter in the central nervous system, and its activation of the $\text{GABA}_A$R results in anion movement through the integral ion channel pore. Benzodiazepines are used clinically for their sedative, anxiolytic and anti-convulsant effects. These drugs act at an allosteric site of the $\text{GABA}_A$R to positively modulate the channel when activated by an agonist acting at the orthosteric site. Several hypotheses have been suggested to explain the molecular mechanisms of this benzodiazepine enhancement of function, including an increase in the $\text{GABA}$ binding affinity of the receptor (1-3), an increase in $\text{GABA}$ efficacy (4,5) or a shift of the receptor towards a ‘pre-activated’ state (6).
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Different α-subunit containing GABA<sub>A</sub> receptors, account for the various therapeutic indications of benzodiazepines. GABA<sub>A</sub>Rs containing α<sub>1</sub> subunits are thought to be primarily responsible for the sedative and anti-convulsive effects of benzodiazepines, while α<sub>2</sub>-containing GABA<sub>A</sub>Rs are responsible for their anxiolytic effects (7-10). The inability of classic benzodiazepines to distinguish between receptors comprised of different α subtypes suggests a conserved molecular mechanism of action. Histidine residue 101 in the α<sub>1,2,3</sub> subunits (103 in α<sub>3</sub>) plays an important role in benzodiazepine binding, with substitution of this residue with arginine rendering receptors less sensitive to benzodiazepines (11). However, the conformational changes in the GABA<sub>A</sub>R that occur subsequent to benzodiazepine binding are less well understood.

Inter- and intra-subunit electrostatic interactions play important roles in cys-loop receptor function. For example, electrostatic interactions between residues of adjacent alpha subunits in the glycine receptor play an important role in its activation (12). Specifically, the aspartate 97 residue is thought to interact with arginine 119 to stabilize the closed state of the glycine receptor, and once this bond is broken after agonist binding, the channel opens. Additionally, electrostatic interactions between aspartic acid 149 and lysine 279 within the same α subunit, as well as between aspartic acid 146 and lysine 215 within the same β subunit are implicated in the coupling of GABA binding to the opening of the GABA<sub>A</sub>R (13,14). Further, glutamic acid 153 and lysine 196 within the same β subunit of the GABA<sub>A</sub>R may be involved in stabilizing the open state of the receptor (15). Disulfide trapping experiments have led to insights into the conformational changes that benzodiazepines produce in the GABA<sub>A</sub>R after binding (16); however, thus far an electrostatic interaction has not been identified in the GABA<sub>A</sub>R that occurs because of this conformational change.

In the current study, we used homology modeling with published structures to produce models of α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> GABA<sub>A</sub>R. We used these models to identify potential electrostatic interactions occurring before or after the conformational changes produced by benzodiazepine binding, identifying a pair of residues that appear to be interacting in a manner specific for benzodiazepine modulation of the GABA<sub>A</sub>R.

RESULTS

Molecular modeling identifies possible electrostatic interactions present before and after benzodiazepine binding at the α<sub>1</sub>-γ<sub>2</sub> subunit interface of the GABA<sub>A</sub>R.

As a starting point for our studies, we used two different models to identify potential electrostatic interactions at the α<sub>1</sub>-γ<sub>2</sub> subunit interface. The first was based on molecular dynamic modeling performed by Yoluk et al. (17) on the GluCl ligand-gated cys-loop receptor, in the absence of ivermectin (Fig. 1, left). This first model corresponds to the closed, GABA- and benzodiazepine-unbound state of the GABA<sub>A</sub>R in our studies. The second model (Fig. 1, right) is based on the GABA- and diazepam-bound GABA<sub>A</sub>R receptor model described by Bergmann et al. (18). Choosing to investigate only charged residues predicted to be 6Å or less apart, we identified seven interactions that could occur before benzodiazepine binding, as well as four interactions that could occur after benzodiazepine binding. Two of these pairs, aspartic acid 56 of the α<sub>1</sub> subunit (α<sub>1</sub>D56) with arginine (R) 197 of the γ<sub>2</sub> subunit (γ<sub>2</sub>R197), as well as glutamic acid (E) 58 of the α<sub>1</sub> subunit (α<sub>1</sub>E58) with γ<sub>2</sub>R197, were predicted to form electrostatic pairs both before and after diazepam binding. In the present study, we focused on the electrostatic interactions that were predicted to interact closest to the benzodiazepine binding site (Fig. 1, interactions B-F and H-K).

Effects of cysteine substitution on diazepam potentiation of GABA<sub>A</sub>R function

Diazepam (1 μM) enhancement of the effects of a GABA concentration required to produce 5-10% of the maximal response (EC<sub>5-10</sub>), was tested on a series of cysteine mutants. Cysteine substitution of residues of the α<sub>1</sub> or γ<sub>2</sub> subunit predicted to be involved in electrostatic interactions before and/or after diazepam binding resulted in a significant effect of mutation on diazepam potentiation (see Fig. 2 legend for statistics). Replacing α<sub>1</sub> E58 with cysteine [α<sub>1</sub>(E58C)] resulted in a significant increase in diazepam potentiation, while the α<sub>1</sub>(K104C), α<sub>1</sub>(E137C), γ<sub>2</sub>(D75C) and γ<sub>2</sub>(R197C)
substitutions all resulted in significant decreases in diazepam enhancement (Fig. 2). Six of the other residues substituted with cysteine, α₁(D56C), α₁(K105C), α₁(E165C), γ₂(R97C), γ₂(D120C) and γ₂(R194C), resulted in no significant changes in receptor enhancement by diazepam, compared to wildtype GABA₄R. Of the pairs probed, the only hypothesized pair that produced similar changes in diazepam effects upon mutation to cysteine were α₁(K104C) and γ₂(D75C) (Fig. 1, interaction 1). If an electrostatic interaction was occurring between two residues, one would expect similar changes in receptor function if that bond was broken by mutating either residue. For this reason we focused on the α₁K104 - γ₂D75 pair. Before diazepam binding α₁K104 was predicted to be approximately 9Å from γ₂D75 (Figs. 3A and 3B), but after diazepam binding these residues were predicted to move much closer together, to approximately 5Å apart (Fig. 3C).

