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Identification of an Inhibitory Alcohol Binding Site in $GABA_A$ ρ1 Receptors

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Abstract

Alcohols inhibit γ-aminobutyric acid type A ρ1 receptor function. After introducing mutations in several positions of the second transmembrane helix in ρ1, we studied the effects of ethanol and hexanol on GABA responses using two-electrode voltage clamp electrophysiology in *Xenopus laevis* oocytes. The 6′ mutations produced the following effects on ethanol and hexanol responses: small increase or no change (T6′M), increased inhibition (T6′V) and small potentiation (T6′Y and T6′F). The 5′ mutations produced mainly increases in hexanol inhibition. Other mutations produced small (3′ and 9′) or no changes (2′ and L277 in the first transmembrane domain) in alcohol effects. These results suggest an inhibitory alcohol binding site near the 6′ position. Homology models of ρ1 receptors based on the X-ray structure of GluCl showed that the 2′, 5′, 6’ and 9′ residues were easily accessible from the ion pore, with 5′ and 6′ residues from neighboring subunits facing each other; L3′ and L277 also faced the neighboring subunit. We tested ethanol through octanol on single and double mutated ρ1 receptors [ρ1(I15′S), ρ1(T6′Y) and ρ1(T6′Y,I15′S)] to further characterize the inhibitory alcohol pocket in the wild-type ρ1 receptor. The pocket can only bind relatively short-chain alcohols and is eliminated by introducing Y in the 6’ position. Replacing the bulky 15′ residue with a smaller side chain introduced a potentiating binding site, more sensitive to long-chain than to short-chain alcohols. In conclusion, the net alcohol effect on the ρ1 receptor is determined by the sum of its actions on inhibitory and potentiating sites.

Keywords

ligand-gated ion channel; transmembrane; homology model; 6′ position; pore; oocyte

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SUPPORTING INFORMATION**:** Figure 1: GABA concentration-response curves for ρ1T6′ mutants. Figure 2: Linear correlation between alcohol effects on ρ1T6′ mutants and several variables. Figure 3: GABA concentration-response curves for ρ1I5′ mutants. Figure 4: Linear correlation between alcohol effects on ρ1I5′ mutants and several variables. Figure 5: GABA concentration-response curves for neighboring ρ1 mutants in TM2 and TM1. Figure 6: GABA concentration-response curves for ρ1T6′Y and/or I15′S mutants.

INTRODUCTION

Pentameric ligand-gated ion channels (pLGICs), a superfamily that includes γ-aminobutyric acid type A ($GABA_A$), nicotinic acetylcholine (nACh), and glycine (Gly) receptors, are crucial for neurotransmission in the central nervous system and are considered likely targets for alcohol action. $GABA_A$ are ionotropic receptors composed of five subunits arranged around a central pore. There are multiple $GABA_A$ receptor subunits: α1-6, β1-3, γ1-3, δ, ε, π, θ and ρ1-3[1]. $GABA_A$ receptors composed of homomeric ρ subunits provide distinct characteristics that differentiate them from receptors with other subunit arrangements, and were previously termed $GABA_C$ receptors[2].

Previous research[3-5] identified amino acids in the transmembrane (TM) domains of inhibitory pLGICs that are critical for alcohol action. For $GABA_A$ receptors, these correspond to I307 (15′, TM2) and W328 (TM3) in ρ1 and S270 and A291 in α1 subunits. GABA ρ1 subunits were useful in this identification process because of their distinct alcohol pharmacology[5]. The ρ subunits were identified in retina[6, 7] but detected in much lower levels in specific regions of the central nervous system[8], and thus were initially not considered relevant to alcohol action themselves. However, deletion of ρ1 in mice altered the behavioral responses to alcohol[9], suggesting that these receptors are important for alcohol action, even though their levels in brain are low. Also, a genetic correlation of the level of ρ1 expression in nucleus accumbens with ethanol consumption and motor activation was found using BxD recombinant inbred mice [r= 0.77, ethanol preference[10] and r= −0.48, ethanol-induced motor response[11], from genenetwork.org]. Furthermore, single nucleotide polymorphisms (SNPs) in the human genes that encode ρ1 and ρ2 were associated with alcohol dependence[12].

Alcohols potentiate other GABA- and glycine-gated inhibitory pLGICs via binding sites located in TM domains[13, 14], while alcohols inhibit wild-type ρ1 receptors[15]. Single mutants, ρ1(I307S) and ρ1(W328A), and the double mutant ρ1(I307S,W328A) were potentiated by long-chain alcohols, but the ability of ethanol to inhibit these mutants was conserved[16]. This suggested that there was an inhibitory binding site for short-chain alcohols distinct from the binding site at TM2 15'-TM3. An inhibitory binding site for short-chain alcohols was also suggested by a tryptophan scan of α1 TM2 in $\alpha1\beta2\gamma2$ $GABA_A$ receptors[17] and was described in αβ and αβγ $GABA_A$ receptors at the 6' position of TM2[18], the α-helix that lines the channel[19]. Multiple alcohol binding sites that possess opposing effects have been described in pLGICs. For instance, both potentiating and inhibitory sites for alcohols were identified in TM2 of nACh receptors, the effects of which depended on the alcohol chain length[20].

A series of crystal structures from pLGICs such as GluCl[21] and a homomeric $GABA_A$ receptor[22] have paved the way for molecular models of related pLGICs, allowing the study of binding sites for agonists and modulators located at interfaces in the extracellular domain[23]. Another technique that has shed light on binding sites was receptor photolabeling using modified allosteric modulators, especially in the TM domains, where intersubunit sites were shown to be of particular interest[24].

The objective of this study was to define the inhibitory ethanol binding site in the ρ1 subunit through the introduction of mutations in critical sites. We hypothesized that if a residue forming part of an inhibitory binding site was replaced by a larger residue, n-alcohols would not be able to bind due to the bulkier residue occupying the cavity, and therefore the inhibitory alcohol effect would be decreased or even eliminated. We focused on the 6' position, but also studied neighboring amino acids. We then combined a mutation that eliminates ethanol inhibition (T6'Y) with a previously described mutation that may provide a potentiating binding site for alcohols (I15'S), and studied the effects of alcohols of different chain length on these mutants. We also built homology models of wild-type and mutated ρ1 receptors, based on the the wild-type GluCl, which has been identified as the best template to model the ρ1 receptor out of five different X-ray crystal structures[25].

