Supplementary Information

Experimental Design

Radioligand Binding Assay

Competition binding assays were performed in a final assay volume of 201 μL. Appropriate volumes of membrane were made up in assay buffer (50 mM Tris-Cl, 0.5 % F127 Pluronic acid). The radioligand [^{3}H] Ro15-1788 was diluted in assay buffer to approximately K_d for the given membrane type, as determined by saturation binding experiments. Compounds were incubated with [^{3}H] Ro15-1788, and reactions initiated by addition of the membrane. Bretazenil was used to determine non-specific binding at an assay concentration of 0.9 μM. The plates were centrifuged briefly, and left to reach equilibrium at room temperature for 2 hours. The reaction was terminated by rapid filtration using a vacuum harvester with four 0.8 mL washes of ice-cold wash buffer (50 mM Tris-Cl pH 7.4). The filters were soaked in 50 μL of scintillation fluid, and the amount of radioactivity present was determined by liquid scintillation counting. Raw data was analyzed using SiGHTS proprietary software, using a four parameter logistic equation to determine $IC_{50,}$ with K_i, determined by using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

Electrophysiology Assay Protocols

Recording solutions

The recording solutions used in these experiments were as follows: extracellular solution (in mM) 137 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 10 Glucose, pH 7.4 with NaOH, osmolarity 303-308 mOsm; intracellular solution (in mM) 90 KCl, 50 KF, 1 $MgCl_2$, 10 HEPES, 11 EGTA, 2 Mg-ATP, pH 7.35 with KOH, osmolarity 295-300 mOsm.

Cell lines

The following stable cell lines were used for these experiments, all in a HEK293 host cell: Human $GABA_A$ $\alpha1\beta3\gamma2$, $\alpha2\beta2\gamma2$. The cells were kept under 80% confluency during routine cell culture to maintain expression of the $GABA_A$ receptor at sufficient levels for QPatch recordings.

Compound preparation

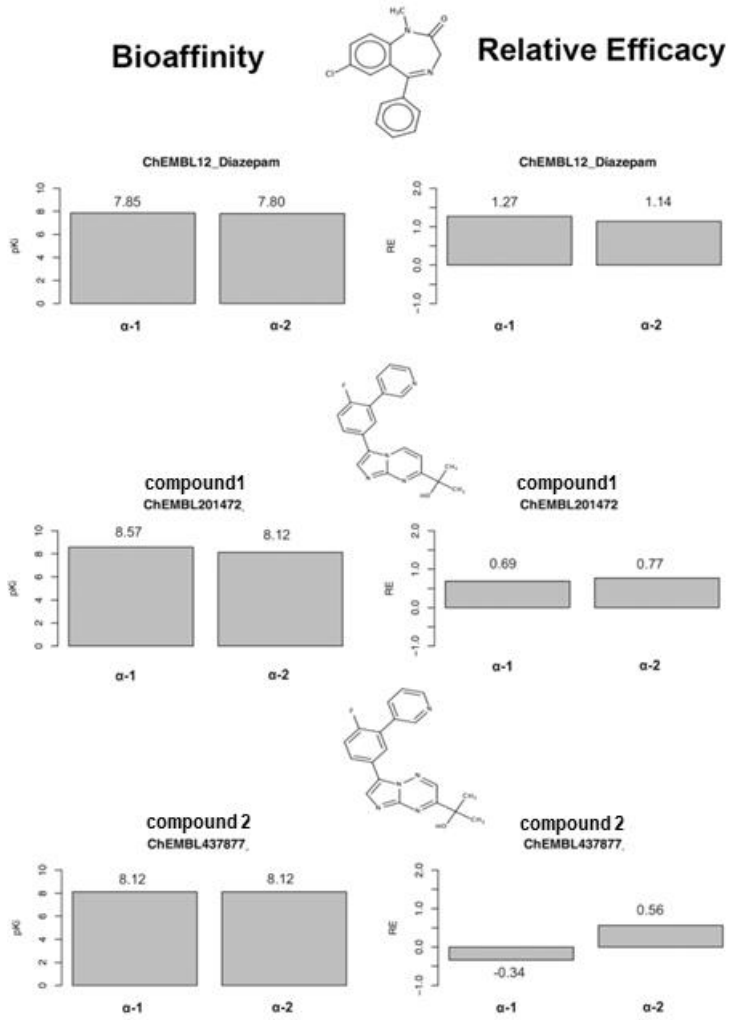
Compounds were diluted in 100% DMSO, and then diluted 1 in 1000 in the external solution, giving a final DMSO concentration of 0.1% in all solutions.