Effects of cysteine substitution on GABA sensitivity at α₁K104 and γ₂D75 residues

GABA concentration-response curves for α₁(K104C)β₂γ₂, α₁β₂γ₂(D75C) and α₁(K104C)β₂γ₂(D75C) receptors did not significantly differ from those of wildtype receptors (Fig. 4A). However, one-way ANOVAs revealed that lower GABA concentrations (3 μM and 10 μM) produced greater responses in α₁(K104C)β₂γ₂(D75C) receptors compared to the single mutants and wildtype receptors (see Fig. 4 legend for statistics). Despite the model-based hypothesis that the electrostatic interaction between α₁K104 and γ₂D75 is predicted to occur after diazepam binding, substituting these residues with cysteines could allow a disulfide bond to form spontaneously, which would be able to form between residues at greater distances apart than an electrostatic bond. Therefore, we tested if the disulfide bond between α₁K104C and γ₂D75C had spontaneously occurred. The reducing agent dithiothreitol (DTT) is able to break accessible disulfide bonds. Application of 2 mM DTT to the α₁(K104C)β₂γ₂(D75C) receptor resulted in an increase in the GABA EC₅₀, from 3.6 ± 0.4 μM before DTT application to 10.5 ± 0.35 μM after DTT (Fig. 4B). This is due to the breakage of a single inter-subunit disulfide bond as shown in Fig. 5A.

To further probe if α₁K104C and γ₂D75C spontaneously form a disulfide bond in the α₁(K104C)β₂γ₂(D75C) receptor, we tested PMTS for its effects. PMTS is able to covalently bind to free cysteine residues to which it has access. PMTS caused a significant decrease in GABA EC₅₀ current in the single and double mutant receptors (Fig 5B, hollow bars with open circles). In the wildtype and both single cysteine mutant receptors, the effect of PMTS remained unchanged after a prior DTT application (Fig. 5B, hollow bars with triangles). This indicates that in single mutant receptors the cysteine substituted residues do not form disulfide bonds with endogenous cysteines in GABA₄R. Since these single mutant and wildtype receptors exhibited similar changes in response to PMTS before and after DTT application, we did not test these receptors again 60 minutes after DTT treatment. For the α₁(K104C)β₂γ₂(D75C) receptor, a one-way ANOVA revealed a significant effect of PMTS treatment before, 5 minutes after, and 60 minutes after DTT treatment [F(2,13)=108.363, p<0.001]. Without prior exposure to DTT, application of PMTS resulted in a decrease in current (Fig. 5B, white bar, open circles). However, DTT application before PMTS resulted in an increase in current (solid bar). Waiting 60 minutes after DTT washout, and then applying PMTS, resulted in a decrease in current similar to that seen with PMTS application before DTT application. For the double cysteine mutant receptor the white bar with open circles represents PMTS binding to the single available cysteine residue situated between the α and β subunit interfaces as shown in the illustration on the left in Fig. 5A. When DTT breaks the sole disulfide bond between α and γ subunits, PMTS can now bind to up to three free cysteines. Since there was no significant difference between PMTS application before DTT application and 60 minutes after DTT application, we hypothesize that the disulfide bond breakage produced by DTT is only temporary, and that the receptor spontaneously returns to its pre-DTT form within an hour. The reformation of the disulfide bond in the double mutant receptor was also seen experimentally by repeatedly applying the GABA EC₅₀ to the DTT-treated receptor and observing a gradual increase in current (Figs. 5C and 5D). The current produced by a maximally-effective concentration of GABA was not changed by applying DTT (data not shown).
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There were no effects of DTT application on the modulation produced by 1 μM diazepam, flunitrazepam, Ro 15-4513 or zolpidem on wildtype receptors, as expected. This was also the case for $\alpha_i$(K104C)$\beta_2\gamma_2$ and $\alpha_i\beta_2\gamma_2$(D75C) receptors. However, application of DTT to the $\alpha_i$(K104C)$\beta_2\gamma_2$(D75C) receptor produced an increase in diazepam potentiation (from 76.6 ± 6.3% to 136.7 ± 9%) and flunitrazepam potentiation (from 121.2 ± 9.1% to 201 ± 22.3%), and a decrease in potentiation by Ro 15-4513 (from 1 ± 3.2 to -17.8 ± 1.9%) (Fig. 6A-C). DTT treatment rescued responses of $\alpha_i$(K104C)$\beta_2\gamma_2$(D75C) receptors to wildtype levels by flunitrazepam and Ro 15-4513, but not by diazepam. Application of the non-classical benzodiazepine, zolpidem, did not produce a significant interaction between receptor mutant and DTT treatment, but a Tukey’s post-hoc test revealed a small significant difference between pre- and post-DTT treatment in $\alpha_i$(K104C)$\beta_2\gamma_2$(D75C) receptors ($p$<0.05) (Fig. 6D). Interestingly, Ro 15-4513 produced a greater inhibitory response in the $\alpha_i\beta_2\gamma_2$(D75C) mutant compared to wildtype receptors, both before and after DTT treatment (Fig 6C). Treatment with 0.3% H$_2$O$_2$ for 90 sec, which would favor cysteine bond reformation, before the benzodiazepine application reversed the effects of DTT in $\alpha_i$(K104C)$\beta_2\gamma_2$(D75C) receptors, but produced no changes in responses by wildtype receptors (Figs. 7A and 7B).

Effects of cysteine substitution on non-benzodiazepine modulators of the GABA$_A$R

Allosteric modulators of the GABA$_A$R acting at sites other than the benzodiazepine binding site were next tested to determine the specificity of the electrostatic interactions between $\alpha_i$(K104C) and $\gamma_2$(D75C). Ethanol and the neurosteroid allopregnanolone produced similar potentiation of the effects of GABA on wildtype, $\alpha_i$(K104C)$\beta_2\gamma_2$, $\alpha_i\beta_2\gamma_2$(D75C) and $\alpha_i$(K104C)$\beta_2\gamma_2$(D75C) GABA$_A$Rs (Fig. 8). There was no significant effect of DTT treatment on the enhancement of wildtype or mutant receptors by 200 mM ethanol, 100 nM allopregnanolone (Fig. 8) or 1 μM allopregnanolone (data not shown).