RESULTS AND DISCUSSION

Our approach to identify the inhibitory ethanol binding site in ρ1 was to introduce mutations in the TM2 6' site, which had been identified as a putative inhibitory binding site for short-chain alcohols in other $GABA_A$ receptors[17, 18]. We hypothesized that a mutation in the region near the inhibitory alcohol binding site would modify the ethanol effect. In addition, we mutated neighboring amino acids in TM2 and TM1. The positions studied are color-coded in the molecular model shown in Fig. 1. The 6′ position is occupied by a threonine in the wild-type receptor. In the model, this residue leans towards the 5′ position in TM2 of the neighboring subunit (7.5 Å Cα-Cα distance), which is essentially facing towards the interface with the neighboring subunit. The leucine in the 9′ position is one turn of the α-helix above T6′; these two residues are quite close (4.4 Å intrasubunit and 7.6 Å intersubunit Cα-Cα distance). The proline in the 2′ position is also lining the pore, and one turn of the α-helix below T6′ (5.7 Å intrasubunit and 9.1 Å intersubunit Cα-Cα distance). The leucine in the 3′ position (TM2) and L277 (TM1) are both facing towards the interface with the neighboring subunit and proximal to I5′ (7.1 Å and 8.7 Å Cα-Cα distance, respectively). Recently, a crystal structure of human α3 glycine receptor bound to strychnine was described[26] and the zebrafish α1 glycine receptor was studied using electron cryo-microscopy[27]. In both cases, the location of the residues homologous to the ones we studied was similar to the one in our model.

The GABA concentration-response curves for the ρ1 6′ mutant receptors can be found in Supp. Fig. 1, and the corresponding parameters are in Table 1. Only the T6′F and T6′V mutations significantly changed the GABA sensitivity of the ρ1 receptors.

Increasing the residue volume in the 6' position drastically changed the alcohol effect on GABA-mediated currents in ρ1 receptors (representative tracings can be found in Fig. 2). Instead of inhibition, significant potentiation by both ethanol and hexanol was observed in mutants T6'Y and T6'F (Fig. 3A and C). In the T6'Y mutant model, after optimization of all the side chains in the mutations and relaxation with molecular dynamics, three of the Y residues are protruding into the pore (Fig. 3B) while in the T6'F mutant model, only two F residues are protruding into the pore; the balance of the mutated 6' residues are tucked between TM2 helices (Fig. 3D). Replacing the T6' with valine residues had the opposite effect on the alcohol action on GABA currents, increasing the ethanol and hexanol inhibitory

effects (Fig. 3E). In the molecular model of this mutant, the small 6' valine residues are lining the pore (Fig. 3F), similar to the 6′ threonine residues (Fig. 1A). Replacing T6' with a methionine residue produced small or no changes in alcohol effects compared with wild-type receptors (Fig. 3G). The M6' residues also line the pore in the molecular model (Fig. 3H). A summary of alcohol effects on 6′ mutants can be found in Table 2.

When introducing a mutation, there is the possibility of modifying the binding or gating effects of the agonist. To confirm that GABA is still a full agonist in ρ1(T6′Y), we tested 100 mM ethanol on maximal GABA responses in wild-type and mutant ρ1 receptors. If GABA had become a partial agonist at the mutant receptors, we would have seen a differential effect of ethanol. The ethanol modulation at maximal GABA was small and similar in both wild-type and mutant (20 ± 10 and 10 ± 3 % potentiation, respectively; n=4, data not shown).

When the alcohol effect (expressed as % change of the EC_{20} GABA responses) for each of these mutants was graphed as a function of the amino acid volume present in the 6' position, about 50-80% of the variance is explained by the size of the amino acids. A positive linear correlation was established for ethanol (r^2= 0.79, p< 0.05, Supp. Fig. 2A), but it did not reach significance for hexanol (r^2= 0.50, p=0.18, Supp. Fig. 2B). In contrast, there was no correlation between the alcohol effect and the hydrophobicity (r^2= 0.24, Supp. Fig. 2C and r^2= 0.36, Supp. Fig. 2D). The ethanol effect had no linear correlation with the GABA EC_{50} for any of the 6'mutants (r^2= 0.52, Supp. Fig. 2D), but the hexanol effect was correlated with the GABA EC_{50} (r^2= 0.81, p=0.03, Supp. Fig. 2F). However, this correlation is not meaningful, as it heavily depends on the GABA EC_{50} value for T6′V, which is markedly different from all the other mutants.

These results define an inhibitory binding site for alcohols at T6' in the ρ1 receptor; mutation of ρ1T6' changed both the ethanol and hexanol inhibitory effects (larger amino acids decreased alcohol inhibition, and a smaller amino acid increased alcohol inhibition). This is consistent with larger residues occupying the pocket and preventing alcohol binding and subsequently inhibiting receptor function. There was a correlation between the amino acid residue size and ethanol effect, but not between the GABA affinity (apparent EC_{50}) and ethanol effect. These results suggest a direct effect of the size of amino acids lining an alcohol binding pocket and not an indirect effect of ethanol on GABA binding and channel gating.

The GABA concentration-response curves for the ρ1 5′ mutant receptors are shown in Supp. Fig. 3, and the corresponding parameters appear in Table 1. All the mutations changed the ρ1 receptor GABA sensitivity.

Only the replacement of I5′ with the large residue tryptophan increased the ethanol inhibition; the other mutations at the 5′ position did not alter ethanol action (Fig. 4A, C, E and G). In contrast, almost all substitutions significantly increased the hexanol inhibition (Fig. 4A, C, E and G). In the I5′A, I5′V and I5′F mutant models, after optimization of the side chains in the mutations and relaxation with molecular dynamics, all the residues are tucked into the interface between TM2 helices (Fig. 4B, D and F). In the I5′W mutant

model, the residues are still located at the interface, but the larger bulk of the tryptophan causes part of the residue chains to protrude out into the channel. A summary of alcohol effects on 5′ mutants can be found in Table 2.

When the alcohol effect (expressed as % change of EC_{20} GABA responses) for each of these 5′ mutants was graphed as a function of the amino acid volume present in the 5' position, there was no correlation for either ethanol (r^2= 0.45, Supp. Fig. 4A) or hexanol (r^2= 0.35, Supp. Fig. 4B); however, about 35-45% of the variance was explained by the size of the amino acids. For hydrophobicity, there was no correlation for ethanol (r^2= 0.41, Supp. Fig. 4C) but a positive linear correlation was established for hexanol (r^2= 0.85, $p<0.05$, Supp. Fig. 4D). In addition, there was no correlation between the alcohol effects and the GABA EC_{50} for each of the 5' mutants (r^2= 0.34, Supp. Fig. 4E and r^2= 0.00, Supp. Fig. 4F).

The GABA concentration-response curves for the neighboring mutations in ρ1 receptors are shown in Supp. Fig. 5, and the corresponding parameters can be found in Table 1. All the mutations modified the ρ1 receptor GABA sensitivity.

Some of the amino acids near the 5′ and 6' positions also affected alcohol action on ρ1 receptors, but to a lesser degree. A decrease in ethanol inhibition was observed in the L9'F mutant (this particular mutation was chosen because the resulting receptor is not spontaneously open[28]), but the effect of hexanol was not changed (Fig. 5A). In the molecular model, all 9'F residues were lining the pore (Fig. 5B). The L3'F mutant showed a decrease both in ethanol and hexanol inhibition (Fig. 5C). All 3'F residues were located in the center of the four α-helix bundle within a single subunit (Fig. 5D). Lastly, there were two mutants that did not change either ethanol or hexanol inhibition: ρ1(L277F) and P2'G (Fig. 5E and G). The residue at position 277 is located in TM1, in the interface between α-helices from neighboring subunits for both wild-type (leucine, Fig. 1A) and the mutant (phenylalanine, Fig. 5F). The P2′G mutant lacks a side chain, but it remains lining the pore (Fig. 5H).