The QPatch automated electrophysiology assay was run on QPatch HT instruments (Mathes *et al.*, 2009). The assay was set up to maximize the recording success rate by using an assay of the shortest possible duration. The open channel assay format was used for generating these data. In this assay format, GABA was first applied in the presence of 0.1% DMSO for 3 or 9 seconds to allow the GABA current to stabilize (the 3 second application time was changed to 9 seconds to more accurately measure the GABA current rundown). This was followed by addition of a positive allosteric modulator (PAM) in the presence of the same GABA concentration for 15 seconds. This application was washed off using the extracellular solution containing 0.1% DMSO.

The GABA concentration used in this assay was dependent on the GABA EC_{50} of each receptor subtype. The selected GABA concentration was approximately EC_{10} to EC_{20} for each receptor subtype, to enable activation of the $GABA_A$ receptor at a low enough level to provide a sufficient assay window size and prevent rundown of the current. The following GABA concentrations were used: α2 containing receptors 0.8 μM-1 μM, α1 containing receptors 0.4 μM-0.5 μM. A complete protocol of solution preparation, compounds preparation and cell lines used is provided in the Supplementary Information.

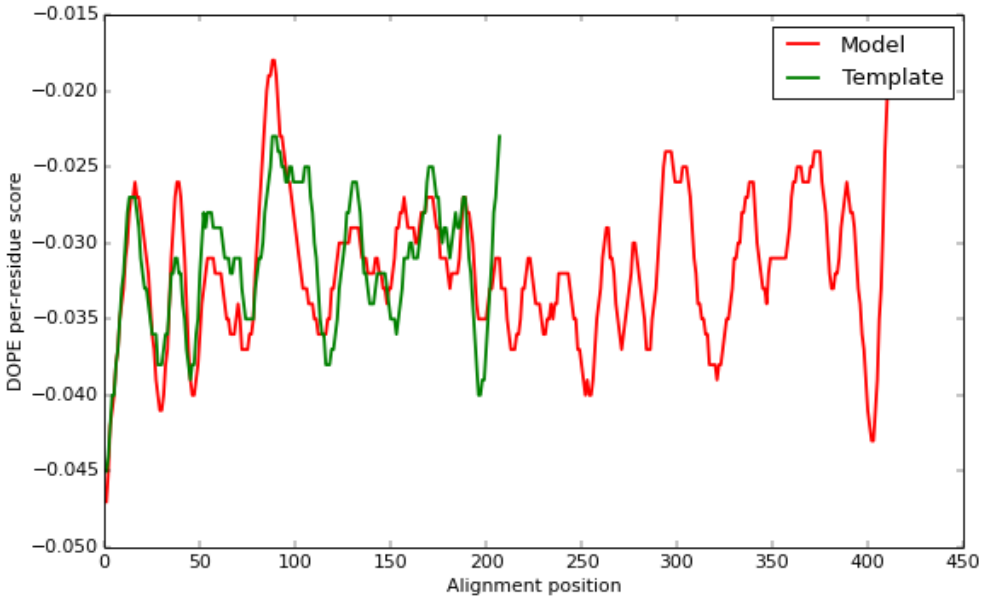
The following quality control criteria were applied to all raw data on the QPatch software: minimum current amplitude 80pA (with leak current subtracted), maximum series resistance 16MΩ, rundown ± 20%, maximum leak current 150pA. The effect of each PAM was calculated from the baseline GABA current as follows:

$$\%Enhancement = [\frac{((peakPAM_{currentamplitude} - leakcurrent) - (GABA_{currentamplitude} - leakcurrent))}{(GABA_{currentamplitude} - leakcurrent)}] \times 100$$