Effects of alanine substitution at $\alpha_i$K104 and $\gamma_2$D75 on GABA and benzodiazepine responses

To examine the effects of alanine substitutions at the $\alpha_i$K104 and/or $\gamma_2$D75 residues, we compared receptors containing these alanine residues to wildtype receptors in their responses to GABA, 1 μM diazepam and 1 μM flunitrazepam. The GABA concentration-response curve for $\alpha_i\beta_2\gamma_2$(D75A) was slightly right shifted (EC$_{50}$ 133.6 ± 19.4 μM), while that of the $\alpha_i$(K104A)$\beta_2\gamma_2$ receptor was slightly left shifted (EC$_{50}$ 61.9 ± 12.2 μM) compared to the wildtype receptor curve (EC$_{50}$ 77 ± 8.8 μM) (Fig. 9A). A repeated-measures ANOVA revealed a significant difference among the four concentration-response curves (see Fig. 9A legend for statistics). Interestingly, the $\alpha_i$(K104A)$\beta_2\gamma_2$(D75A) GABA concentration-response curve (EC$_{50}$ 87.3 ± 12.4 μM) was not left-shifted at lower concentrations (3 μM and 10 μM), unlike the $\alpha_i$(K104C)$\beta_2\gamma_2$(D75C) receptor (Fig. 9A compared to Fig. 4A). One-way ANOVAs revealed that there was a significant effect of alanine substitution on the enhancement of GABA EC$_{50}$ by 1 μM diazepam or flunitrazepam. While the single substitution $\alpha_i$(K104A)$\beta_2\gamma_2$ and $\alpha_i\beta_2\gamma_2$(D75A) receptors exhibited a decreased response to diazepam and flunitrazepam compared to wildtype receptors, the $\alpha_i$(K104A)$\beta_2\gamma_2$(D75A) receptors displayed a level of potentiation not significantly different from that of wildtype receptors (Fig. 9B).

Effects of charge reversal of $\alpha_i$K104 and $\gamma_2$D75 residues on GABA and GABA receptor modulator responses

To test if reversing the charges of $\alpha_i$K104 and $\gamma_2$D75 would restore GABA sensitivity, GABA concentration-response curves of $\alpha_i$(K104D)$\beta_2\gamma_2$(D75K) were compared to those of wildtype receptors (Fig. 10A). A repeated-measures ANOVA found a significant difference between wildtype and $\alpha_i$(K104D)$\beta_2\gamma_2$(D75K) concentration response curves (see Fig. 10A legend for statistics). The average EC$_{50}$ value for wildtype receptors was 86.8 ± 16.5 μM while the EC$_{50}$ for $\alpha_i$(K104D)$\beta_2\gamma_2$(D75K) was increased to 146.3 ± 23.1 μM. The charge reversal did not restore to wildtype levels receptor potentiation by 1 μM diazepam or Ro 15-4513, but did restore potentiation by 1 μM flunitrazepam and zolpidem (Fig. 10B). Other GABA$_A$ receptor modulators (200 mM ethanol and 100 nM allopregnanolone) displayed no changes in potentiation of GABA.
EC₅₋₁₀ after charge reversal, compared to wildtype receptors (data not shown).

**DISCUSSION**

Signal transduction of ligand-gated ion channels after neurotransmitter binding to its orthosteric site is believed to involve a wave of structural rearrangements (19) in the receptor, and this rearrangement is thought to be separate from the signal transduction pathway produced by allosteric modulators (20). Using molecular modeling to identify potential electrostatic interactions between the α₁ and γ₂ subunits, we identified an interaction between α₁K104 and γ₂D75 that occurs after diazepam binding (Fig. 3). It is likely that these residues interact to stabilize the positively-modified state of the receptor, and that this interaction is specific for the benzodiazepine signal transduction pathway.

Low concentrations of GABA produced greater responses in α₁(K104C)β₂γ₂(D75C) than in wildtype receptors, but this was not seen at higher GABA concentrations (Fig. 4A). This increased response at low GABA concentrations is similar to what one would expect to see in response to co-application of GABA with a benzodiazepine in wildtype receptors. Benzodiazepine site agonists increase the effects of low but not higher concentrations of GABA, since the ion channel approaches its maximal open probability at saturating GABA concentrations (21).

One might hypothesize that a receptor that is behaving as though a benzodiazepine molecule has already bound would exhibit a decreased response to a co-application of benzodiazepine with GABA. In the α₁(K104C)β₂γ₂(D75C) receptor, the two cysteine residues spontaneously formed a disulfide bond. Accordingly, we saw the expected decrease in diazepam, flunitrazepam, and zolpidem potentiation in the double cysteine substituted receptors (Figs. 6A, 6B and 6D). After the disulfide bond is broken with DTT, responses to these benzodiazepines increase, suggesting that an electrostatic bond between these residues in wildtype receptors formed in response to benzodiazepine binding. After DTT application, the response of α₁(K104C)β₂γ₂(D75C) receptors to flunitrazepam potentiation was rescued to wildtype levels (Fig. 6B). One reason why potentiation by flunitrazepam, but not diazepam or zolpidem, may be completely rescued following DTT application is that the latter two show weaker modulatory responses after α₁K104C-γ₂D75C disulfide bond formation than flunitrazepam; i.e., lower potentiation of GABA responses before DTT application. The conformational rearrangement within the GABA₄ receptor after benzodiazepine binding most likely depends on the formation of multiple bonds, not just α₁K104-γ₂D75. Thus, the α₁K104 - γ₂D75 bond may be less important for flunitrazepam potentiation than for diazepam or zolpidem.

Zolpidem is a non-classical benzodiazepine and at low concentrations is selective for the α₁ subunit-containing GABA₄R over those containing other α subunits (22). Disulfide trapping within the γ₂ subunit has shown that the conformational change produced by classical benzodiazepines may not be the same as that produced by zolpidem (16). Similarly, there are mutations in the α₁ and γ₂ subunits that affect classical but not non-classical benzodiazepines, or vice versa (23-26). The magnitude of the increase in α₁(K104C)β₂γ₂(D75C) receptor potentiation by zolpidem after DTT application was far smaller than the increase seen with the classical benzodiazepines diazepam and flunitrazepam (Fig. 6). We speculate that this may be due to classical and non-classical benzodiazepines producing overlapping yet distinct conformational changes in the GABA₄R after binding.