In summary, neighboring amino acids also participate in alcohol action sites, and mutation of ρ1L9' decreased ethanol but not hexanol inhibition. Mutation of ρ1I5' mainly modified the hexanol effect. Mutation of L3'decreased ethanol inhibition, and to a lesser degree, hexanol inhibition.

The mutations affected alcohol inhibition in a specific manner, indicating that the changes in alcohol effects are not due to general changes in receptor function produced by the mutation itself. In fact, two nearby mutations did not alter alcohol effects: one in TM2 (P2'G) and one located near I5' [ρ1(L277F) in TM1]. Several of the residues studied (2′, 6′ and 9′) line the pore of the channel in the ρ1 receptor, and had been shown to have an important role in receptor function and pharmacology. For instance, residues at the 6′ and 9′ positions are important for channel gating[29]. Also, residues at the 2′ and 6′ positions are critical for inhibition by picrotoxinin[30, 31] and some neurosteroids[32, 33]. However, even though P2′ has a profound effect on the ion selectivity, conductance, and pharmacology of ρ1 receptors[34-37], its mutation to glycine had no effect on alcohol inhibition, suggesting that the changes observed in alcohol effects due to the studied mutations are not due simply to non-specific changes in the receptor function, but to modifications in sites specific to alcohol action.

The residue in the 15’ position has been shown to be critical for alcohol action, both in ρ1 as well as other inhibitory receptors[5, 16], but it appears to fulfill a different role in nACh receptors[38]. Based on previous research[17, 18], we hypothesized that an inhibitory alcohol site in ρ1 involved the 6′ position and a stimulatory alcohol binding site could be introduced in 15′. We tested this hypothesis by introducing mutations at 6’ and/or 15’ (Fig. 6A and B show T6′ and I15′ in the wild-type ρ1, and Fig. 6C and D show Y6′ and S15′ in the ρ1 double mutant). Four different ρ1 receptors were examined: wild-type, single mutants T6’Y and I15’S, and the double mutant T6’Y, I15’S. The GABA concentration-response curves for these mutants can be found in Supp. Fig. 6, and the corresponding parameters in Table 1.

The alcohol effect on EC_{20} GABA responses was measured in these four receptors (Fig. 7). The wild-type $GABA_A$ ρ1 receptor was inhibited by alcohols containing up to six carbons. The ρ1(I15’S) mutant was potentiated by alcohols with chains longer than five carbons, but still inhibited by ethanol and propanol; butanol produced no net effect. The ρ1(T6’Y) mutant showed moderate potentiation by ethanol through pentanol; hexanol had no effect, and heptanol and octanol produced small inhibition. The double mutant ρ1(T6’Y, I15’S) displayed potentiation across all of the *n*-alcohols tested. A summary of the alcohol effects on these receptors can be found in Table 3.

Previous studies[16] have shown that if the residue in the 15’ position is large (e.g., isoleucine in the wild-type), all alcohols inhibit the ρ1 receptor; in contrast, if the residue is small (e.g., serine, as in the α1 $GABA_A$ and Gly receptor subunits), ethanol inhibits but longer chain alcohols potentiate the GABA responses in the ρ1 mutant. Considering those results together with the present ones, the existence of two different binding sites for alcohols is a strong possibility: an inhibitory binding site for alcohols lined by the 6’ residue and the possibility of introducing an excitatory binding site (where mainly long-chain alcohols act) when the 15’ residue is replaced by a smaller one. Replicating earlier results[15], alcohols up to six carbons long inhibited the wild-type $GABA_A$ ρ1 receptor in our study. This could be the result of alcohols up to hexanol binding in the inhibitory binding site at 6’. Alcohols showed a dual effect on the ρ1(I15’S) mutant, with short-chain alcohols inhibiting and long-chain alcohols potentiating the responses; the crossover point was for butanol, which produced no net effect. In our model, reducing the size of the 15’ residue creates a potentiating binding site where alcohols can also bind, in addition to the inhibitory site at 6’; therefore, the alcohol effect will be the combined result of its effect on both sites. Alcohols like ethanol and propanol act mainly at the 6’ inhibitory site, while butanol has an equal effect on both sites, and pentanol and longer alcohols act mainly at the 15’ potentiating site. Short-chain alcohols produced moderate potentiation in the ρ1(T6’Y) mutant, with long-chain alcohols producing no effect or small inhibition.

In terms of our model, a large residue at 6’ would eliminate the inhibitory site, leaving only a small and/or not very efficient potentiating site at 15’. Therefore, the combined effects are very small and pertain only to short-chain alcohols. In support of our hypothesis, all the *n*-alcohols tested produced potentiation of the double mutant ρ1(T6’Y, I15’S). According to our model, the 6’ inhibitory site has been eliminated in this mutant and a 15’ potentiating site has been introduced; therefore, all alcohols act through the 15’ potentiating site, including the long-chain alcohols like hexanol, that have little to no effect on the wild-type

and other mutant receptors. Evidence for a similar situation, with dual and opposing sites of action for alcohols, was found in $GABA_A$[18], GLIC[39-41] and nACh receptors[20].

The molecular simulations often yielded a pentamer without symmetry in the pore region, where the homologous side chains are located in different rotamer positions even though the backbone of TM2 is conserved. In the case of the residues of interest, some of the side chain residues are in the channel and others are between the subunits. Different approaches were tested, including using an algorithm to find the “best” rotamer for each side chain versus not adjusting rotamers, and using both short and long optimizations, etc.; however, the asymmetry persisted. Because of this, any pore profile or evaluation of changes in intrasubunit dimension spaces would be arbitrary.

This molecular modeling provides a static model of the protein, but it is worth keeping in mind that conformational movements result in very dynamic pLGICs, even in regions such as TM2[39, 42]. The mobility of TM2 in response to different agonists and modulators has been explored using labeling with fluorophores[43] and crosslinking of mutated cysteines[44], including asymmetric rotations in the area of the 6′ position[45]. These studies also revealed a surprising degree of mobility in this domain.

A recent study in ELIC (a bacterial pLGIC) showed that isoflurane, a volatile anesthetic that presents functional similarities to alcohol, inhibited this receptor[46]. Co-crystallization of ELIC with isoflurane revealed that the anesthetic occupied sites inside the pore near the 6′ and 13′ positions. Electrophysiological recordings showed that mutations at these positions modify isoflurane inhibition, providing further support to the idea of an inhibitory binding site located in the pore.

In summary, we have identified an inhibitory alcohol binding site on the $GABA_A$ receptor near the pore of the channel. Not only it is possible to eliminate this inhibitory binding site through mutagenesis, it is also possible to introduce a potentiating alcohol binding site analogous to those present in other $GABA_A$ and Gly receptors, such that a net effect of alcohol on the ρ1 receptor is determined by the sum of its actions on inhibitory and potentiating sites. This suggestion is consistent with dual sites found in other pLGICs, such as $GABA_A$[18], GLIC[39-41] and nACh receptors[20]. The present results warrant construction of mice with mutated ρ1 subunits that either lack ethanol inhibition or display ethanol potentiation, enabling behavioral studies of the role of ethanol on ρ1 function in vivo.

