Supplementary material for "An epilepsy-related region in the $GABA_A$ receptor mediates long-distance effects on GABA and benzodiazepine binding sites," in Molecular Pharmacology.

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In kinetic modeling, it is common to insist that all processes must follow the principle of "microscopic reversibility" or "detailed balance" (Tolman, 1925). This principle is a restatement of the law of conservation of energy, and asserts that for any closed system at thermodynamic equilibrium, reactions are equally likely in the forward or backward direction. For ion channel kinetics, the principle predicts that any kinetic scheme containing a loop will be traversed with equal probability in the clockwise and counterclockwise directions (i.e., the products of clockwise and counterclockwise rate constants will be equal, hence the sum of changes in potential energy, ΔG, in both directions will be equal; Colquhoun et al., 2004). However, ion channels normally operate within ionic and voltage gradients, and are free to exchange heat with the bathing medium. They are therefore not in a closed system, nor can they be assumed to be at thermodynamic equilibrium. Indeed, some ion channels are known to operate at steady-state with a net movement in one kinetic direction (Richard and Miller, 1990).

Ultimately, it is desirable to use the simplest model that makes the fewest assumptions, while simultaneously giving the best fit to the data. Our default model of $GABA_A$ receptor kinetics does not adhere to microscopic reversibility. Because a similar slow desensitization is prominent at both low and saturating agonist concentrations (Overstreet et al., 2000), the receptor must have access to a slow desensitized state from both singly and doubly liganded states. Allowing a net counterclockwise movement around the loop in the model in figure 4A from the main text permits this behavior, with a minimum of formal assumptions. However, another possibility is that there are multiple similarly slow desensitized states, accessed separately from singly and doubly liganded states. Here we show that a) these two assumptions are essentially indistinguishable for fitting our data with GABA and diazepam, and b) both assumptions yield identical conclusions about cooperativity between binding sites and the effects of the mutation γ_2R43Q and allosteric modulation by diazepam.

For the model in figure 4A, allowing the loop containing the states B1, D1, D2 and B2 to violate microscopic reversibility has been useful to simulate macroscopic currents with a minimum of kinetic states (Jones et al., 1998; Wagner et al., 2004). This means that for each cycle around the loop, energy is lost or gained, the amount of which is defined by the ratio of the products of the transition rates in either direction around the loop using the Arrhenius equation as $\Delta G = -RT\ ln\left((k_{+2}\ d_2\ p\ r_1/\left(2k_{-2}\ d_1\ q\ r_2\right)\right)$, where R is the universal gas constant and T is temperature. Using the optimized rate constants for $\alpha_1\beta_2\gamma_2$ receptors (Fig. 4C), ΔG is approximately -7 kcal/mol at room temperature for each counterclockwise cycle, which given the rate limiting step r_1 can occur