One might hypothesize that the conformational changes produced by benzodiazepines would be different than those produced by inverse agonists such as Ro 15-4513. Indeed, previous studies have shown that this might be the case, where disulfide trapping at the α-γ interface of the GABA₄R, that affected benzodiazepine potentiation, had no effect on inverse benzodiazepine inhibition (16). Our work supports this hypothesis, as the α₁(K104C)β₂γ₂(D75C) mutant GABA₄R, which traps the receptor in a ‘positively modified’ state, was not inhibited by Ro 15-4513 as much as wildtype receptors (Fig. 6C). Once DTT is applied, the α₁(K104C)β₂γ₂(D75C) receptor is relieved of this ‘positively modified state’, and Ro 15-4513 is able to produce inhibition to levels similar to that of wildtype receptors (Fig. 6C). Ro 15-4513 produced more inhibition in the
αβγ2(D75C) receptor than in the wildtype, α1(K104C)βγ2(D75C) and α1(K104C)βγ2(D75C) receptors. Substituting the γ2D75 residue with a lysine or alanine residue increased the inhibition by Ro 15-4513 even more so than the cysteine replacement at that residue (data not shown). This decrease suggests that the γ2D75 residue may be involved in the conformational change produced by Ro 15-4513 as well as a distinct conformational change produced by potentiating benzodiazepines.

After DTT breaks the disulfide bond in α1(K104C)βγ2(D75C) receptors, one might expect the receptor to behave similarly to the α1(K104A)βγ2(D75A) receptor. While the α1(K104A)βγ2(D75A) receptor exhibited levels of diazepam and flunitrazepam potentiation similar to wildtype receptors (Fig. 9B), DTT treatment to α1(K104C)βγ2(D75C) receptors did not fully restore levels of diazepam potentiation (Fig. 6A). One possible explanation for this is that DTT breaking the disulfide bond by reducing each mutant cysteine) results in two hydrogen-bond cysteine residues that would occupy more volume than alanine residues at those positions, preventing the conformational change produced by diazepam from occurring. Another possibility is that in the α1(K104C)βγ2(D75C) receptor, the spontaneous reformation of a disulfide bond after DTT treatment (Fig. 5C and 5D) prevents one from experimentally capturing the maximal amount of enhancement produced by diazepam.

The α1(K104D)βγ2(D75K) receptor, bearing two charge-reversing substitutions, displayed a right-shifted GABA concentration-response curve compared to wildtype receptors (Fig. 10A). Additionally, the α1(K104D)βγ2(D75K) receptor did not restore GABA, diazepam, or Ro 15-4513 sensitivity to wildtype levels (Fig. 10B). This is likely because the α1K104 and γ2D75 residues lie within a pocket of charges, and that modifying these residues is preventing other interactions from occurring; i.e., although K104D and D75K substitutions may restore the electrostatic interaction between these residues, there are other charged residues near these sites that may now interact differently with the reversed charge residues, compared to the original wildtype amino acids. Evidently, the α1K104 - γ2D75 interaction is not the only interaction that is important for producing the positively modified state of the receptor. If it were, one would see no benzodiazepine potentiation of the receptor after mutating the α1K104 and γ2D75 residues.

One might argue that the data obtained from the alanine substitution experiments at α1K104 and γ2D75 do not fit our overall hypothesis that formation of a bond between these two residues facilitates benzodiazepine effects at the GABA receptor. Perhaps what is happening is that during the conformational changes produced by benzodiazepine site agonists at wildtype receptors these two charged residues come close enough together to at least partially neutralize each others' charges. This hypothesis is supported by results obtained using the single alanine substitutions, which retain single charged residues in each pair, and display weaker effects of benzodiazepines than those seen in the double alanine mutant (Fig. 9B). A possible explanation may be that the retained charged residue in the single mutants may still be interacting with other nearby charged residues (e.g., α1K105, γ2D148 or γ2R197), thus retarding the ability of the receptor to adopt the benzodiazepine-activated conformational state. This would also apply to the single cysteine substitutions which also display decreased responses to diazepam and flunitrazepam. In scenarios in which the α1K104 and γ2D75 residues are in close proximity (e.g., cysteines crosslinked) the receptor has already adopted a benzodiazepine positively-modified state and thus adding exogenous benzodiazepine does not have much effect. In cases where these two residues are not initially close together, but are capable of moving closer together, (e.g., the double alanine substitutions or the double uncrosslinked cysteines) a greater effect of applied benzodiazepine will be seen. Lastly, in scenarios in which one or the other of these residues is constrained in its movement (e.g., single substitutions), benzodiazepine effects would be smaller due to the remaining charged residue.

An initial concern was that the receptor mutants were not being incorporated correctly on cell surfaces and that oocytes were expressing primarily α1β2 receptors, not α1βγ2 receptors. Previous studies used ZnCl2 to test for γ2 subunit incorporation, as zinc inhibits α1β2 receptors to a greater extent than α1βγ2 GABA Rs (27).
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However, using this test may not be the most accurate way to test for \(\alpha\beta\) contamination, as even a small fraction of \(\alpha_1\beta_2\) receptors present may produce a significant inhibitory effect by zinc (28). Interestingly, the \(\alpha_1\beta_2\) receptors display an increase in their GABA-evoked currents after DTT treatment, but \(\alpha_1\beta_2\gamma_2\) receptor currents are unchanged (29). In our study, we saw no change in GABA-evoked currents after DTT treatment in wildtype and mutant receptors, except for \(\alpha_1\beta_2\gamma_2\) (D75C) receptors, where we actually saw a decrease in GABA-evoked currents (Fig. 5D). This, together with the fact that we injected receptors cDNAs in a 1\(\alpha_1\):1\(\beta_2\):10\(\gamma_2\) cDNA ratio and we still saw a benzodiazepine effect, engenders confidence that the receptors are incorporating wildtype and mutated \(\gamma_2\) subunits.

