An epilepsy-related region in the GABA<sub>A</sub> receptor mediates long-distance effects on GABA and benzodiazepine binding sites.¹

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Abstract

The GABA<sub>A</sub> receptor mutation γ<sub>2</sub>R43Q causes absence epilepsy in humans. Homology modeling suggests that γ<sub>2</sub>R43, γ<sub>2</sub>E178 and β<sub>2</sub>R117 participate in a salt-bridge network linking the γ<sub>2</sub> and β<sub>2</sub> subunits. Here we show that several mutations at these locations exert similar long-distance effects on other intersubunit interfaces involved in GABA and benzodiazepine binding. These mutations alter GABA evoked receptor kinetics by slowing deactivation, enhancing desensitization, or both. Kinetic modeling and nonstationary noise analysis for γ<sub>2</sub>R43Q reveal that these effects are due to slowed GABA unbinding and slowed recovery from desensitization. Both γ<sub>2</sub>R43Q and β<sub>2</sub>R117K also speed diazepam dissociation from the receptor’s benzodiazepine binding interface, as assayed by the rate of decay of diazepam-induced potentiation of GABA evoked currents. These data demonstrate that γ<sub>2</sub>R43 and β<sub>2</sub>R117 similarly regulate the stability of both the GABA and benzodiazepine binding sites at the distant β/α and α/γ intersubunit interfaces, respectively. A simple explanation for these results is that γ<sub>2</sub>R43 and β<sub>2</sub>R117 participate in interactions between the γ<sub>2</sub> and β<sub>2</sub> subunits, disruptions of which alter the neighboring intersubunit binding sites in a similar fashion. In addition, γ<sub>2</sub>R43 and γ<sub>2</sub>E178 regulate desensitization, probably mediated within the transmembrane domains near the pore. Therefore, mutations at the γ/β intersubunit interface have specific long-distance effects that are propagated widely throughout the GABA<sub>A</sub> receptor protein.
The γ-aminobutyric acid type A (GABA$_A$) receptor, a member of the cys-loop superfamily of ligand-gated ion channels (LGICs), is the major mediator of inhibition in the central nervous system. The receptor is composed of five subunits arranged in a ring enclosing a central chloride ion channel. The most abundant subunits are α$_1$, β$_2$ and γ$_2$ (McKernan and Whiting, 1996), which form receptors with the counterclockwise arrangement β$_2$α$_1$γ$_2$β$_2$α$_1$ when viewed from the synapse (Fig. 1A) (Baumann et al., 2002). GABA binding at the interface between β$_2$ and α$_1$ subunits triggers channel opening (Amin and Weiss, 1993; Wagner and Czajkowski, 2001), whereas binding of benzodiazepines (BZDs) such as diazepam (DZ) at the interface between α$_1$ and γ$_2$ subunits potentiates the effects of GABA (Sigel and Buhr, 1997; Boileau et al., 1998).

A third interface between the γ$_2$ and β$_2$ subunits is the site of a mutation (γ$_2$R43Q) that causes childhood absence epilepsy and febrile seizures in humans (Wallace et al., 2001; Cromer et al., 2002). Similar symptoms occur in γ$_2$R43Q heterozygous knock-in mice (Tan et al., 2007; and unpublished data). These mice have reduced cell surface expression of the γ$_2$ subunit and reduced miniature inhibitory postsynaptic currents (mIPSCs). In heterologous expression systems, the mutation alters receptor kinetics (Bowser et al., 2002; but see Bianchi et al., 2002) and impairs receptor assembly or trafficking (Kang and Macdonald, 2004; Sancar and Czajkowski, 2004; Hales et al., 2005; Frugier et al., 2007; Eugène et al., 2007). Either kinetic or trafficking effects could result in hyperexcitability and seizures: the former by accumulation of desensitized receptors during high frequency transmission, and the latter by decreasing the number of functional receptors.

Modeling based on homology with the crystallized acetylcholine binding protein suggests that γ$_2$R43, γ$_2$E178, and β$_2$R117 participate in a salt-bridge network linking the γ$_2$ and β$_2$ subunits (Fig. 1B) (Cromer et al., 2002). This region conforms to a highly conserved motif that may mediate intersubunit communication across the LGIC superfamily (Hales et al., 2005).
Interestingly, interactions linking the $\gamma_2$ and $\beta_2$ subunits are in a position to transmit their effects to multiple distant regions of the protein: either through the $\beta_2$ subunit which participates in GABA binding at the $\beta/\alpha$ interface some 30 Angstroms away, or through the $\gamma_2$ subunit which participates in BZD binding at the equally distant $\alpha/\gamma$ interface (see Fig. 1A). In addition, both $\beta_2$ and $\gamma_2$ subunits contribute to the transmembrane domains containing the channel pore and gate.

Although our observations may ultimately shed light on the role of the mutation $\gamma_2$R43Q in epilepsy, which continues to be studied in knock-in mice (Tan et al., 2007), this study primarily addresses the biophysical implications of this and related mutations on GABA$\alpha$ receptor function. Here we demonstrate that mutations at $\gamma_2$R43, $\gamma$E178 and $\beta_2$R117 similarly alter channel gating, stabilize GABA binding and destabilize BZD binding. Therefore interactions at the $\gamma/\beta$ interface influence specific events occurring at distant intersubunit interfaces and regions involved in channel gating.
Materials and Methods

Cell Culture and Transfection

Human embryonic kidney (HEK293) cells were cultured in Minimum Essential Medium with Earle’s salts (Mediatech, Inc., Herndon, VA) containing 10% bovine calf serum (Sigma-Aldrich, St. Louis, MO) in a 37°C incubator under a 5% CO2 atmosphere. Cells were transfected using a calcium phosphate precipitation method, or with the LipofectAMINE2000™ reagent (Invitrogen, Gaithersburg, MD) using the prescribed protocol, with 1-4 μg total of either αβγ (1:1:1 ratio) or αβ (1:1 ratio) from α1, β2, β2R117K, β2R117E, γ2, γ2R43K, γ2R43Q, γ2R43E, γ2E178K and γ2E178Q human GABA<sub>A</sub> receptor subunit cDNAs in vector pcDNA3.1 (Invitrogen). The mutant constructs were made using recombinant PCR, and verified by double stranded sequencing of the entire coding region. Recordings were performed 24-80 hours after transfection.

Patch Clamp Electrophysiology

Recordings from outside-out patches excised from HEK293 cells were made at room temperature using borosilicate glass pipettes filled with (in mM): 140 KCl, 10 EGTA, 2 MgATP, 20 phosphocreatine and 10 HEPES, pH 7.3, osmolarity 315 mOsm. Patches were voltage-clamped at -60 mV and placed in the stream of a multibarreled flowpipe array (Vitrodynamics, Rockaway, NJ) mounted on a piezoelectric bimorph (Morgan Electro Ceramics Inc, Bedford, OH). GABA, zinc, and diazepam were dissolved in the perfusion solution, which contained (in mM) 145 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 4 mM Glucose, pH 7.3, osmolarity 320 mOsm adjusted with sucrose. All reagents were from Sigma-Aldrich Chemicals, St. Louis, MO. A computer-controlled constant current source (WPI, Sarasota, FL) drove the bimorph to move solution interfaces over the patch with 10-90% exchange times of <200 μs, as measured by the liquid junction current at the open pipette tip after each experiment. Junction currents were
generated by altering the ionic strength with an additional 5 mM NaCl or 1% H2O in solutions containing GABA or zinc/diazepam, respectively. Currents were low-pass filtered at 5 kHz with a four-pole Bessel filter, and digitized at a rate no less than twice the filter frequency. Data were collected using an Axopatch 200B amplifier and Digidata 1320A digitizer (Molecular Devices, Sunnyvale, CA), controlled by AxoGraph software (Axograph Scientific, Sydney, AUS) running on a Macintosh G4 (Apple Computer Inc., Cupertino, CA). Curve fitting was performed using AxoGraphX, Prism 4 (GraphPad Software Inc., San Diego, CA), or homwritten routines in either C++ or Matlab 7 (The Mathworks Inc., Natick, MA).