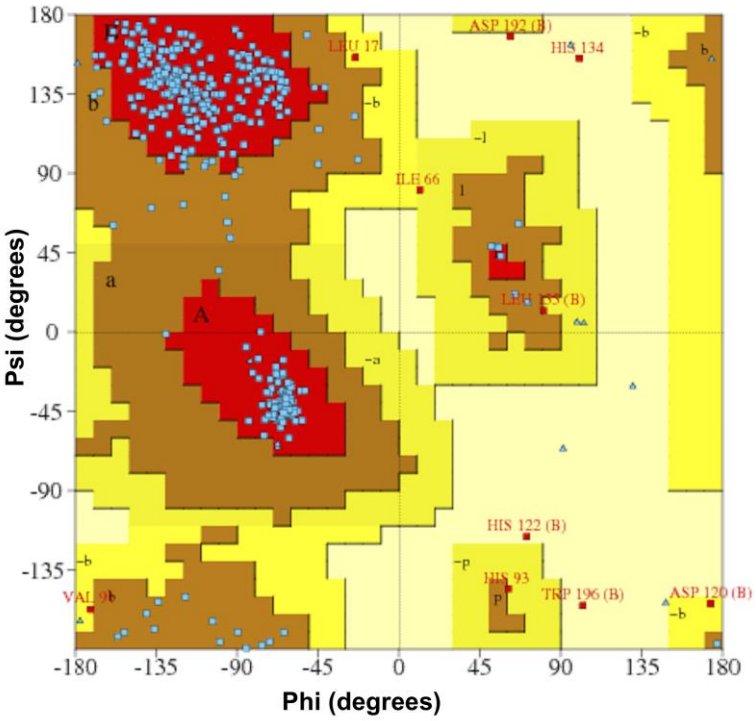


Supplementary Figure 1: Binding affinity and efficacy of Diazepam, compound 1 and compound 2 against GABA-A α1 and α2 subtype receptors

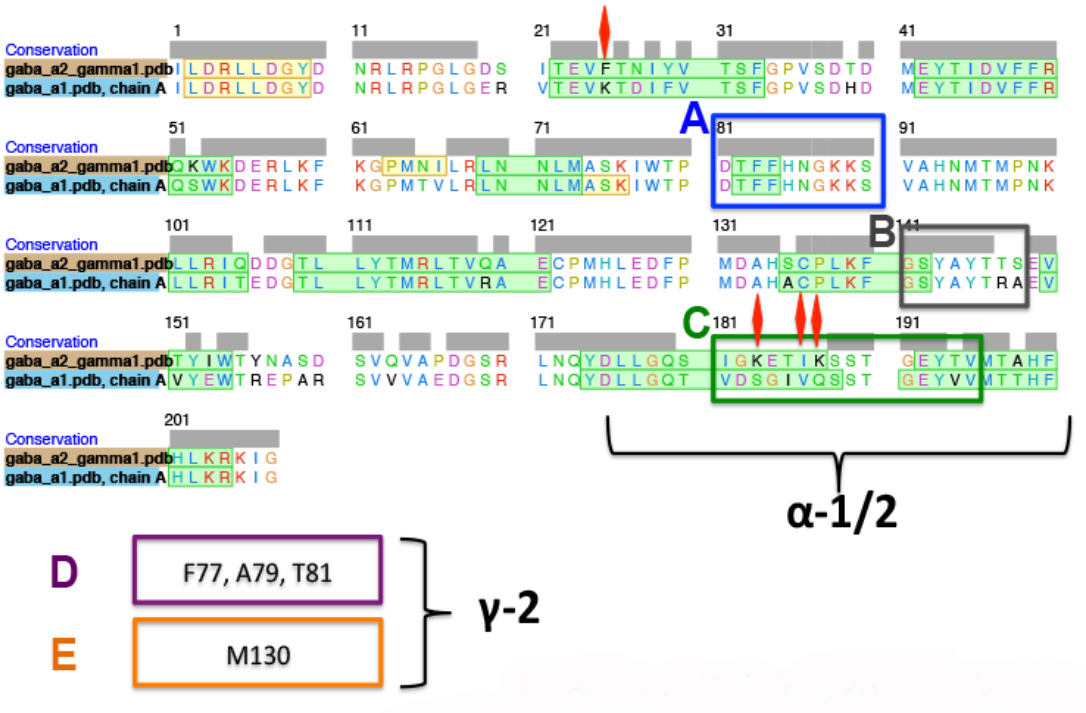
Quality Checks for $GABA_A$ α2 γ2 modeled structure