One interesting and clinically-relevant aspect of this study revolves around the additive and synergistic properties of GABA\(_A\) modulators. Benzodiazepines are often co-abused with ethanol (30), and the two classes of compounds are thought to act additively or synergistically as central nervous system depressants. While ethanol is thought to act at the \(\alpha+/\beta-\) interface in \(\alpha \beta \delta\) GABA\(_A\)Rs, it is not clear if this is necessarily the case in \(\alpha \beta \gamma\) receptors (31). We tested if mutations that affect both GABA and benzodiazepine responses also produced changes in ethanol responses. In the \(\alpha_1\beta_2\gamma_2\) ‘positively modified’ receptor, no changes in ethanol potentiation were observed (Fig. 8). Similarly, the charge reversal \(\alpha_1\beta_2\gamma_2\) receptor exhibits similar ethanol potentiation to that of wildtype receptors (data not shown). These data suggest that the conformational changes in the GABA\(_A\)R produced by ethanol are experimentally separable from the conformational changes produced by benzodiazepines, and that both can occur simultaneously to further enhance receptor function. This provides a possible molecular mechanism for the synergistic/additive effects of benzodiazepines and alcohol.

The neurosteroid allopregnanolone acts as a potent modulator of the GABA\(_A\)R, as well as a direct activator at high concentrations. The binding site for this enhancing action is thought to be within a cavity formed by transmembrane domains 1 and 4 within a single \(\alpha\) subunit (32,33).

The \(\alpha_1\beta_2\gamma_2\) (D75C) ‘positively modified’ receptor and \(\alpha_1\beta_2\gamma_2\) (D75K) charge reversal receptor displayed no differences in their sensitivities to the potentiating effects of allopregnanolone, compared to wildtype receptors (Fig. 8). As well as having distinct binding sites, our data suggest that allopregnanolone and benzodiazepines produce distinct conformational changes in the GABA\(_A\)R.

In summary, our study suggests that an inter-subunit electrostatic interaction between \(\alpha_1\)K104 and \(\gamma_2\)D75 occurs after benzodiazepine site agonist binding to help stabilize the GABA\(_A\)R in a positively modified state. This interaction seems to be more important for classical (non-selective between GABA\(_A\)R \(\alpha\) subunits) benzodiazepines than non-classical (\(\alpha_1\) selective) compounds. Additionally, this interaction does not seem to be important for modulators of the GABA\(_A\)R acting at non-benzodiazepine sites, suggesting that the \(\alpha_1\)K104-\(\gamma_2\)D75 interaction is specific for benzodiazepine site agents.

**EXPERIMENTAL PROCEDURES**

**Reagents**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated below.

**Structural modeling**

Two homology models of the GABA\(_A\)R were generated using the Modeler module of Discovery Studio 2016 (DS 2016, Biovia, San Diego, CA) as previously described (34). The first model was of the GABA\(_A\)R in the benzodiazepine-unbound state. This was built using the GluCl X-ray structure in the absence of ivermectin (17) as a template. This template was produced by starting with the structure of GABA\(_A\)R with five ivermectin molecules bound (PDB ID 3RHW), removing the five ivermectin molecules, and then running extensive constrained molecular dynamics simulations using GROMACS 4.5. The resulting model was judged to be in the closed/resting state because the subunits moved closer by 2.0 Å and the pore diameter decreased by 1.2 Å (17). The second homology model illustrated GABA\(_A\)R after diazepam was bound. This model was based on a GluCl/ELIC X-ray structure which modeled diazepam binding (18). It should be noted that other investigators have proposed a different
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The orientation of diazepam docking at this α/γ interface (35). Since the latter template was built using a novel method of combining coordinates from two X-ray structures, it deserves some comment. The model is based primarily on the glutamate-bound GluCl crystal structure (PDB ID 3RIF) with a contribution of the ELIC crystal structure (PDB ID 2VLO) that the authors identified as leading to the best alignment and the best composite structure. Of interest for the present results; in GABA<sub>A</sub>R α<sub>2</sub>, lysine 104 is in beta strand 4 and all coordinates are from GluCl. However, in GABA<sub>A</sub>R α<sub>1</sub>, aspartatic acid 75 is in beta strand 2, a residue that is conserved in ELIC but not in GluCl. As a result, Bergman et al (18) used the ELIC structure as a template for residues 75-77.

Since both templates are homopentamers and our goal was to measure intersubunit interactions, we prepared a composite sequence by linking GABA<sub>A</sub>R α<sub>1</sub>/β<sub>2</sub>/α<sub>1</sub>/β<sub>2</sub>/γ<sub>2</sub> and aligning the composite with the sequence of the two templates (36). Then the GABA<sub>A</sub>R sequences were trimmed to match the length of the template sequences as needed. The two pairs of aligned sequences were submitted to the Modeler module of DS 2016. Both of the resulting homology models were assigned the CHARMM force field in DS 2016, minimized, and then subjected to molecular dynamics simulations at 300K as previously described (34). These two models were analyzed for possible electrostatic interactions using DS 2016.

Site-directed mutagenesis
Human cDNAs encoding α<sub>1</sub>, β<sub>2</sub> and γ<sub>2</sub> GABA<sub>A</sub>R subunits, subcloned into a pBK-CMV vector, were used in this study. Point mutations were introduced in the α<sub>1</sub> and γ<sub>2</sub> subunits using a QuikChange site-directed mutagenesis kit (Agilent Technologies, CA). These mutations were confirmed with double-stranded DNA sequencing.

Harvesting, isolation and injection of Xenopus laevis oocytes
Xenopus laevis (Nasco, WI) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility in a room kept at 17°C and a 12h light/dark cycle, in tanks monitored for water pH and conductivity. Oocytes were surgically removed in accordance with the National Institute of Health guidelines under a protocol approved by the IACUC of the University of Texas at Austin, and placed in a hypertonic solution (108 mM NaCl, 1 mM EDTA, 2 mM KCl, and 10 mM HEPES). The thecal and epithelial layers of Stage V and VI oocytes were manually removed using forceps. Isolated oocytes were transferred to a solution (83 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub> and 5 mM HEPES) containing 0.5 mg/mL collagenase from Clostridium histolyticum for 10 minutes in order to enzymatically remove the follicular layer of the oocytes. The animal poles of oocytes were then injected using a Nanoject II (Drummond Scientific Co., PA) with 1.5 ng/30nL of human α<sub>1</sub>, β<sub>2</sub> and γ<sub>2</sub> GABA<sub>A</sub>R subunit cDNAs in a 1:1:10 ratio. Oocytes were stored singly in 96-well plates containing incubation media (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 10 mM HEPES, 0.82 mM MgSO<sub>4</sub>*7H<sub>2</sub>O, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.91 mM CaCl<sub>2</sub>, 2 mM sodium pyruvate, 0.5 mM theophylline, 10 U/L penicillin, 10 mg/L streptomycin). The oocytes were kept at room temperature (20°C) and away from light.