METHODS

Clones

Mutations in the ρ1 cDNAs were made through site-directed mutagenesis using QuikChange (Agilent Technologies, Santa Clara, CA, USA).

Transcription and oocyte injection

The in vitro transcription of wild-type and mutant ρ1 subunits was performed using mMessage mMachine (Life Technologies, Grand Island, NY, USA). Surgery of *Xenopus laevis* frogs was performed according to an approved institutional protocol. After manual

isolation of *Xenopus laevis* oocytes, they were injected with complementary RNAs (cRNAs, 5 ng/oocyte) encoding wild-type or mutant ρ1 subunits. The injected oocytes were incubated at 15°C in sterilized Barth's solution for 3-7 days before recording.

Electrophysiological recordings

The responses of $GABA_A$ ρ1 receptors expressed in oocytes were studied through two-electrode voltage clamp. The oocyte was placed in a chamber perfused with ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5), and voltage-clamped at −70 mV. GABA applications lasted for 30-150 s and the interval between applications was 5-15 min.

Concentration-response curves

Increasing concentrations of GABA were applied (0.1-100 μM) and responses were expressed as percentages of the maximal current.

Alcohol application protocol

Alcohols were first pre-applied for 1 min and then co-applied with GABA. The application sequence for the wild-type and mutants ρ1 receptors was as follows: maximal GABA (to obtain maximal response, EC_{100}), EC_{20} GABA, EC_{20} GABA, pre-application of ethanol (100 mM) immediately followed by a co-application with EC_{20} GABA, EC_{20} GABA, pre-application of hexanol (1.14 mM) immediately followed by a co-application with EC_{20} GABA, EC_{20} GABA. The response to GABA in the presence of the modulator was expressed as a % change compared to the mean of the previous and subsequent GABA responses. In the past, we have used EC_5 to assess alcohol effects, as the alcohol effect is larger in the presence of lower EC values. However, we used EC_{20} in these studies because currents in some of the mutant receptors were greatly diminished.

Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, CA, USA). Pooled data are represented as mean ± S.E.M. Statistical significance was determined using two-way analysis of variance (ANOVA). Nonlinear regression analysis for the concentration-response curves was performed after normalizing the agonist responses in each cell to the maximal current that could be elicited by GABA. The concentration-response curves were fitted to the following equation:

$$I/I_{MAX} = \frac{1}{1+10^{(logEC_{50}-log[GABA])\times n_H}}$$

where I/I_{MAX} is the fraction of the maximally-obtained GABA response, EC_{50} (effective concentration 50) is the concentration of GABA producing a half-maximal response, $[GABA]$ is GABA concentration, and n_H is the Hill coefficient. Linear correlation analysis was also carried out using Prism 7, using the following sources for the independent variables: amino acid volume (calculated by J.R. Trudell), hydrophobicity[47], and EC_{50} GABA (determined in this study, Table 1).

Molecular models

Homology models of ρ1 $GABA_A$ receptors were built by threading the primary sequence of five subunits onto the X-ray structure of GluCl (Protein Data Bank ID code 3RHW[21]). The Modeler module of Discovery Studio 4.1 (DS 4.1Biovia, San Diego, CA, USA) was used to build 50 models of the wild-type and each mutation. We chose the 'best' models based on the force field potential energies measured by the Discovery Studio version of CHARMm. The 'side-chain refinement' module of DS 4.1 was used to optimize the side chain rotamers in the model while the backbone atoms were fixed. Then all backbone atoms were tethered with a quadratic restraint of 10 kcal/$Å^2$ and optimized the resulting models to a gradient of 0.001 kcal/ (mol × Å). We performed an optimization of each model and then a molecular dynamics 'relaxation' at 300 K using the default parameters of DS 4.1. All of the molecular graphics were created using the UCSF Chimera package version 1.7[48].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

EC	effective concentration
cRNA	complementary RNA
GABA	γ-aminobutyric acid
GLIC	*Gloeobacter violaceus* ligand-gated ion channel
GluCl	*Caenorhabditis elegans* glutamate-gated chloride channel
Gly	glycine
nACh	nicotinic acetylcholine
pLGIC	pentameric ligand-gated ion channel
TM	transmembrane

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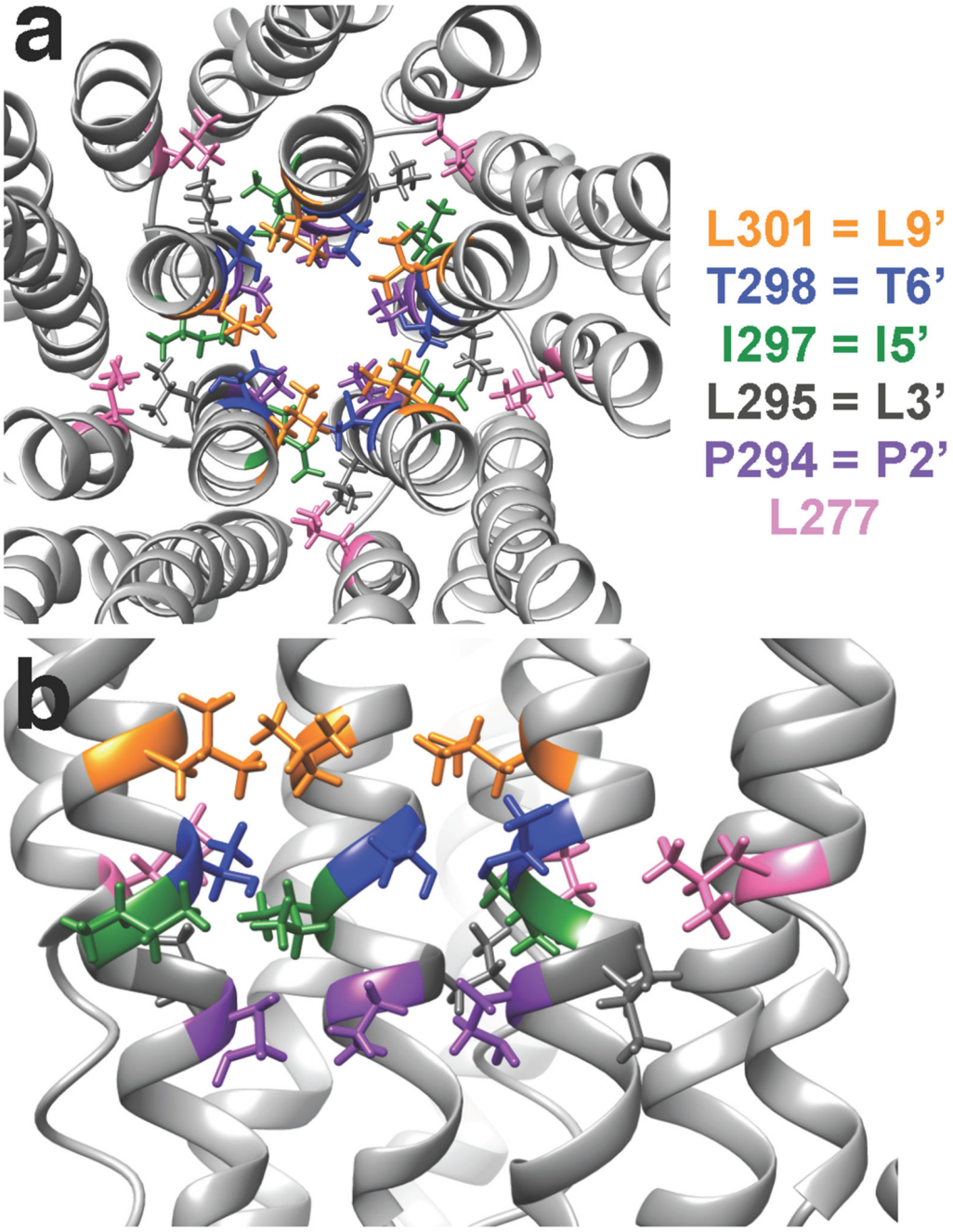

Figure 1.