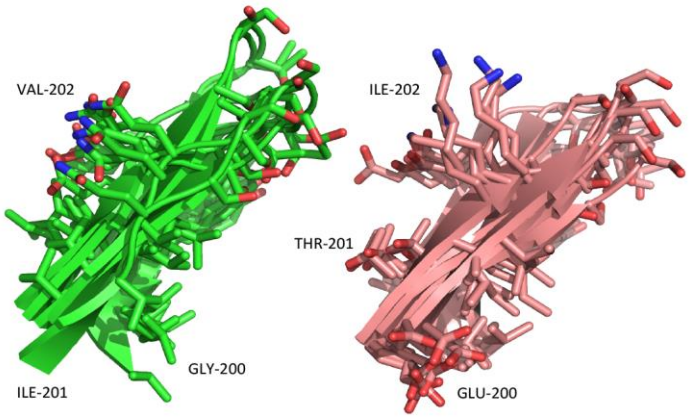
Supplementary Figure 2: Discreet Optimized Potential Energy (DOPE) profile of the template structure (GABA α1γ2) and the model (GABA α2γ2). The per-residue energy profile of modeled α2 shows the stability of the homology model. As the modeling was only performed for α subunit (green), the DOPE profile is only shown for the residues of α subunit; γ2 subunit was same in both complexes.



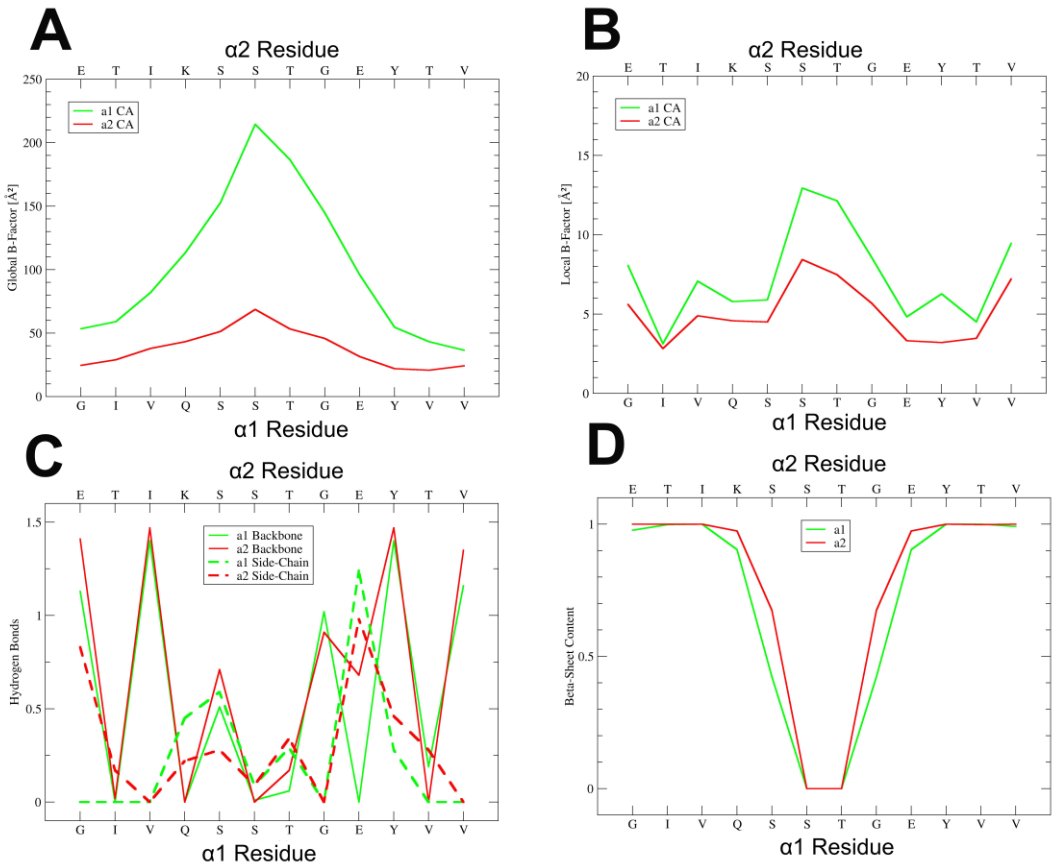
Supplementary Figure 3: Ramachandran plot of modeled GABA α2γ2 structure with 86.8% residues in the most favored regions and only 1.1% residues in disallowed regions. These statistics suggest a good quality of the model.