Statistical Analysis

Significant differences were tested using either a Student’s t-test or one-way ANOVA with post-hoc Dunnett’s test, p < 0.05 (Prism 4). P-values are reported in the figures, but not the text. Weighted time constants (τw) for biexponential fits to macroscopic kinetics (i.e. I(t) = Σaiexp(-t/τi)) were calculated as τw = Σaiτi, where ai and τi are the fractional amplitude and time constant of the ith component, I is current and t is time.

Nonstationary Variance Analysis

Nonstationary variance analysis (Sigworth, 1980) was performed on responses to repeated pulses of saturating GABA (10 mM) from which ensemble mean current (I) and variance (σ²) were calculated at each time point. The mean current was divided into 100 equally sized bins, and the variances in each bin were averaged. Plots of binned variance versus current were fit with the equation: σ² = il - ²N⁻¹, where i is the single channel current, and N is the number of channels. Conductance was computed by dividing i by the holding potential of -60 mV. Variance resulting from slow drift (i.e., rundown or runup) was corrected by local linear fitting of the drift, calculating the variance due to this trend at each point, and subtracting this
drift variance (scaled by the squared current amplitude) from the total variance before fitting. This method yields accurate estimates of $i$ and $N$ when tested on simulated data with drift (Wagner et al., 2004).

**Kinetic Modeling**

Kinetic modeling was performed with homwritten software using the Q-matrix method (Colquhoun and Hawkes, 1995). We considered two simplified models of GABA_A receptor behavior, each including two ligand binding steps, channel opening and desensitization (Fig. 4A and 4B). Although more complex models are needed to explain all of the observed macroscopic and microscopic behavior of the receptor (e.g. single channel data suggests the existence of at least three open states, whereas we include only two; Fisher and Macdonald, 1997; Keramidas and Harrison, 2008), these simpler models benefit from having fewer unconstrained parameters while still being able to describe multiple aspects of receptor behavior. The model in figure 4A has been described previously (Jones et al., 1998; Wagner et al., 2004). Because this model contains a loop that was not constrained to follow microscopic reversibility, we also explored a similar model that lacked such a loop (Fig. 4B; see supplemental data). Our overall conclusions were the same for both models (compare figures 4 and S1).

Before optimization the closing rate constants $\alpha_1$ and $\alpha_2$ were constrained based on the single channel open times of $\alpha_1\beta_3\gamma_2L$ and $\alpha_1\beta_3\gamma_2L$R43Q receptors. Although $\alpha_1\beta_3\gamma_2L$ receptors exhibit three distinct open durations, the similarly increased fraction of openings to the two longer open states with increasing GABA concentration suggests that these openings are likely to occur from doubly liganded states (Fisher and Macdonald, 1997). Therefore, $\alpha_2$ was set to the inverse of the weighted average of the two longer reported open times at 600 $\mu$M GABA (2.7 ms), and $\alpha_1$ to the inverse of the shortest open time (0.3 ms) (Fisher and Macdonald, 1997).
After constraining the closing rates, the maximal open probability ($P_{o-max}$) was set to 0.69 based on nonstationary variance analysis (Fig. 3), and the remaining unconstrained rate constants were optimized for $\alpha_1\beta_2\gamma_2$ or $\alpha_1\beta_2\gamma_2R43Q$ receptors by fitting current responses to 2-5 ms, 500 ms and paired pulses of saturating (10 mM) GABA (paired pulse data was from Bowser et al., 2002). We then fixed $d_1$ and $r_1$ based on the initial fits so as to qualitatively reproduce the observed paired pulse data, and optimized again by simultaneously fitting current responses to both 2-5 ms and 500 ms pulses for each individual patch. We have found that simultaneous fitting of multiple protocols stressing different aspects of receptor behavior (e.g. deactivation or desensitization) is often a strong constraint for model optimization. However, responses to saturating GABA are not sensitive to the binding rates. We therefore chose to optimize $k_{\pm 1}$ and $k_{\pm 2}$ for responses to subsaturating (30 $\mu$M) GABA (Fig. 7D-E and S3D-E), during which we kept all other rates fixed and set the peak open probability ($P_o$) to 0.25 based on predictions from the initial optimization (such a low peak $P_o$ cannot be estimated by noise analysis because the data produces only the linear region of a parabola; Sigworth, 1980). Finally, we optimized the model again by simultaneously fitting current responses to both 2-5 ms and 500 ms pulses of 10 mM GABA with $k_{\pm 1}$ and $k_{\pm 2}$ now fixed to their respective mean optimized values. The results of this final round were accepted as the most reliable rate constants (Fig. 4C and S1B).

Although the closing rates $\alpha_1$ and $\alpha_2$ above were constrained based on data from channels containing the $\beta_3$ subunit, whereas our data is from $\beta_2$ subunit-containing receptors, we chose to use these rates initially so as to incorporate the reported reduction in mean single channel open time conferred by $\gamma_2R43Q$ in 1 mM GABA (Bianchi et al., 2002) (i.e. we increased the closing rate $\alpha_2$ 1.5-fold for the mutant). To address whether differences in open time distributions between $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_3\gamma_2L$ receptors would affect our conclusions, we repeated the modeling described above with the closing rates constrained based on the three single channel open time components reported for $\alpha_1\beta_2\gamma_2$ receptors (Keramidas and Harrison, 2008;
we used open times for the most frequently observed bursting mode, M-Mode) in an analogous fashion to that for $\alpha_1\beta_2\gamma_2L$ receptors. Because we observed different kinetic effects for the mutation $\gamma_2R43Q$ than did Bianchi and colleagues (2002), we also tested whether our conclusions depended on the speeding of the closing rate $\alpha_2$ by constraining it to its wild type value. Figure S3 shows that neither constraining the closing rates based on open time distributions from $\alpha_1\beta_2\gamma_2$ receptors, nor abolishing the speeding of the doubly liganded closing rate for the mutation $\gamma_2R43Q$, affected our overall conclusions.

To fit the current traces over the diazepam (DZ) dissociation timecourse (Fig. 5), the models in figure 4A and 4B were extended to allow DZ binding/unbinding from each state (Fig. 7A and S2A, respectively). For each patch, the DZ-unbound rates were first fixed by fitting the control response to 30 $\mu$M GABA alone (the fitting procedure for these currents is described above). To test the idea that altered GABA binding/unbinding can explain all of our observed effects for DZ, all of the DZ-bound rates $(x^{DZ})$ were constrained to be identical to their DZ-unbound counterparts $(x)$, except for $k_{\pm1}^{DZ}$ and $k_{\pm2}^{DZ}$. For the model in figure 4A, we assumed that DZ binding did not change the energy associated with a complete cycle around the loop (see supplemental data) by constraining $q^{DZ} = q k_{+2}^{DZ} / k_{-2}$ and $p^{DZ} = p k_{-2}^{DZ} / k_{+2}$. To both simplify the model and allow for GABA binding to influence BZD affinity (Sieghart, 1995; Boileau et al., 1998), we split the DZ binding and unbinding transitions into three groups: those occurring between states with no bound GABA molecule ($k_{\pm DZ}$), one bound GABA molecule ($k_{\pm DZ1}$) or two bound GABA molecules ($k_{\pm DZ2}$). For each construct, $k_{DZ}$ was set to the inverse of the observed mean decay time constant of DZ-induced potentiation following washout (Fig. 5B-C) and $k_{DZ}$ was approximated as $10^8$ M$^{-1}$s$^{-1}$ by dividing $k_{DZ}$ by the affinity constant found in radioligand binding and current potentiation experiments (Sieghart, 1995; Boileau et al., 1998). Microscopic reversibility was enforced for all loops in the model containing DZ binding steps by setting $k_{\pm DZ1} = k_{DZ1} k_1 k_{DZ} k_{+1}^{DZ} / (k_{-1}^{DZ} k_{DZ} k_{+1})$ and $k_{\pm DZ2} = k_{DZ2} k_2 k_{DZ1} k_{+2}^{DZ} / (k_{-2}^{DZ} k_{DZ1} k_{+2})$, in that order.
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(Colquhoun et al., 2004). The models were then optimized to fit the series of traces illustrating the decay in potentiation by starting in the state $U^{\beta_2}$ with time zero being the time of DZ washout (Fig. 7C and S2C). We reached the same overall conclusions for both the models with and without a loop (compare figures 7 and S2).