Two-electrode voltage clamp electrophysiology
Oocytes expressed GABA<sub>A</sub>Rs 1-3 days post injection with cDNA, and all electrophysiological recordings were completed within this time. An oocyte was placed in a 100 μL bath containing ND-96 buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 5 mM HEPES, pH 7.5). The bath was continuously perfused with ND-96 buffer at a rate of 2 mL/minute through 18-gauge polyethylene tubing connected to a Masterflex peristaltic pump (Cole Parmer Instruments, IL). The tips of two KCl-filled borosilicate glass electrodes, with a resistance of 0.5-10 MΩ, were placed into the animal pole of the oocyte, and it was voltage-clamped at -80mV using an OC-725C oocyte clamp (Warner Instruments, CT). Electrophysiological data were collected at a rate of 1 kHz using a digitizer (PowerLab ML866) and LabChart (v. 7.4.7) software (both from ADInstruments, Australia).

Concentration-response curve generation and analysis
Concentration-response data were collected for wildtype α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> GABA<sub>A</sub>R or the α<sub>1</sub>(K104C)β<sub>2</sub>γ<sub>2</sub>, α<sub>1</sub>(K104A)β<sub>2</sub>γ<sub>2</sub>, α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>(D75C), α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub>(D75A), α<sub>1</sub>(K104C)β<sub>2</sub>γ<sub>2</sub>(D75C), α<sub>1</sub>(K104A)β<sub>2</sub>γ<sub>2</sub>(D75A) or α<sub>1</sub>(K104D)β<sub>2</sub>γ<sub>2</sub>(D75K) mutants. Once voltage clamped, the oocyte was exposed to a maximally-
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Effective concentration of GABA (100 mM) for 10 sec. Following a ten-min washout with ND-96 buffer to allow re-sensitization of the receptors, increasing concentrations of GABA (3 µM - 10 mM) were applied for 20-30 seconds, allowing 5-10 minutes of washout between applications. Another maximally-effective concentration of GABA (100 mM) was applied at the end of the experiment, so that any drift (up or down) of current throughout the experiment could be corrected for. The responses to increasing concentrations of GABA were fit to the Hill equation using SigmaPlot 11.0 (Systat Software, CA).

GABAₐR modulator responses

Responses to modulators (1 µM diazepam, 1 µM flunitrazepam, 1 µM flumazenil, 1 µM Ro 15-4513, 1 µM zolpidem, 100 nM and 1 µM allopregnanolone, 200 mM ethanol) were recorded in oocytes expressing wildtype or mutant receptors. 10 mM stock solutions of all modulators (made with 0.1% DMSO in ND-96 buffer), except ethanol, were stored at -20°C and diluted in ND-96 before use. The GABA EC₅₋₁₀, the concentration of GABA that produces 5-10% of the maximal response, was first determined and then repeatedly applied for 30 seconds, followed by 3 minute ND-96 washouts, until responses were stable. Once stable, oocytes were pre-incubated for 30 seconds with a modulator, followed immediately by a co-application of modulator plus GABA EC₅₋₁₀. The allosteric modulation was calculated as \[\left(\frac{I_{\text{GABA after PMTS}}}{I_{\text{GABA before PMTS}}}\right) - 1\] *100. This was repeated after a 2-min treatment with 2 mM DTT, waiting 5 or 60 minutes after application before applying PMTS.

Dithiothreitol (DTT) and hydrogen peroxide (H₂O₂) treatment

DTT and H₂O₂ were made fresh in ND-96 buffer before each experiment. The GABA EC₅₋₁₀ was determined and applied at three minute intervals until stable responses were obtained. This was repeated after a 2-min DTT (2 mM) application, during which the oocyte was unclamped from -80 mV during the 5-min washout, and after a 90s application of 0.3 % H₂O₂ (oocyte unclamped during the 7 min washout). To measure the effects of DTT and H₂O₂ on allosteric modulation, the GABA EC₅₋₁₀ was determined and ensured to be stable. GABA was then applied in the presence of allosteric modulator, as described previously.

Propyl methanethiosulfonate (PMTS) treatment

A 300 mM PMTS (Toronto Research Chemicals, Canada) stock solution in DMSO was stored at -20°C and diluted to 0.5 mM in ND-96 before each experiment. The GABA EC₅₋₁₀ was determined and applied at 3-min intervals until stable responses were observed. Oocytes were then unclamped from -80 mV and treated with 0.5 mM PMTS for 60 seconds. After a 2-minute wash, oocytes were re-clamped to -80 mV and the same GABA EC₅₋₁₀ was reapplied. Percent changes in current were calculated as \[\left(\frac{I_{\text{GABA after PMTS}}}{I_{\text{GABA before PMTS}}}\right) *100\]. This was repeated after a 2-min treatment with 2 mM DTT, waiting 5 or 60 minutes after application before applying PMTS.

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Conflicts of interest:

The authors of this paper declare that they have no conflicts of interest.¹

Author contributions:

NCP, SJM and GLC participated in study design. JRT provided the structural models of the GABAₐR; NCP and SJM analyzed these models for potential electrostatic interactions. NCP and AWD conducted experiments. NCP, SJM, JRT, and GLC contributed to the writing and editing of this manuscript.