$GABA_A$ ρ1 receptor homology model based on GluCl (PDB ID: 3RHW). The second TM helix of each subunit lines the pore of the channel. The amino acids that were mutated are shown as color-coded sticks. All amino acids in the TM2 at the top of panel A and the center of panel B are shown as sticks. A. Extracellular view of the pore from the plane between the extracellular and the TM domains. B. View of three subunits from within the pore.

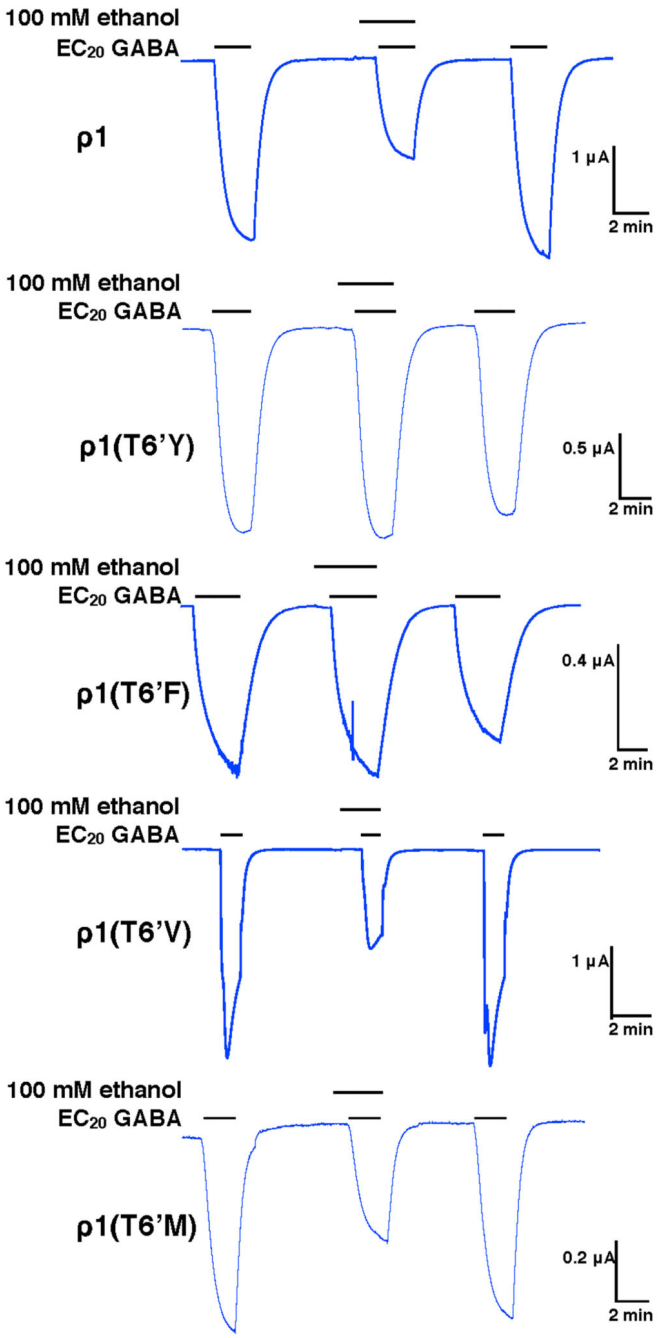

Figure 2.
Representative tracings of GABA responses in the presence of ethanol in ρ1T6' mutants expressed in *Xenopus laevis* oocytes.

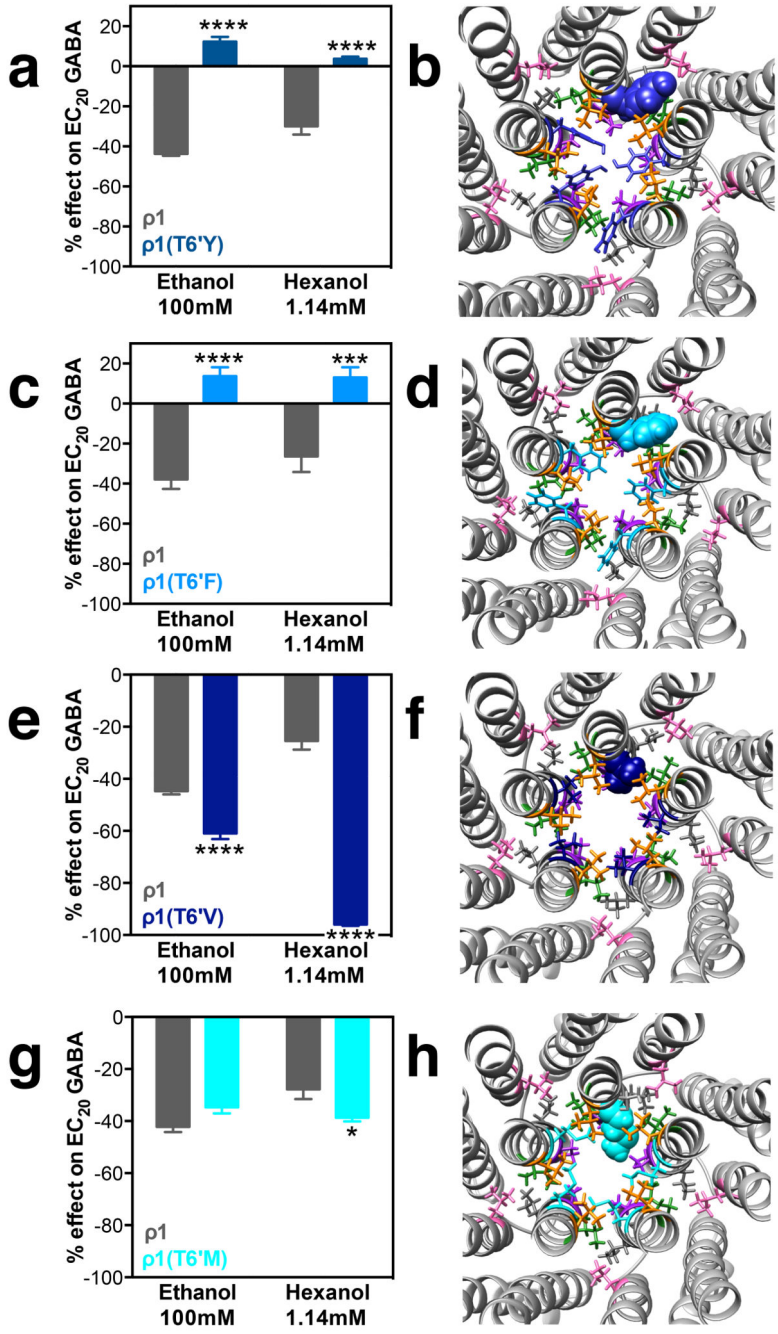

Figure 3.