Optimization used a Nelder-Mead simplex algorithm to minimize the amplitude-weighted sum of squared errors between actual and simulated currents. In all cases, significant differences in fitted parameters between constructs were tested using a two-tailed unpaired Student’s $t$-test, $p < 0.05$.

**Results**

*Mutations to lysine or glutamine at the $\gamma_2/\beta_2$ subunit interface enhance desensitization, slow deactivation, or both.*

Responses to rapid application of 10 mM GABA were recorded in outside-out patches from HEK293 cells transfected with either $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2\text{R}43K$, $\alpha_1\beta_2\gamma_2\text{R}43Q$, $\alpha_1\beta_2\gamma_2\text{R}43E$, $\alpha_1\beta_2\gamma_2\text{E}178K$, $\alpha_1\beta_2\gamma_2\text{E}178Q$, $\alpha_1\beta_2\text{R}117\gamma_2$ or $\alpha_1\beta_2\text{R}117\text{E}\gamma_2$ GABA$_A$ receptor subunit cDNA combinations. Kinetics were characterized with biexponential fits to macroscopic deactivation following brief 2-5 ms pulses, or to desensitization during 500 ms pulses (Table 1).

Individual mutations to lysine (K) or glutamine (Q) at $\gamma_2\text{R}43$, $\gamma_2\text{E}178$ or $\beta_2\text{R}117$ always enhanced desensitization, slowed deactivation, or both. Mutations of the $\gamma_2$ residues had qualitatively equivalent effects: lysine ($\gamma_2\text{R}43K$, $\gamma_2\text{E}178K$) conferred deeper desensitization, whereas glutamine ($\gamma_2\text{R}43Q$, $\gamma_2\text{E}178Q$) sped desensitization and slowed deactivation (Fig. 1C-D). In contrast, the $\beta_2$ subunit mutation $\beta_2\text{R}117K$ slowed deactivation with little effect on desensitization (Fig. 1E).

Arginine to glutamate charge reversals in either the $\gamma_2$ or $\beta_2$ subunit ($\gamma_2\text{R}43E$, $\beta_2\text{R}117E$) essentially abolished currents in response to 10 mM GABA (peak current amplitude and fraction...
of patches with detectable current, $\alpha_1\beta_2\gamma_2R43E = 7 \pm 2 \text{ pA}$, 4 of 12 patches; $\alpha_1\beta_2R117E\gamma_2 = 22 \pm 16 \text{ pA}$, 2 of 9 patches). These small currents precluded kinetic analysis, but are consistent with a role for these residues in receptor assembly or trafficking (Cromer et al, 2002; Hales et al, 2005).

At least one mutation at each of $\gamma_2R43$, $\gamma_2E178$ or $\beta_2R117$ slowed receptor deactivation, which is shaped in part by the GABA unbinding rate (Jones et al., 1998). Thus, despite being distant from the GABA binding site, all three residues appear to participate in regulating the stability of the GABA-bound complex. Additional evidence supporting changes in the GABA unbinding rate is presented in a later section. By similar reasoning, both $\gamma_2$ subunit residues also influence desensitization while GABA is bound.

**Mutations to lysine or glutamine at the $\gamma_2/\beta_2$ subunit interface do not preclude $\gamma_2$ subunit incorporation into functional, surface expressed receptors.**

Currents recorded from receptors containing mutations at $\gamma_2R43$ or $\gamma_2E178$ appear kinetically similar to currents from $\alpha_1\beta_2$ receptors in that they have slower deactivation and/or deeper desensitization than currents from $\alpha_1\beta_2\gamma_2$ receptors (Fig. 2A and compare with Fig. 1C-D). Thus, the kinetic changes conferred by these mutations could potentially reflect an impaired ability to incorporate the $\gamma_2$ subunit into functional receptors. However, because $\alpha_1\beta_2$ receptors have a smaller conductance than $\alpha_1\beta_2\gamma_2$ receptors, a mixture of the two subtypes would require a larger fraction of $\alpha_1\beta_2$ receptors for the kinetics to appear $\alpha_1\beta_2$-like. Simulations of mixed populations of $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ receptors demonstrate that most of the receptors in the population must lack the $\gamma_2$ subunit in order for impaired $\gamma_2$ subunit incorporation to explain the observed kinetics of these mutants (Fig. 2A).

To verify that a $\gamma_2$ subunit was present in functional mutant receptors, we observed the effects of 10 $\mu$M zinc on peak GABA-evoked responses. Currents from $\alpha_1\beta_2\gamma_2$ receptors are
fairly insensitive to this concentration of zinc, whereas $\alpha_1\beta_2$ receptors are almost completely blocked (Fig. 2B-C) (% zinc block: $\alpha_1\beta_2\gamma_2 = 16 \pm 5, n = 14; \alpha_1\beta_2 = 87 \pm 2, n = 7$) (Hosie et al., 2003). All of the mutants were less sensitive to zinc than $\alpha_1\beta_2$ receptors, suggesting that none of the mutations precluded $\gamma_2$ subunit incorporation (Fig. 2B-C) (% zinc block: $\alpha_1\beta_2\gamma_2R43K = 31 \pm 12, n = 6; \alpha_1\beta_2\gamma_2R43Q = 9 \pm 2, n = 9; \alpha_1\beta_2\gamma_2E178K = 17 \pm 1, n = 4; \alpha_1\beta_2\gamma_2E178Q = 20 \pm 6, n = 7; \alpha_1\beta_2\gamma_2R117K\gamma_2 = 55 \pm 5, n = 6$). Zinc blocked $\gamma_2$ subunit mutants to a similar degree as $\alpha_1\beta_2\gamma_2$ receptors, further suggesting that a $\gamma_2$ subunit was present in a large fraction of these mutant receptors. This is consistent with previous reports for $\gamma_2R43Q$ (Wallace et al., 2001; Bowser et al., 2002). Though the charge reversal mutant $\gamma_2R43E$ did not express well in outside-out patches, sufficient current was obtained from two whole cells to suggest that this mutation severely reduces $\gamma_2$ subunit incorporation into functional receptors (% zinc block = 69 ± 16, data not shown). Zinc block of the $\beta_2$ subunit mutant $\alpha_1\beta_2\gamma_2\gamma_2R117K\gamma_2$, however, was intermediate to that of $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$, which could reflect impaired assembly with the $\gamma_2$ subunit, or alternatively, a direct effect on the receptor’s interaction with zinc. However, the mutation $\beta_2R117K$ also exhibited kinetics that differed the most with that expected for a simple mixture of subtypes (i.e. $\alpha_1\beta_2\gamma_2\gamma_2R117K\gamma_2$ receptors desensitized like $\alpha_1\beta_2\gamma_2$ receptors and deactivated like $\alpha_1\beta_2$ receptors; compare figures 1E and 2A).

Thus, both their kinetic profiles and zinc sensitivity demonstrate that the functional effects of mutations at $\gamma_2R43$, $\gamma_2E178$ or $\beta_2R117$ are unlikely to be artifacts caused by assembly deficits. Additional support for this conclusion is provided by changes in the kinetics of diazepam dissociation, described in a later section.

*The mutation $\gamma_2R43Q$ does not alter single channel conductance or peak open probability.*
We used nonstationary variance analysis (Sigworth, 1980) to estimate single channel conductance ($\gamma$) and maximal open probability ($P_{o\text{-max}}$) of the receptor (Fig 3). Because $P_{o\text{-max}}$ is a measure that depends on the interplay between numerous microscopic transitions, it is useful not only as a general measure of microscopic gating changes, but also as a constraint on any kinetic model of the receptor (see below).