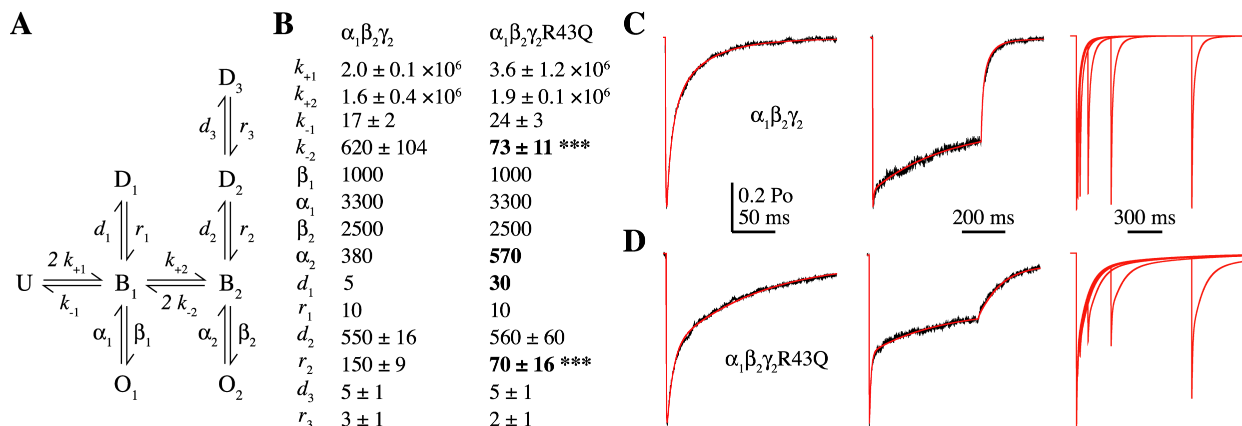

Figure S1: The conclusions that the kinetic effects of the mutation γ_2R43Q can be explained by faster channel closure, slower recovery from desensitization and slower unbinding are unchanged for a model without a loop (compare with Fig. 4 in the main text). A) The Markov model used to simulate GABA responses (U = unbound, B = bound, O = open, D = desensitized). B) Rate constants used to simulate $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2$R43Q responses to 10 mM GABA (units are s^{-1} except for GABA binding steps, which are $M^{-1}s^{-1}$). The values of $k_{\pm 1}$, $k_{\pm 2}$, d_2, r_2, d_3, and r_3 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). k_{-2} and r_2 were the only unconstrained rate constants that significantly differed when comparing mutant and wild type models (two-tailed unpaired Students t-Test, ***p $<$ 0.0001). C-D) Current responses (black) evoked by 2 ms (left) or 500 ms (middle) pulses of 10 mM GABA from two individual patches containing $\alpha_1\beta_2\gamma_2$ (C) or $\alpha_1\beta_2\gamma_2$R43Q (D) receptors overlaid with simulated responses (red). The model qualitatively reproduces the slowing of paired pulse recovery for $\alpha_1\beta_2\gamma_2$R43Q (D, right) as compared to $\alpha_1\beta_2\gamma_2$ (C, right) observed by Bowser et al. (2002).

at most three times per second. To put this in perspective, this energy is approximately equivalent to the energy associated with the binding of two GABA molecules (Jones et al., 1998). However, we have not experimentally verified whether or not the $GABA_A$ receptor undergoes such an unbalanced process, nor to which source the receptor may be energetically coupled. Therefore, we explored enforcing microscopic reversibility in the loop by constraining $q = (k_{+2}\, d_2\, p\, r_1) / (2k_{-2}\, d_1\, r_2)$. With this constraint we were required to add a third doubly liganded desensitized state (D3) to describe the slow phase of desensitization during 500 ms pulses of saturating (10 mM) GABA. With the additional state the loop was no longer required, and thus microscopic reversibility is obeyed by default because no unbalanced loops exist (Fig. S1).

We used simulation and fitting methods identical to those described in the main text, to evaluate whether a model that lacked a loop, but included an additional desensitized state, could account for our kinetic observations. Our conclusions that the mutation γ_2R43Q slows both recovery from desensitization and unbinding from the double bound state were the same for both models (compare Fig. 4 and S1), and did not depend on the loop in the model in figure 4A. Therefore, we cannot differentiate between these two models. In addition, although D3 needed to be connected to one of the doubly bound states (D2, B2 or O2) in the model in figure S1A, we obtained good fits for all three of these cases, and thus cannot differentiate between them either (Fig. S1C-D, fits for the latter two cases not shown). We also explored an extension of the model in figure S1A allowing diazepam (DZ) binding/unbinding to each state (Fig. S2). The model in figure S2A was able to

describe all of our observed effects of DZ with DZ-induced speeding of GABA binding and slowing of GABA unbinding. These results are identical to those obtained by a similar extension of the model containing a loop described in the main text (compare Fig. 7 and S2).

Therefore, our conclusions concerning the effects of mutations on GABA and diazepam binding sites do not rely on any specific assumptions about microscopic reversibility, and are independent of the choice of the two models tested, both of which are sufficiently detailed to capture the complexity of the observed macroscopic kinetics, namely having multiple binding steps and both slow and fast desensitization.

References

[1] Colquhoun D, Dowsland KA, Beato M, Plested AJR (2004) How to impose microscopic reversibility in complex reaction mechanisms. *Biophys J* 86: 3510-3518.

[2] Overstreet LS, Jones MV, Westbrook GL (2000) Slow desensitization regulates the availability of synaptic $GABA_A$ receptors. *J Nerusoci* 20: 7914-7921.

[3] Richard EA, Miller C (1990) Steady-state coupling of ion-channel conformations to a transmembrane ion gradient. *Science* 247: 1208-1210.

[4] Tolman RC (1925) The principle of microscopic reversibility. *PNAS* 11: 436-439.