¹The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
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**FOOTNOTES**

DTT, dithiothreitol; EC, effective concentration; GABA, gamma-aminobutyric acid; GABA\textsubscript{A}R, \(\gamma\)-aminobutyric acid receptor type A; H\textsubscript{2}O\textsubscript{2}, hydrogen peroxide; PMTS, propyl methanethiosulfonate; WT, wildtype
Fig. 1. Two different homology models of the \( \alpha_1 \) (orange) - \( \gamma_2 \) (green) interface of the GABA\(_A\)R. The model on the left is based on a modified ivermectin-unbound GluCl crystal structure (17) and represents the GABA-unbound closed state of the channel. The model on the right is based on the glutamate-bound GluCl crystal structure with a contribution of the ELIC crystal structure (18) and represents the diazepam (in red) bound receptor. Both models depict the inside of the interface. Labeled interactions represent putative electrostatic interactions of residues 6Å or less apart that are predicted to occur between residues in the \( \alpha_1 \) and \( \gamma_2 \) subunits before (A-F) or after (H-K) diazepam binding. A: \( \alpha_1 \)R28-\( \gamma_2 \)D26; 5Å. B: \( \alpha_1 \)E165-\( \gamma_2 \)R97; 4Å. C: \( \alpha_1 \)E137-\( \gamma_2 \)R194; 5Å. D: \( \alpha_1 \)E58-\( \gamma_2 \)R197; 5Å. E: \( \alpha_1 \)D56-\( \gamma_2 \)R197; 5Å. F: \( \alpha_1 \)K278-\( \gamma_2 \)D161; 5Å. G: \( \alpha_1 \)K311-\( \gamma_2 \)D260; 3Å. H: \( \alpha_1 \)K105-\( \gamma_2 \)D120; 5Å. I: \( \alpha_1 \)K104-\( \gamma_2 \)D75; 5Å. J: \( \alpha_1 \)E58-\( \gamma_2 \)R197; 5Å. K: \( \alpha_1 \)D56-\( \gamma_2 \)R197; 6Å. Black dashed lines represent inter-subunit bonds.
Fig. 2. Diazepam enhancement of \( \text{GABA}_A \)R function is altered in some cysteine mutations of residues predicted to form electrostatic interactions at the \( \alpha_1-\gamma_2 \) subunit interface. EC\textsubscript{5.10} GABA was applied alone, as well as in the presence of 1 \( \mu \)M diazepam, to wildtype and multiple cysteine substituted receptors. The horizontal dashed line indicates the level of potentiation produced by diazepam in wildtype receptors. A one-way ANOVA showed a significant effect of cysteine substitution on receptor enhancement by 1 \( \mu \)M diazepam, \( [F(11,60)=26.310, p<0.001] \). A post-hoc Tukey’s test showed a significant change (\( p<0.001 \)) in potentiation by 1\( \mu \)M diazepam in \( \alpha_1\text{E58C}, \alpha_1\text{K104C}, \alpha_1\text{E137C}, \gamma_2\text{D75C} \) and \( \gamma_2\text{R197C} \) containing \( \text{GABA}_A \)R. Each symbol represents the percent potentiation of the GABA EC\textsubscript{5.10} by one oocyte, and each bar represents the mean percent potentiation \( \pm \) SEM.
Fig. 3. Homology models of the $\alpha_1$ (orange) - $\gamma_2$ (green) interface inside the GABA$\alpha$ receptor in both the GABA-unbound closed state of the channel and the diazepam-bound open state of the channel. These models predict that the nitrogen atom of $\alpha_1$ lysine 104 (orange residue) and oxygen atom of $\gamma_2$ aspartic acid 75 (green residue) are within 9Å of each other before GABA and diazepam bind (A and enlarged in B) but move to within 5Å of each other after GABA and diazepam (in red) bind to the receptor (C).
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Fig. 4. Formation and breakage of the disulfide bond between α1(K104C) and γ2(D75C) affects responses to GABA. A. GABA concentration-response curves were generated in wildtype α1β2γ2, single mutant α1(K104C)β2γ2 and α1β2γ2(D75C), and double mutant α1(K104C)β2γ2(D75C) GABA A receptors. A repeated-measures ANOVA revealed no difference in the concentration-response curve between wildtype and mutant receptors. However, one-way ANOVAs showed significant effects of mutation at 3 µM [F(3,26)=15.504, p<0.001] and 10 µM GABA [F(3,26)=18.163, p<0.001], with a Tukey’s post-hoc test at both concentrations showing a significant increase in response in α1(K104C)β2γ2(D75C) receptors compared to the other three receptors (***, p<0.001). Some symbols are hidden behind other symbols. B. DTT (2 mM, dark symbols and bars) increased the absolute concentration of GABA required to produce an EC50 response in α1(K104C)β2γ2(D75C) but not wildtype receptors. A two-way ANOVA followed by a Tukey’s post-hoc test revealed a significant effect of DTT treatment on α1(K104C)β2γ2(D75C) receptors (***, p<0.001). Each symbol represents the GABA EC50 of one oocyte, and each bar represents the mean GABA EC50 ± SEM.
Fig. 5. \(\alpha_1(K104C)\beta_2\gamma_2(D75C)\) receptors spontaneously crosslink, and reform this crosslink after DTT application. A. Illustration depicting the disulfide bond that spontaneously crosslinks between the \(\alpha_1\) and \(\gamma_2\) subunits of the \(\alpha_1(K104C)\beta_2\gamma_2(D75C)\) GABA\(_A\)R, and that is broken after DTT application, slowly reforming over time. B. Effect of PMTS application on currents elicited by the GABA EC\(_{5,10}\) of wildtype, \(\alpha_1(K104C)\beta_2\gamma_2\), \(\alpha_1\beta_2\gamma_2(D75C)\) and \(\alpha_1(K104C)\beta_2\gamma_2(D75C)\) GABA\(_A\) receptors. The change in GABA EC\(_{5,10}\) currents by PMTS was decreased in single mutant receptors both before \([F(3,18) = 14.56, p < 0.05]\) and after \([F(3,18) = 45.45, p < 0.001]\) DTT application compared to those seen in wildtype receptors. The change in EC\(_{5,10}\) currents produced by PMTS was not significantly altered after DTT application to wildtype or single mutant receptors, but did significantly change in double mutant receptors \([F(3,37) = 41.698, p < 0.001]\). A one-way ANOVA showed a significant effect of time after DTT treatment (pre-DTT treatment, 5 