The mutation $\gamma_2$R43Q had no effect on either the single channel conductance or maximal open probability. The estimated number of channels was lower on average for $\alpha_1\beta_2\gamma_2$R43Q than for $\alpha_1\beta_2\gamma_2$ receptors, although patch-to-patch variability precluded significance ($\alpha_1\beta_2\gamma_2$: $\gamma = 33 \pm 2$ pS, $P_{o\text{-max}} = 0.70 \pm 0.07$, $N = 341 \pm 105$, $n = 12$; $\alpha_1\beta_2\gamma_2$R43Q: $\gamma = 30 \pm 3$ pS, $P_{o\text{-max}} = 0.68 \pm 0.06$, $N = 126 \pm 33$, $n = 7$). These results are consistent with the single channel conductances observed for $\alpha_1\beta_3\gamma_2L$ and $\alpha_1\beta_3\gamma_2L$R43Q receptors (Bianchi et al., 2002). Since $\alpha_1\beta_2$ receptors have both lower conductance and $P_{o\text{-max}}$ ($\alpha_1\beta_2$: $\gamma = 11$ pS, $P_{o\text{-max}} = 0.40$, Wagner et al., 2004), this result again suggests that $\gamma_2$R43Q does not significantly affect $\gamma_2$ subunit incorporation into functional receptors.

The kinetic effects of the epilepsy-related mutation $\gamma_2$R43Q are explained by fast channel closure, slow recovery from desensitization and slow unbinding.

To explore the microscopic changes underlying the macroscopic kinetic effects of $\gamma_2$R43Q, we fit responses from $\alpha_1\beta_2\gamma_2$ (16 patches) and $\alpha_1\beta_2\gamma_2$R43Q (8 patches) receptors with a kinetic model previously shown to describe multiple aspects of neuronal and recombinant GABA$_A$ receptor function (Fig. 4A) (Jones et al., 1998; Wagner et al., 2004). The model was optimized for individual patches by simultaneously fitting current responses to 2-5 ms and 500 ms pulses of 10 mM GABA (Fig. 4D-E left, middle; see methods). We were able to quantitatively replicate all observed effects of $\gamma_2$R43Q by incorporating three specific changes: 1) increasing the channel closing rate $\alpha_2$ as reported by Bianchi et al. (2002), 2) decreasing the
resensitization rate $r_2$ and 3) decreasing the unbinding rate of GABA from the doubly liganded state $k_{-2}$. The final rate constants (mean ± SEM) are listed in figure 4C. The same conclusions were reached for a similar model lacking a loop (see supplemental data; Fig. S1). Also, a faster closing rate was not required to explain our macroscopic observations (Fig. S3).

These simulations suggest that a residue at the $\gamma/\beta$ intersubunit interface ($\gamma_2 R43$) participates in regulating both the stability of the desensitized state, which probably involves transmembrane regions near the channel pore (Revah et al., 1991), and GABA unbinding from the distant $\beta/\alpha$ interface. The similar kinetic profile of mutations at $\gamma_2 E178$ suggests that this residue may play a similar role. Also, because a change in deactivation with no change in desensitization or $P_{o\text{-max}}$ strongly suggests an effect on GABA binding/unbinding (Jones et al., 1998; Wagner et al., 2004), it is very likely that $\beta_2 R117K$ slows GABA unbinding as well.

**The mutations $\gamma_2 R43Q$ and $\beta_2 R117K$ speed diazepam dissociation from the GABA_\text{A} receptor.**

Our results with kinetics and modeling strongly suggest that mutations at $\gamma_2 R43$, $\gamma_2 E178$ or $\beta_2 R117$ affect processes that occur at the $\beta/\alpha$ GABA binding site such that GABA unbinding is slowed. To test whether these mutations also influence processes that occur at the $\alpha/\gamma$ interface we examined the rate at which the BZD agonist diazepam (DZ) dissociates from the receptor. Because BZD binding requires a $\gamma_2$ subunit (Sigel and Buhr, 1997), the rate of DZ dissociation depends only on the function of $\gamma_2$ subunit-containing receptors, independent of any putative effects on $\gamma_2$ subunit incorporation.

To measure the kinetics of DZ dissociation, we pre-equilibrated receptors in 10 µM DZ, rapidly jumped to wash solution to allow DZ to unbind, then tested the remaining degree of potentiation at various wash intervals with 20-40 ms pulses of sub-maximal (30 µM) GABA (Fig. 5A). The degree of potentiation was expressed as a percentage of the control response without
DZ pre-equilibration. Only a single pulse of GABA was delivered on any sweep, and wash intervals were interleaved to compensate for any rundown.

DZ potentiated responses from $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2\text{R43Q}$ and $\alpha_1\beta_2\text{R117K}\gamma_2$ receptors, confirming the ability of the mutant receptors to incorporate a $\gamma_2$ subunit, although the amount of potentiation was less for both mutants than for wild-type (% potentiation immediately following washout of DZ: $\alpha_1\beta_2\gamma_2 = 36 \pm 5$, $n = 7$; $\alpha_1\beta_2\gamma_2\text{R43Q} = 17 \pm 5$, $n = 5$; $\alpha_1\beta_2\text{R117K}\gamma_2 = 10 \pm 2$, $n = 6$).

Considering our kinetics, zinc, and noise analysis data, the lesser DZ-induced potentiation of both mutants compared to wild-type is likely due to a reduction in the affinity or efficacy of DZ. However, we cannot rule out that it could be due to contamination of the current by some receptors that cannot be potentiated because they lack a $\gamma_2$ subunit.

Interestingly, the timecourse of potentiation following washout of DZ was biphasic, consisting of a fast rise (i.e., increase in potentiation) followed by a slower decay (i.e., decrease back to control amplitude) (Fig. 5B). Both mutants sped the decay phase time constant by about 2-fold ($\tau_{\text{decay}}$ s) of DZ potentiation: $\alpha_1\beta_2\gamma_2 = 3.2 \pm 0.3$, $n = 7$; $\alpha_1\beta_2\gamma_2\text{R43Q} = 1.8 \pm 0.5$, $n = 5$; $\alpha_1\beta_2\text{R117K}\gamma_2 = 1.4 \pm 0.4$, $n = 6$). The biphasic timecourse of potentiation following washout of DZ suggests two distinct transitions. The initial increase in potentiation may reflect rapid DZ unbinding from a low affinity blocking site whose occupancy counteracts potentiation caused by occupancy of a higher affinity site. Consistent with this idea, the fractional amplitude of the rising phase was reduced by $74 \pm 14\%$ ($n = 8$) following pre-equilibration in lower concentrations (1-100 nM) of DZ (data not shown). Also, previous studies have shown that BZD effects on single channel properties (e.g. opening frequency) and macroscopic kinetics (e.g., peak current potentiation) decrease at higher BZD concentrations (Rogers et al., 1994; Perrais and Ropert, 1999; Baur et al., 2008). In contrast, the slow monoexponential decay of potentiation suggests a single transition from a high affinity DZ-bound potentiated state to an unpotentiated state. The simplest interpretation is that this component reflects the dissociation of DZ from a single high
MOL #58289

affinity potentiating site, with a dissociation rate equal to the inverse of the potentiation decay time constant \( (k_{DZ} = 1/\tau_{\text{decay}}) \). However, we cannot rule out more complicated interpretations, such as the existence of multiple kinetic steps between potentiation and dissociation that allow for a slow transition from potentiated to unpotentiated states while DZ is bound. In either case, the faster decay of potentiation demonstrates that the mutations have altered the function of \( \gamma_2 \) subunit-containing receptors, and destabilized the DZ-bound state of the receptor.

These data show that mutations at the \( \gamma/\beta \) intersubunit interface not only regulate the stability of the GABA binding site at the \( \beta/\alpha \) interface, but also that of the BZD binding site at the \( \alpha/\gamma \) interface.