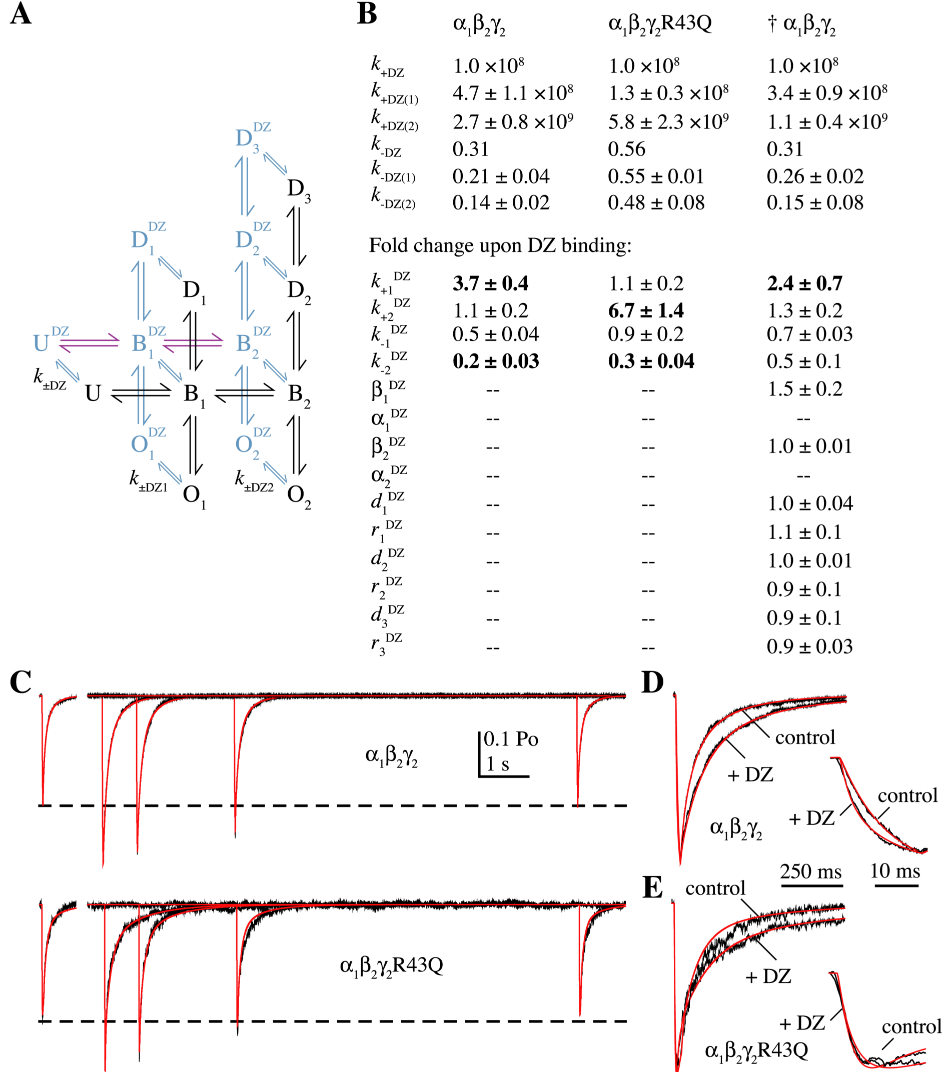

Figure S2: The conclusion that the effects of diazepam (DZ) can be explained by speeding GABA binding and slowing GABA unbinding are unchanged for a model without a loop (compare with Fig. 7 in the main text). A) An extension of the kinetic model shown in figure S1A allowing DZ binding/unbinding from 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 S1A) except for the binding and unbinding rates shown in purple, which were allowed to vary (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. †Fits were repeated for $\alpha_1\beta_2\gamma_2$ receptors where all of the DZ-bound rates were allowed to vary except the closing rates α_1 and α_2 as single channel open times are not altered by DZ (Vicini et al., 1987; Rogers et al., 1994). Our overall conclusion that DZ alters GABA binding/unbinding was not changed, and there was also a DZ-induced increase in the singly-bound opening rate constant β_1, suggesting that DZ could have both binding and gating effects. C) Current responses (black) evoked by 20-40 ms pulses of 30 μM GABA alone (offset left) or at varying times following washout of 10 μM DZ (right) for $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2\gamma_2$R43Q 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).