minutes after and 60 minutes after) on \(\alpha_1(K104C)\beta_2\gamma_2(D75C)\) GABA\(_A\)R \([F(2,13) = 108.363, p < 0.001]\), and a Tukey’s post-hoc test showed a significant difference between pre-DTT and 5 minutes after DTT application and a significant difference between 5 minutes after DTT and 60 minutes after DTT (***, \(p < 0.001\); each symbol represents an oocyte, and bars represent the mean ± SEM). C. Sample tracing showing spontaneous reformation of the \(\alpha_1\gamma_2\) inter-subunit disulfide bond in the \(\alpha_1(K104C)\beta_2\gamma_2(D75C)\) GABA\(_A\)R. The GABA EC\(_5\) measured in the oocyte before DTT application was 3\(\mu\)M GABA, but after DTT application 3\(\mu\)M GABA elicited a much smaller response. After approximately 60 minutes the response to 3\(\mu\)M GABA had returned to pre-DTT levels. D. Time courses of EC\(_5\) values plotted for 5 oocytes expressing \(\alpha_1(K104C)\beta_2\gamma_2(D75C)\) receptors returning to their pre-DTT values. This can also be interpreted as the time required to re-form the disulfide bond after DTT application. The average time to return to half of the pre-DTT EC\(_5\) was 26.9 ± 2.5 minutes.
Fig. 6. Benzodiazepine responses of wildtype and mutant GABA\textsubscript{A} receptors before (white symbols and bars) and after (dark symbols and bars) DTT application. Bar graphs showing the percent potentiation of GABA EC\textsubscript{5-10} in wildtype, $\alpha_{1}(K104C)\beta_{2}\gamma_{2}$, $\alpha_{1}\beta_{2}\gamma_{2}(D75C)$ and $\alpha_{1}(K104C)\beta_{2}\gamma_{2}(D75C)$ GABA\textsubscript{A} receptors produced by 1µM (A) diazepam, (B) flunitrazepam, (C) Ro 15-4513, and (D) zolpidem. A two-way ANOVA followed by a Tukey’s multiple comparison post-hoc test showed a significant increase in all benzodiazepine-site responses after DTT application to $\alpha_{1}(K104C)\beta_{2}\gamma_{2}(D75C)$ GABA\textsubscript{A} receptors but not wildtype or single mutant receptors (*, p<0.05; ***, p<0.001, with each symbol representing the percent potentiation of the GABA EC\textsubscript{5-10} seen in one oocyte, and each bar representing the mean percent potentiation ± SEM).
Fig. 7. Sample tracings showing the effects of DTT and H₂O₂ treatment on potentiation by 1µM diazepam and 1µM flunitrazepam. The top panels show tracings obtained from oocytes expressing wildtype receptors while the bottom panel shows tracings of oocytes expressing α₁(K104C)β₂γ₂(D75C) GABAₐ receptors. DTT application to α₁(K104C)β₂γ₂(D75C) receptors increased both diazepam (A) and flunitrazepam (B) potentiation, and hydrogen peroxide application reversed this increase back to pre-DTT levels.
Fig. 8. Modulators acting at sites other than the benzodiazepine site at wildtype and cysteine substituted GABA<sub>A</sub> receptors are unaffected by DTT treatment. Before modulator effects were tested, the EC<sub>5-10</sub> concentration of GABA was determined in each oocyte. Effects of 200mM ethanol and 100nM allopregnanolone were measured in wildtype and α<sub>1</sub>(K104C)β<sub>2</sub>γ<sub>2</sub>(D75C) GABA<sub>A</sub> receptors before (white symbols and bars) and after (dark symbols and bars) application of DTT, with no significant changes being observed (Two-way ANOVA’s: 200mM ethanol [F(3,43)=0.031]; 100nM Allopregnanolone [F(3,45)=0.176]. Each symbol represents the percent potentiation of the GABA EC<sub>5-10</sub> seen in one oocyte, and each bar represents the mean percent potentiation ± SEM.
Fig. 9. Effect of alanine substitution at $\alpha_1$K104 and $\gamma_2$D75 on GABA sensitivity and benzodiazepine responses. A. GABA concentration-response curves of wildtype, $\alpha_1$(K104A)$\beta_2\gamma_2$, $\alpha_1$$\beta_2\gamma_2$(D75A), and $\alpha_1$(K104A)$\beta_2\gamma_2$(D75A) receptors. The concentration-response curves were significantly different [$F(21,132)=1.937, p<0.05$]. Each symbol represents the data from 3-6 oocytes ± SEM. In some cases error bars fall within symbols. B. Bar graph comparing levels of diazepam and flunitrazepam potentiation between wildtype and alanine substituted receptors. Potentiation of GABA $EC_{50}$ by 1µM diazepam and 1µM flunitrazepam was decreased for single but not double alanine substitution mutants compared to wildtype receptors. A one way ANOVA revealed a significant effect of mutant on receptor potentiation by diazepam [$F(3,23)=51.407, p<0.001$] and flunitrazepam [$F(3,19)=26.926, p<0.001$]. Each symbol represents the percent potentiation observed in one oocyte, and each bar represents the mean percent potentiation ± SEM.
Fig. 10. Charge reversal at α$_1$K104 and γ$_2$D75 does not rescue GABA sensitivity or benzodiazepine responses to wildtype responses. **A.** The α$_1$(K104D)β$_2$γ$_2$(D75K) receptor GABA concentration-response curve is significantly right-shifted compared to wildtype receptors [F(8,105)=2.8, p<0.01]. The EC$_{50}$ for wildtype receptors was 86.8 ± 16.5µM, increasing to 146.3 ± 23.1µM for the α$_1$(K104D)β$_2$γ$_2$(D75K) GABA$_A$R. Each symbol represents the mean ± SEM from 5-6 oocytes. **B.** Bar graph comparing levels of benzodiazepine enhancement between α$_1$(K104D)β$_2$γ$_2$(D75K) and wildtype receptors. The α$_1$(K104D)β$_2$γ$_2$(D75K) GABA$_A$R was unable to fully rescue responses to wildtype levels of potentiation by 1µM diazepam and Ro 15-4513, but was able to rescue the responses to 1µM flunitrazepam and zolpidem. Each symbol represents the percent potentiation of the GABA EC$_{5.10}$ seen in one oocyte, and each bar represents the mean potentiation ± SEM.
An inter-subunit electrostatic interaction in the GABA<sub>A</sub> receptor facilitates its responses to benzodiazepines
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