The effects of diazepam are explained by faster GABA binding and slower unbinding.

Both \( \alpha_1\beta_2\gamma_2R43Q \) and \( \alpha_1\beta_2R117K\gamma_2 \) had faster 20-80% rise times (\( \tau_{\text{rise}} \)) and slower deactivation (\( \tau_{\text{deact}} \)) than \( \alpha_1\beta_2\gamma_2 \) receptors in response to 30 \( \mu \)M GABA (Fig. 6) \( \alpha_1\beta_2\gamma_2: \tau_{\text{rise}} = 8.1 \pm 0.3 \) ms, \( \tau_{\text{deact}} = 90 \pm 8 \) ms, \( n = 14 \); \( \alpha_1\beta_2\gamma_2R43Q: \tau_{\text{rise}} = 6.1 \pm 1.3 \) ms, \( \tau_{\text{deact}} = 141 \pm 21 \) ms, \( n = 4 \); \( \alpha_1\beta_2R117K\gamma_2: \tau_{\text{rise}} = 5.5 \pm 0.8 \) ms, \( \tau_{\text{deact}} = 148 \pm 14 \) ms, \( n = 11 \)). In addition to potentiating the amplitude of these responses, DZ sped their rise times and prolonged their deactivation, although the faster rise was not significant for \( \gamma_2R43Q \) (Fig. 6). In addition, DZ did not potentiate the amplitude of responses from \( \alpha_1\beta_2\gamma_2 \) receptors to higher (100 \( \mu \)M) GABA concentrations, but did prolong deactivation (two patches, data not shown). These results are consistent with previous studies on the effects of BZDs (Segal and Barker, 1984; Lavoie and Twyman, 1996; Mellor and Randall, 1997; Perrais and Ropert, 1999; Mercik et al., 2007) that suggest DZ mainly modulates GABA binding and unbinding rates.

To examine whether or not our observed kinetic effects of DZ could be attributed entirely to changes in GABA binding and unbinding, we fit the traces shown in figure 5A using an extension of the model in figure 4A where DZ was allowed to bind and unbind from each of the
original states (Fig. 7A). For both $\alpha_1\beta_2\gamma_2$ (5 patches) and $\alpha_1\beta_2\gamma_2R43Q$ (3 patches) receptors, our data were well described by allowing DZ to modulate only GABA binding and unbinding rates (see methods). The DZ binding and unbinding rates, and the DZ-induced fold change in GABA binding and unbinding rate constants (mean ± SEM), are listed in figure 7B. For $\alpha_1\beta_2\gamma_2$ receptors, DZ primarily sped binding of the first GABA molecule ($k_{+1}$), whereas for $\alpha_1\beta_2\gamma_2R43Q$ receptors binding of the second GABA molecule was increased the most ($k_{+2}$). For both constructs, DZ slowed unbinding largely from the doubly liganded state ($k_{-2}$). The differential effects of DZ on binding/unbinding of the first or second GABA molecule could reflect a preferential interaction between the DZ binding site and one of the two GABA binding sites, or altered cooperativity between GABA binding sites. In addition, the model in figure 7B predicts that the affinity for DZ should increase sequentially with GABA binding. This is consistent with the observed increase in BZD radioligand binding affinity with increasing GABA concentration (Sieghart, 1995; Boileau et al., 1998). The same conclusions were reached for a similar model lacking a loop (see supplemental data; Fig. S2).

To address whether or not our observations could be explained by DZ-induced gating changes (Downing et al., 2005; Rüsch and Forman, 2005; Campo-Soria et al., 2006) instead of changes to GABA binding/unbinding, we refit the DZ unbinding timecourse for $\alpha_1\beta_2\gamma_2$ receptors using the model in figure 7A and allowing only the DZ-bound channel opening rates $\beta_1$ and $\beta_2$ to vary ($k_{+1}$ and $k_{+2}$ were constrained to be equal to their DZ-unbound counterparts). Although DZ-modulation of $\beta_1$ and $\beta_2$ was able to describe the decay in potentiation of the peak current amplitudes, we were unable to simulate the speeding of the rising phase to the degree observed (not shown), similar to the conclusion reached by Lavoie and Twyman (1996) for a much simpler kinetic scheme. Thus, for the models in figures 7A and S2A, modulation of GABA binding/unbinding rates was necessary to explain all of our observed effects of DZ on detailed nonequilibrium kinetics of both $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2R43Q$ receptors, including the timecourse of the
decay in potentiation, speeding of the rising phase for $\alpha_1\beta_2\gamma_2$ receptors, and prolongation of deactivation. However, it is possible that the mutation $\gamma_2R43Q$ could confer both binding and gating effects. Indeed, when we let all of the DZ-bound rates vary except for the channel closing rates (channel open times have been shown to not depend on DZ; Vicini et al., 1987; Rogers et al., 1994), we found that although the GABA binding and unbinding rates were affected the most, the singly liganded opening rate $\beta_1$ was also increased (Fig. S2B).
Discussion

There is some discrepancy regarding the effects of the epilepsy-related mutation γ2R43Q. Bowser et al. (2002) reported similar kinetic alterations to those shown here, whereas Bianchi et al. (2002) found no changes in macroscopic kinetics, although they did observe a reduction in mean single channel open time. Despite differences in methodology, such as whether β2 or β3 subunits were used, the explanation for the qualitatively different results remains unclear. However, our demonstration here of similar kinetic effects of multiple mutations clearly shows that γ2R43 and other residues at the γ2/β2 interface do indeed regulate the kinetics of α1β2γ2 receptors.

Structural interactions at the γ/β intersubunit interface.

The similarity in effects of mutations at γ2R43, γ2E178 or β2R117 (i.e. desensitization was enhanced or not, but never reduced, deactivation was slowed or not, but never sped, and DZ dissociation was sped for the two mutations tested) suggests that these residues are similarly involved in these kinetic processes. However, we cannot prove or disprove the existence of the specific salt-bridge network proposed by Cromer et al. (2002). Indeed, the prototypical mutant cycle analysis experiment (Carter et al., 1984), which involves the mutation of two residues to determine their interaction energy, is difficult to interpret in a three-residue network because mutating any of the residues may alter the interactions between the other two residues. Despite this, our data do illustrate the importance of charge at these positions in receptor function (and possibly assembly/trafficking), and indicate that an arginine specifically is needed for normal function at γ2R43 and β2R117. For example, reversing the charge of either native arginine (γ2R43E, β2R117E) drastically reduced currents in outside-out patches, suggesting that a non-negative charge is needed at both of these locations for either normal function or assembly/trafficking. However, the charge conserving arginine to lysine mutations γ2R43K and
β2R117K both altered receptor kinetics, demonstrating that positive charge alone is not sufficient for normal function. This could be because lysine, whose side chain is slightly shorter than that of arginine, cannot position itself correctly. Lysine also has a different charge distribution than arginine, which could influence electrostatic interactions with nearby residues. Because neutralizing or reversing the charge of the central link in the putative salt-bridge network (γ2E178Q, γ2E178K) did not greatly impact current amplitudes, this residue may not play a large role in assembly/trafficking, but kinetic changes show that it does regulate the function of fully assembled channels.

Functional mutant receptors containing a γ2 subunit.