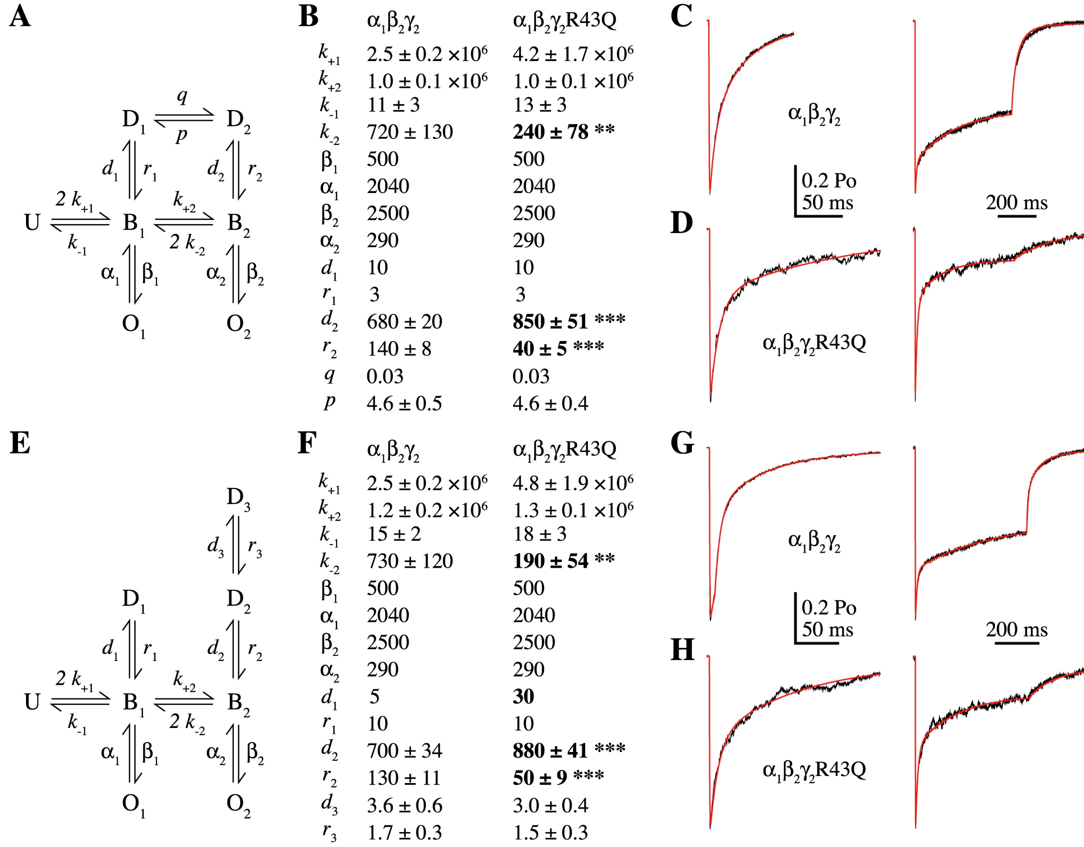

Figure S3: The overall conclusions from the models in figures 4A and S1A that the kinetic effects of the mutation γ_2R43Q can be explained by a stabilizing of the doubly-liganded desensitized state and slower unbinding do not depend on whether the closing rates α_1 and α_2 are based on single channel data from $\alpha_1\beta_2\gamma_2$ or $\alpha_1\beta_3\gamma_{2L}$ receptors, or on whether the doubly liganded closing rate α_2 is sped by the mutation. A, E) The Markov model used to simulate GABA responses. B, F) Rate constants for the models in (A) and (E) optimized to simultaneously fit 2-5 ms and 500 ms current responses to 10 mM GABA (see methods). The closing rates were constrained based on single channel open time distributions for $\alpha_1\beta_2\gamma_2$ receptors (Keramidas and Harrison, 2008; see methods). Differences between the mutant and wild type were assessed by a two-tailed unpaired Students t-Test, **p $<$ 0.01, ***p $<$ 0.0001. Although speeding of d_2 was required to compensate for the longer open times as compared to models with closing rates based on $\alpha_1\beta_3\gamma_{2L}$ receptors, the conclusion that the doubly liganded desensitized state D2 was stabilized by the mutation γ_2R43Q was unchanged (compare with figures 4 and S1). C-D, G-H) Current responses (black) evoked by 2-5 ms (left) or 500 ms (right) pulses of 10 mM GABA from two individual patches containing $\alpha_1\beta_2\gamma_2$ (C, G) or $\alpha_1\beta_2\gamma_2$R43Q (D, H) receptors overlaid with simulated responses (red) for the models in (A) (C-D) and (E) (G-H).