ρ1T6' mutants: alcohol effects and molecular models. Left panels show ethanol (100 mM) and hexanol (1.14 mM) effects on EC_{20} GABA responses. Right panels are molecular models of the pore and TM domains of the corresponding ρ1 mutant, viewed from the plane between the extracellular and the TM domains. The graph bars and the corresponding amino acid in the model are the same blue color. A and B, T6'Y; C and D, T6'F; E and F, T6'V; G and H, T6'M. Bars represent means ± SEM, n= 4-9. Two-way ANOVA, Sidak's multiple comparisons test: *$p< 0.05$, ***$p\leq 0.001$, ****$p\leq 0.0001$.

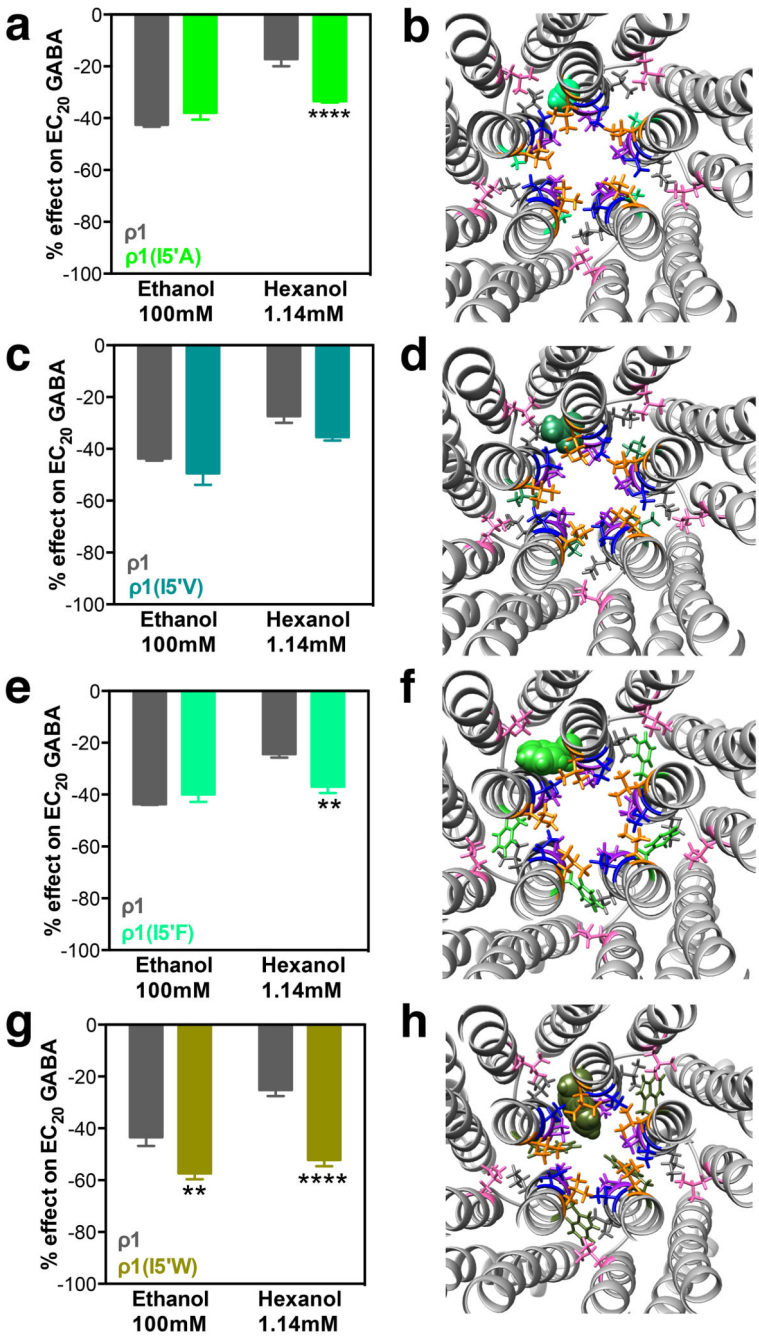

Figure 4.

ρ1I5' mutants: alcohol effects and molecular models. Left panels show ethanol (100 mM) and hexanol (1.14 mM) effects on EC_{20} GABA responses. Right panels are molecular models of the pore and TM domains of the corresponding ρ1 mutant, viewed from the plane between the extracellular and the TM domains. The graph bars and the corresponding amino acid in the model are the same green color. A and B, I5′A; C and D, I5′V; E and F, I5′F; G and H, I5′W. Bars represent means ± SEM, n= 4-9. Two-way ANOVA, Sidak's multiple comparisons test: **p≤ 0.01, ****p≤ 0.0001.