Immunofluorescence studies have shown that mutation of γ2R43 to glutamine, lysine or alanine impairs surface expression of the γ2 subunit, probably by trapping it in the endoplasmic reticulum (Kang and Macdonald, 2004; Sancar and Czajkowski, 2004; Frugier et al., 2007; Tan et al., 2007; Eugène et al., 2007). It remains unclear whether the reduced γ2 subunit expression reflects the loss of all the constituent subunits in those receptors, such as would occur if the trapped γ2 subunits were part of fully assembled channels (Kang and Macdonald, 2004; Hales et al., 2005), or whether the removal of γ2 leaves behind αiβ2 receptors (Sancar and Czajkowski, 2004; Frugier et al., 2007; Tan et al., 2007). However, methods such as immunofluorescence labeling or radioligand binding cannot distinguish between a reduction in functional versus non-functional receptors. We show here that 1) all of the lysine and glutamine mutants exhibit kinetics and zinc sensitivity that are inconsistent with a simple mixture of αiβ2 and αiβ2γ2 receptors, 2) γ2R43Q has a similar single channel conductance and Po-max to that of αiβ2γ2 receptors, and 3) both γ2R43Q and β2R117K alter the dissociation rate of DZ (a measure that absolutely requires the presence of a γ2 subunit). Therefore, the responses we studied here mainly reflect receptors that contained a γ2 subunit. These results are not incompatible with an
overall reduction in the total number of surface-expressed functional receptors. Indeed, we did observe a (non-significant) trend towards reduction in the average number of channels present in patches from cells transfected with $\alpha_1\beta_2\gamma_2$R43Q versus $\alpha_1\beta_2\gamma_2$ subunits as determined by noise analysis. Together with the arguments presented above, these data demonstrate that $\gamma_2$R43Q can assemble with $\alpha_1$ and $\beta_2$ subunits. If the mutation affects trafficking it appears to impair expression of the entire $\alpha_1\beta_2\gamma_2$R43Q complex, without leaving behind a significant number of $\alpha_1\beta_2$ receptors.

In addition to GABA-evoked macroscopic kinetics, there is also some discrepancy about the effect of $\gamma_2$R43Q on allosteric modulation by benzodiazepines. Four separate studies found that 1-2 $\mu$M DZ either did not potentiate GABA responses from mutant receptors (Wallace et al., 2001), potentiated them less than wild-type (Bowser et al., 2002; Eugène et al., 2007), or potentiated them as much as wild-type (Bianchi et al., 2002). Here we show that both $\alpha_1\beta_2\gamma_2$R43Q and $\alpha_1\beta_2$R117K$\gamma_2$ form functional receptors containing the $\gamma_2$ subunit whose GABA-evoked responses are potentiated by DZ, although to a lesser degree than $\alpha_1\beta_2\gamma_2$ receptors (Fig 5). If, as our data suggest, a $\gamma_2$ subunit is present in most of the functional mutant receptors, then their reduced DZ potentiation implies either reduced affinity or efficacy of DZ. A reduction in affinity is also consistent with the observed faster dissociation of DZ from $\alpha_1\beta_2\gamma_2$R43Q and $\alpha_1\beta_2$R117K$\gamma_2$ receptors.

**Cooperativity between GABA binding sites.**

Although we initially modeled the GABA binding/unbinding steps as being equal and independent, we were unable to reasonably fit the macroscopic responses of $\alpha_1\beta_2\gamma_2$ receptors to both 2-5 ms and 500 ms pulses of 10 mM GABA with the models in figure 4A and 4B (data not shown). We obtained good fits, however, when we allowed the individual GABA binding and unbinding steps to vary independently, such that the unbinding rates exhibited an apparent
negative cooperativity \((k_2 > k_{-1})\), due to the rapid initial deactivation of our macroscopic responses. Although our motivation for allowing the binding and unbinding rates from the singly and doubly liganded states to vary independently was primarily to best fit the data, there is evidence that either cooperativity exists between GABA binding sites, or the sites are inherently unequal (Lavoie and Twyman, 1996; Baumann et al., 2003; Mozrzymas et al., 2003). However, a more direct model-independent measure of microscopic binding and unbinding rates is likely required to resolve this issue.

**Interactions at the \(\gamma/\beta\) intersubunit interface mediate the stability of both GABA and BZD binding sites.**

We show here that perturbations at the \(\gamma/\beta\) intersubunit interface are propagated to the distant ligand binding \(\beta/\alpha\) and \(\alpha/\gamma\) interfaces, and to regions involved in desensitization. For each of the three residues studied we found a mutation that slowed deactivation (\(\gamma_2R43Q, \gamma_2E178Q, \beta_2R117K\)). Kinetic modeling suggests that this is due to stabilizing the GABA-bound complex. Both arginine mutations \(\gamma_2R43Q\) and \(\beta_2R117K\) also destabilized the DZ-bound and potentiated complex. Therefore, all three residues (\(\gamma_2R43, \gamma_2E178, \beta_2R117\)) participate in communication between the \(\gamma/\beta\) intersubunit interface at which they reside and the GABA binding site at the \(\beta/\alpha\) interface, and at least two of these residues (\(\gamma_2R43\) and \(\beta_2R117\)) are also involved in communication with the BZD binding site at the \(\alpha/\gamma\) interface. Assuming a counterclockwise subunit arrangement of \(\beta_2\alpha_1\gamma_2\beta_2\alpha_1\) (Baumann et al., 2002), the most direct path for the propagation of structural changes between \(\gamma_2R43\) and the neighboring GABA binding site is across the \(\gamma_2/\beta_2\) interface and through the \(\beta_2\) subunit (see Fig. 1A). Similarly, the most direct path from \(\beta_2R117\) to the neighboring BZD binding site is in the opposite direction across the \(\gamma_2/\beta_2\) interface and through the \(\gamma_2\) subunit. Interestingly, if \(\gamma_2R43, \gamma_2E178\) and \(\beta_2R117\) interact, then \(\gamma_2E178\) is in a position to transmit perturbations along it’s backbone \(\beta\)-strand into
loop F, which has been implicated in BZD efficacy (Hanson and Czajkowski, 2008). Thus, a simple explanation for the effects of the mutations $\gamma_2$R43Q and $\beta_2$R117K on both GABA unbinding and DZ dissociation is that $\gamma_2$R43 and $\beta_2$R117 participate in interactions between the $\gamma_2$ and $\beta_2$ subunits that mediate ligand affinities at the two neighboring $\beta/\alpha$ and $\alpha/\gamma$ intersubunit interfaces. If interactions between subunits at one interface influence the structure of other intersubunit interfaces, then communication between these interfaces offers a robust mechanism for GABA or BZD binding to confer widespread conformational changes that may be important for receptor function. Indeed, both radioligand binding studies (Seighart, 1995; Bolieau et al., 1998) and our results show that DZ binding at the $\alpha/\gamma$ interface modulates GABA binding at the $\beta/\alpha$ interface, and vice versa.

We conclude that the region surrounding the epilepsy-related mutation $\gamma_2$R43, including residues from both the $\gamma_2$ and $\beta_2$ subunits, participates in regulating events in the GABA A receptor that are known to occur at distant regions of the protein. Kinetic analysis revealed that changes in only a few out of many possible transitions account for all of our observed effects. Therefore, perturbations at the $\gamma/\beta$ interface affect specific locations throughout the receptor, including ligand binding sites at two separate intersubunit interfaces and the channel gating apparatus in the transmembrane domains.
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Footnotes

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Legends for Figures

**Figure 1.** Mutations at $\gamma_2$R43, $\gamma_2$E178 or $\beta_2$R117 to lysine (K) or glutamine (Q) enhance desensitization and/or slow deactivation. A) Homology model of the GABA$_A$ receptor (Cromer et al., 2002) illustrating the five subunit ring arrangement. View is from the extracellular side looking through the central channel into the cell. Intersubunit interfaces mediating GABA or BZD binding, or containing the mutated residues, are labeled. B) Zoomed in view of a putative three-residue salt-bridge network linking the $\gamma_2$ and $\beta_2$ subunits. C-E) Normalized current responses from outside-out patches to rapid application of 10 mM GABA. Traces above current responses in (C) are liquid junction currents obtained with an open pipette tip by blowing off the patch following the experiment to assay the speed of solution exchange. All four $\gamma_2$ subunit mutations (C-D) enhanced the rate and/or extent of desensitization during 500 ms pulses (right), whereas only mutations to glutamine significantly slowed deactivation following 2-5 ms pulses (left). The $\beta_2$ subunit mutation $\beta_2$R117K slowed deactivation with little effect on desensitization (E). F) Summary of weighted time constants for desensitization and deactivation, and the final extent of desensitization after 500 ms. Differences from $\alpha_1\beta_2\gamma_2$ were evaluated by one-way ANOVA with post-hoc Dunnett’s test at *$p < 0.05$ or **$p < 0.01$.