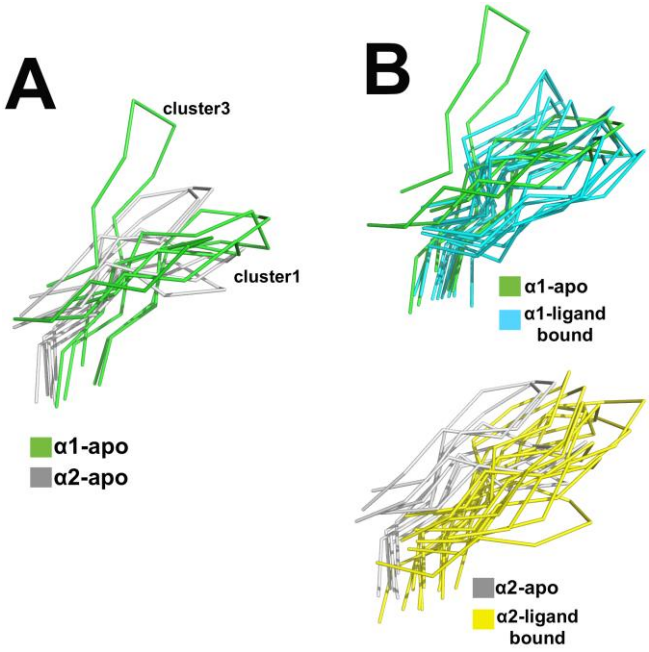
Supplementary Figure 4: Structural alignment of GABA α1 and α2 subtypes colored according to secondary structure elements. The yellow color represents the helix whereas green represent the sheets in 3D structure. The substitutions in loop C annotated inside the box are marked. The γ2 subtype is same in case of both complexes, hence only residues contained in loop D and E are shown.



Supplementary Figure 5: Destabilising variations in loop C region of α1 (green) and α2 (red) subtypes are shown in sticks. The replacement of Gly-200 with Glu-200 decreases the free movement of amino acid by increasing the internal side chain contacts and hence increases the rigidity in α2 subtype. Other variations Ile-201 to Thr-201 and Val-202 to Ile-202 are also known to affect the activity of Zolpidem.



Supplementary Figure 6: 100 ns simulations results for loop C region of both α subtypes. A decrease in **(A)** global and **(B)** local B-factor was observed, suggesting low entropy and hence less flexibility of the loop in α2 subtype as compared to α1. **(C)** An increase in backbone hydrogen bonding was observed for α2. The decreased hydrogen bonding in the side chain of α2 because of increased internal contacts corroborates with less number of hydrogen bonding interactions with the ligand observed in docking experiments. **(D)** An increase in secondary structure elements (β-sheets) was also observed in α2 as compared to α1, which could add to the increased rigidity of this subtype.



Supplementary Figure 7: (A) Conformational clusters of α1 and α2 proteins showing the movement of loop C region of benzodiazepine-binding pocket. During the course of 100ns simulations, it can be seen by the movement of the loop that it fluctuates more in α1 subunit (shown in green) than the α2 subunit (grey). **(B)** Five clusters both from apo and ligand-bound protein structures were extracted from a 100ns simulation to observe the movement of the loop with and without ligand. Freezing of the loop occurring in α1-ligand bound state is observed as compared to α2-ligand bound state. All these results are in correlation with previous findings.