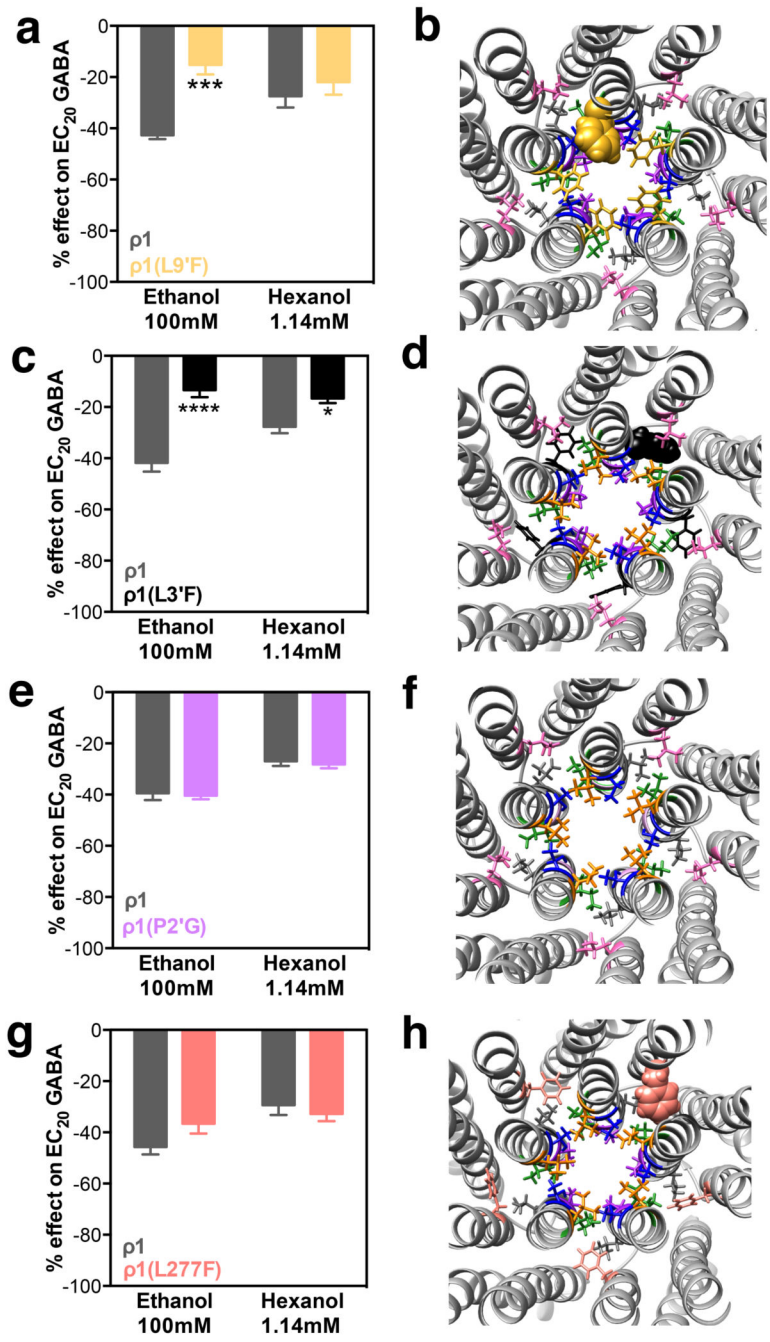

Figure 5.

Neighboring ρ1 mutants in TM2 and TM1: alcohol effects and molecular models. Left panels show ethanol (100 mM) and hexanol (1.14 mM) effects on EC_{20} GABA responses. Right panels are molecular models of the pore and TM domains of the corresponding ρ1 mutant, viewed from the plane between the extracellular and the TM domains. The graph bars and the corresponding amino acid in the model are the same color. A and B, L9'F; C and D, L3'F; E and F, P2′G; G and H, L277F. Bars represent means ± SEM, n= 3-5. Two-way ANOVA, Sidak's multiple comparisons test: *$p < 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

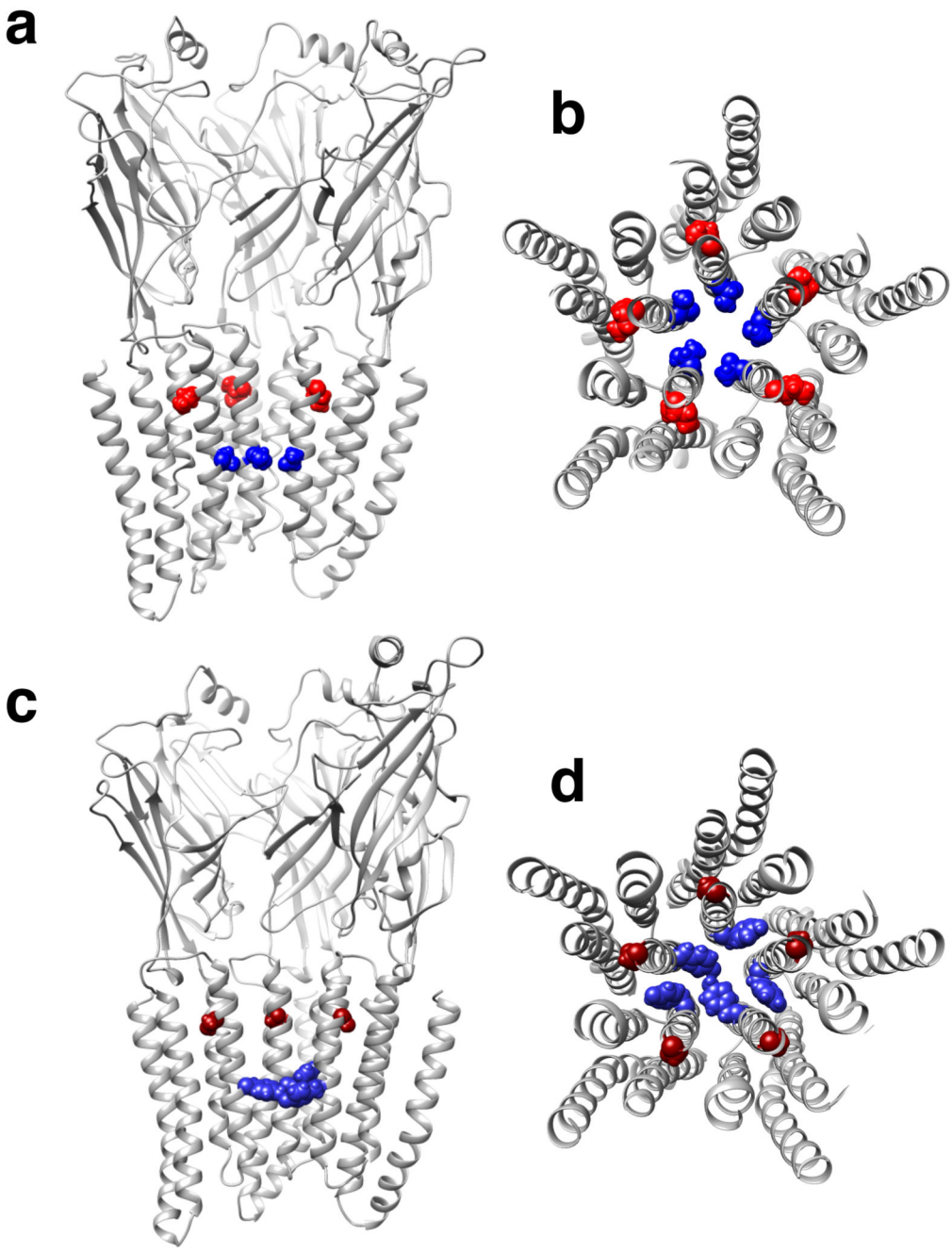

Figure 6.

6’ (blue) and 15’ (red) positions in molecular models of the ρ1 receptor. View of three subunits from the pore for wild-type ρ1 (A) and ρ1(T6′Y,I15′S) (C). Extracellular view of the pore from the plane between the extracellular and the TM domains for wild-type ρ1 (B) and ρ1(T6′Y,I15′S) (D).