**Figure 2.** Mutations at $\gamma_2$R43, $\gamma_2$E178 or $\beta_2$R117 do not preclude $\gamma_2$ subunit incorporation into functional receptors. A) Normalized current responses to 2-5 ms (left) and 500 ms (right) pulses of 10 mM GABA for $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors (black) and weighted mixtures of those responses simulating currents from a heterogeneous receptor population containing 10-90% $\alpha_1\beta_2$ receptors in 10% increments (thin light gray). The contribution of $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors to the resulting current was also weighted by their relative conductances of 33 pS and 11 pS, respectively. Responses from $\alpha_1\beta_2\gamma_2$R43Q receptors are included for comparison (thick dark gray). B) Effect
of zinc on GABA-evoked peak responses. Responses to 10 mM GABA were averaged from interleaved recordings with (gray) and without (black) 10 μM zinc. When present, zinc was both pre- and co-applied for each GABA pulse, which lasted at least 10 ms. Zinc greatly reduced peak currents from α₁β₂ receptors, but did not affect any of the mutants to a similar degree, suggesting that they form functional receptors incorporating a γ₂ subunit (Hosie et al., 2003).

C) Summary of the reduction in peak current by 10 μM zinc. Differences from **α₁β₂ and ††α₁β₂γ₂ were evaluated by one-way ANOVA with post-hoc Dunnett’s test at p < 0.01.

**Figure 3.** The mutation γ₂R43Q does not affect maximal open probability (P_o-max) or single channel conductance. A) Mean (below) and variance (above) of consecutive responses to 500 ms pulses of 10 mM GABA for α₁β₂γ₂ and α₁β₂γ₂R43Q receptors. B) Plots of normalized mean current versus variance for α₁β₂γ₂ (black squares) and α₁β₂γ₂R43Q (gray circles) for the traces shown in (a) fit with a parabola (black line) describing the single channel conductance, P_o-max and the number of channels present in each patch, none of which differed between constructs (two-tailed unpaired Student’s t-test, p ≤ 0.05).

**Figure 4.** Kinetic modeling demonstrates that the kinetic effects of the mutation γ₂R43Q can be explained by faster channel closure, slower recovery from desensitization and slower unbinding. A-B) The Markov models used to simulate GABA responses (U = unbound, B = bound, O = open, D = desensitized; the model in (A) is previously described in Jones et al., 1998). C) Rate constants used to simulate α₁β₂γ₂ and α₁β₂γ₂R43Q responses to 10 mM GABA for the model in (A) (units are s⁻¹ except for GABA binding steps, which are M⁻¹ s⁻¹). The values of k⁺₁, k⁻₂, d₂, r₂ and p are reported as mean ± SEM because they were allowed to vary while the model was optimized to simultaneously fit 2-5 ms and 500 ms current responses from individual patches (see methods). k₂ and r₂ were the only unconstrained rate constants that significantly differed.
when comparing mutant and wild type models (two-tailed unpaired Student’s t-test, **p < 0.01, ***p < 0.0001). See supplemental data (Fig. S1) for simulations and rate constants for the model in (B). D-E) Current responses (black) evoked by 2 ms (left) or 500 ms (middle) pulses of 10 mM GABA from two individual patches containing $\alpha_1\beta_2\gamma_2$ (D) and $\alpha_1\beta_2\gamma_2$R43Q (E) receptors overlaid with simulated responses (red). The model qualitatively reproduces the slowing of paired pulse recovery for $\alpha_1\beta_2\gamma_2$R43Q (E, right) as compared to $\alpha_1\beta_2\gamma_2$ (D, right) observed by Bowser et al. (2002).

*Figure 5.* Mutations $\gamma_2$R43Q or $\beta_2$R117K speed diazepam (DZ) dissociation from the GABA$_A$ receptor. A) Responses from $\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$R43Q and $\alpha_1\beta_2$R117K$\gamma_2$ receptors to 20-40 ms pulses of sub-maximal (30 $\mu$M) GABA at varying times following washout of 10 $\mu$M DZ. Open tip currents (top) illustrate the solution exchange protocol. The control response (no pre-equilibration in DZ) is offset to the left of each set of traces, and the dashed line indicates its amplitude. Each of the other responses were obtained on separate, interleaved sweeps, and involved first pre-equilibrating for $\geq$ 1 s in DZ before rapidly switching to a wash solution, allowing DZ to unbind, and assaying the amount of DZ-induced potentiation remaining after varying time intervals. B) The timecourse of potentiation following washout of DZ had a biphasic shape, with a fast rising phase and slower decay. Solid lines are fits to the mean across patches, which are normalized in the inset to show that both $\alpha_1\beta_2\gamma_2$R43Q and $\alpha_1\beta_2$R117K$\gamma_2$ speed the decay of potentiation by approximately 2-fold. C) Summary of mean and SEMs for the initial and maximal potentiation after DZ washout, and for the decay time constant from biexponential fits to the potentiation timecourse of individual patches. Differences from $\alpha_1\beta_2\gamma_2$ in each case were evaluated with a two-tailed unpaired Student’s t-test at *p < 0.05 or **p < 0.01.
Figure 6. Diazepam (DZ) speeds the rise and prolongs the decay of currents from \( \alpha_1\beta_2\gamma_2 \), \( \alpha_1\beta_2\gamma_2R43Q \) and \( \alpha_1\beta_2R117K\gamma_2 \) receptors. A) Normalized current responses to 20-40 ms pulses of sub-maximal (30 \( \mu \)M) GABA. Comparison of control (gray) and DZ pre-equilibrated (black) currents, either at the time of maximal DZ-induced potentiation (prolonged decay) or 10 seconds after washout of DZ (almost completely overlaps control response). Insets illustrate the rising phase under the same conditions. Traces are the same as those in Fig. 5A. B) Summary of rise times and the weighted time constant from biexponential fits to deactivation. Graphs include control responses, those immediately following DZ washout (initial), and responses for which DZ had a maximal effect after washout. Differences in control responses between mutants and \( \alpha_1\beta_2\gamma_2 \) were assayed with a two-tailed unpaired Student’s \( t \)-test at \(*p < 0.05 \) or \(**p < 0.01 \), whereas for a single receptor type, differences between control and DZ modulated responses were tested with a two-tailed paired Student’s \( t \)-test at †\( p < 0.05 \), ††\( p < 0.01 \) or †††\( p < 0.001 \).

Figure 7. The effects of diazepam (DZ) can be explained by speeding GABA binding and slowing GABA unbinding. A) An extension of the kinetic model shown in figure 4A allowing DZ binding/unbinding from each state (black: DZ-unbound states, blue: DZ-bound states). The rates \( k_{\pm DZ1} \) and \( k_{\pm DZ2} \) are the same for each set of singly or doubly bound states, respectively, and the rates between DZ-bound states are identical to their DZ-unbound counterparts (transition rates are labeled as in figure 4A) except for the binding and unbinding rates shown in purple, which were allowed to vary, and \( p^{DZ} \) and \( q^{DZ} \), which were constrained (see methods). B) Summary of rate constants or their DZ-induced fold change (mean ± SEM, bold indicates greater than 2-fold change) for fits to sub-maximal GABA responses alone and following washout of DZ. C) Current responses (black) evoked by 20-40 ms pulses of 30 \( \mu \)M GABA alone (offset left) or at varying times following washout of 10 \( \mu \)M DZ (right) for \( \alpha_1\beta_2\gamma_2 \) and \( \alpha_1\beta_2\gamma_2R43Q \) receptors overlaid with simulated responses (red). D-E) Expanded view of the fits shown in (C) with the control and
maximally potentiated responses overlaid and normalized to illustrate the DZ-induced slowing of deactivation and speeding of the rising phase for $\alpha_1\beta_2\gamma_2$ receptors (insets). We reached the same overall conclusions for a similar extension of the model shown in figure 4B (see supplemental data, Fig. S2).
Table 1. Summary of biexponential fits to macroscopic kinetics of desensitization and deactivation.

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<td>7 ± 1*</td>
<td>68 ± 2**</td>
<td>166 ± 10*</td>
<td>59 ± 5**</td>
<td>72 ± 3**</td>
<td>20</td>
</tr>
<tr>
<td>α₁β₂γ₂E178K</td>
<td>15 ± 3</td>
<td>49 ± 3</td>
<td>222 ± 20</td>
<td>121 ± 15</td>
<td>58 ± 5*</td>
<td>8</td>
</tr>
<tr>
<td>α₁β₂γ₂E178Q</td>
<td>10 ± 1</td>
<td>55 ± 5**</td>
<td>220 ± 18</td>
<td>99 ± 9**</td>
<td>53 ± 3</td>
<td>10</td>
</tr>
<tr>
<td>α₁β₂R117Kγ₂</td>
<td>9 ± 1</td>
<td>42 ± 5</td>
<td>185 ± 5</td>
<td>111 ± 10</td>
<td>49 ± 2</td>
<td>5</td>
</tr>
<tr>
<td><strong>2-5 ms deactivation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α₁β₂</td>
<td>19 ± 1**</td>
<td>73 ± 3**</td>
<td>198 ± 10**</td>
<td>68 ± 6*</td>
<td>n/a</td>
<td>17</td>
</tr>
<tr>
<td>α₁β₂γ₂</td>
<td>13 ± 1</td>
<td>58 ± 2</td>
<td>88 ± 6</td>
<td>45 ± 4</td>
<td>n/a</td>
<td>24</td>
</tr>
<tr>
<td>α₁β₂γ₂R43K</td>
<td>16 ± 3</td>
<td>53 ± 5</td>
<td>112 ± 13</td>
<td>59 ± 4</td>
<td>n/a</td>
<td>6</td>
</tr>
<tr>
<td>α₁β₂γ₂R43Q</td>
<td>12 ± 1</td>
<td>59 ± 2</td>
<td>170 ± 21**</td>
<td>73 ± 6**</td>
<td>n/a</td>
<td>17</td>
</tr>
<tr>
<td>α₁β₂γ₂E178K</td>
<td>16 ± 3</td>
<td>51 ± 5</td>
<td>115 ± 17</td>
<td>62 ± 7</td>
<td>n/a</td>
<td>8</td>
</tr>
<tr>
<td>α₁β₂γ₂E178Q</td>
<td>17 ± 2</td>
<td>45 ± 2*</td>
<td>146 ± 14*</td>
<td>86 ± 7**</td>
<td>n/a</td>
<td>9</td>
</tr>
<tr>
<td>α₁β₂R117Kγ₂</td>
<td>14 ± 1</td>
<td>41 ± 3*</td>
<td>130 ± 19</td>
<td>83 ± 12**</td>
<td>n/a</td>
<td>5</td>
</tr>
</tbody>
</table>

Mutations at γ₂R43, γ₂E178 and β₂R117 alter the kinetics of responses from outside-out patches to brief (2-5 ms) and longer (500 ms) pulses of 10 mM GABA. Each mutation either enhanced desensitization, slowed deactivation, or both relative to α₁β₂γ₂ receptors. The kinetics of α₁β₂ receptors are listed for comparison. Data are mean ± SEM. Differences from α₁β₂γ₂ were
evaluated by one-way ANOVA with post-hoc Dunnett’s test at *p < 0.05 or **p < 0.01. †Extent of desensitization at the end of a 500 ms pulse.
Figure 1

A) Molecular structure of the GABA receptor with labels for α1, β2, γ2, BZD, and GABA. The scale bar indicates 10 Å.

B) Close-up of the γ2E178 and β2R117 regions with mutations indicated.

C) Current-voltage curves for various mutant receptors: α1β2γ2, γ2R43K, γ2R43Q, γ2R43Q, γ2R43K. The time constants for deactivation (τw) are shown in the histogram, with statistical significance indicated by ** and ***.

D) Current-voltage curves for additional mutant receptors: α1β2γ2, γ2E178K, γ2E178Q, γ2E178K. The time constants for desensitization (τw) are shown in the histogram, with statistical significance indicated by **.

E) Current-voltage curves for β2R117K and γ2R43K. The extent of desensitization (%) is shown in the histogram, with statistical significance indicated by ** and *.
Figure 4

A

\[ \begin{align*}
\text{U} & \xrightleftharpoons[2k_{+1}]{k_{-1}} \text{B} \\
\alpha_1 & \xrightarrow{\beta_1} \alpha_2 \xrightarrow{\beta_2} \text{O}_1 \\
\text{D}_1 & \xrightleftharpoons[p]{d_1} \text{D}_2 \\
\alpha_1 & \xrightarrow{\gamma_2} \alpha_1 \beta_2 g_2 \xrightarrow{\gamma_2 R43Q} \text{O}_2
\end{align*} \]

\[
\begin{array}{ccc}
k_{+1} & 1.9 \pm 0.1 \times 10^6 & 3.1 \pm 0.9 \times 10^6 \\
k_{+2} & 1.2 \pm 0.2 \times 10^6 & 1.0 \pm 0.01 \times 10^6 \\
k_{-1} & 13 \pm 2 & 17 \pm 5 \\
k_{-2} & 550 \pm 110 & 90 \pm 18 ** \\
\beta_1 & 1000 & 1000 \\
\alpha_1 & 3300 & 3300 \\
\beta_2 & 2500 & 2500 \\
\alpha_2 & 380 & 570 \\
d_1 & 10 & 10 \\
r_1 & 3 & 3 \\
d_2 & 550 \pm 15 & 530 \pm 67 \\
r_2 & 150 \pm 10 & 60 \pm 8 *** \\
q & 0.03 & 0.03 \\
p & 5 \pm 1 & 6 \pm 1
\end{array}
\]

B

\[ \begin{align*}
\text{U} & \xrightleftharpoons[2k_{+1}]{k_{-1}} \text{B} \\
\alpha_1 & \xrightarrow{\beta_1} \alpha_2 \xrightarrow{\beta_2} \text{O}_1 \\
\text{D}_1 & \xrightleftharpoons[d_3]{r_3} \text{D}_3 \\
\beta_1 & \xrightarrow{\alpha_1} \beta_2 \xrightarrow{\alpha_2} \text{O}_2
\end{align*} \]

D

E
Figure 6

A

control

10 s

max

200 ms

\( \alpha_1 \beta_2 \gamma_2 \)

\( \gamma_2 \text{R}43\text{Q} \)

\( \beta_2 \text{R}117\text{K} \)

B

- gray: control
- light gray: +DZ initial
- dark gray: +DZ max effect

\( \tau_{\text{w}} \), Deact. (ms)

\( 20-80\% \) Rise (ms)

\( \alpha_1 \beta_2 \gamma_2 \)

\( \gamma_2 \text{R}43\text{Q} \)

\( \beta_2 \text{R}117\text{K} \)
Figure 7

A

B

\[ \begin{array}{ll}
\alpha_{1}\beta_{2}\gamma_{2} & \alpha_{1}\beta_{2}\gamma_{2}\text{R43Q} \\
\hline
k_{+\text{DZ}} & 1.0 \times 10^8 \\
0.27 \pm 0.03 & 0.47 \pm 0.04 \\
0.31 & 0.5 \\
3.0 \pm 1.6 \times 10^9 & 1.7 \pm 0.9 \times 10^9 \\
0.20 \pm 0.05 & 0.29 \pm 0.14 \\
\end{array} \]

Fold change upon DZ binding:

\[ \begin{array}{ll}
k_{+1\text{DZ}} & 4.0 \pm 0.2 \\
1.7 \pm 0.2 & 6.8 \pm 2.3 \\
k_{-1\text{DZ}} & 0.8 \pm 0.3 \\
0.3 \pm 0.04 & 0.3 \pm 0.05 \\
\end{array} \]

C

D

E