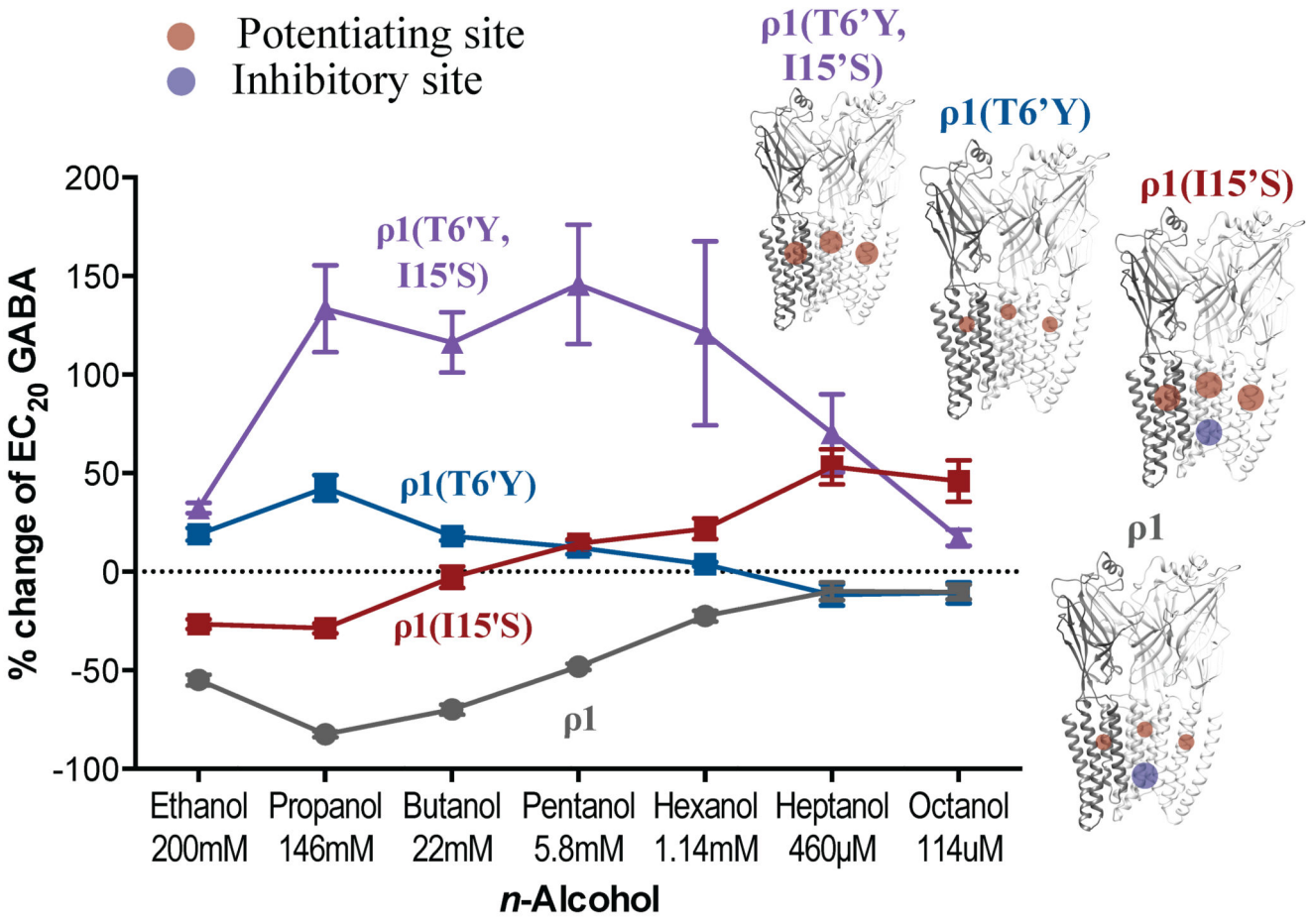

Figure 7.

Alcohol effect on single mutants, ρ1(T6'Y) and ρ1(I15'S), and double mutant ρ1(T6'Y,I15'S). The circles in the molecular models serve only as a visual representation of the approximate position and relative size of the potentiating (red circle) and inhibitory (blue circle) alcohol sites.

Table 1

GABA concentration-response curve parameters. *EC_{50} GABA* (effective concentration producing half-maximal response) values are in μM, and 95% confidence intervals are shown in parentheses. Given the variations observed batch to batch, each mutant was compared to WT receptors expressed in the same oocyte batch. n_{Hill} is the Hill slope value ± S.E.M.; *n* is the number of oocytes per receptor. *p< 0.05, ***p< 0.001, ****p< 0.0001 versus WT for logEC_{50} based on nonlinear regression analysis. The colored text corresponds to the color of the amino acids in the model representations; WT, wild-type.

Receptor	EC_{50} GABA (μM)	n_{Hill}	n
ρ1 WT	1.02 (0.83 to 1.26)	1.8 ± 0.2	4
ρ1(**L9'F**)	0.48 (0.38 to 0.60)***	1.3 ± 0.1	5
ρ1 WT	0.69 (0.65 to 0.75)	2.3 ± 0.1	7
ρ1(**T6'Y**)	0.69 (0.60 to 0.79)	2.1 ± 0.2	7
ρ1 WT	0.78 (0.68 to 0.90)	1.6 ± 0.1	8
ρ1(**T6'F**)	0.36 (0.27 to 0.47)***	1.9 ± 0.3	7
ρ1 WT	1.30 (1.05 to 1.62)	1.5 ± 0.1	7
ρ1(**T6'V**)	6.77 (5.98 to 7.68)****	1.4 ± 0.1	7
ρ1 WT	1.11 (0.92 to 1.33)	1.8 ± 0.2	4
ρ1(**T6'M**)	0.86 (0.60 to 1.24)	1.6 ± 0.3	4
ρ1 WT	0.78 (0.67 to 0.92)	1.7 ± 0.2	5
ρ1(**I5'A**)	2.20 (1.88 to 2.57)****	1.7 ± 0.1	4
ρ1 WT	0.87 (0.74 to 1.03)	1.7 ± 0.2	6
ρ1(**I5'V**)	0.18 (0.13 to 0.24)****	2.5 ± 0.3	6
ρ1 WT	0.69 (0.60 to 0.79)	2.2 ± 0.2	4
ρ1(**I5'F**)	7.47 (6.84 to 8.15)****	1.5 ± 0.1	4
ρ1 WT	1.02 (0.81 to 1.29)	1.8 ± 0.2	3
ρ1(**I5'W**)	0.40 (0.36 to 0.45)****	2.4 ± 0.2	4
ρ1 WT	1.04 (0.75 to 1.45)	1.5 ± 0.2	5
ρ1(**L3'F**)	0.31 (0.24 to 0.39)****	1.5 ± 0.2	5
ρ1 WT	0.78 (0.64 to 0.96)	2.1 ± 0.3	3
ρ1(**P2'G**)	1.62 (1.34 to 1.97)***	1.5 ± 0.1	4
ρ1 WT	1.37 (0.82 to 2.30)	1.2 ± 0.2	4
ρ1(**L277F**)	0.63 (0.42 to 0.94)*	1.1 ± 0.2	6
ρ1 WT	0.88 (0.69 to 1.11)	1.7 ± 0.2	6
ρ1(**I15'S**)	0.27 (0.23 to 0.32)****	2.8 ± 0.4	6
ρ1(**T6'Y,I25'S**)	0.33 (0.29 to 0.37)****	2.2 ± 0.2	6

Table 2

Summary of alcohol effects on EC_{20} GABA responses of mutant ρ1 receptors. Symbols: ↑, potentiation; ↓, inhibition; —, no effect. Numbers indicate the percent change of the EC_{20} GABA responses in the presence of alcohol (+, potentiation; −, inhibition). The colored text corresponds to the color of the amino acids in the model representations; WT, wild-type. The values for ethanol and hexanol effects correspond to the average of all WT receptors in each group. *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.000 1 versus WT expressed in oocytes from the same batch as each mutant, as shown in the figures. Analyzed using Two-Way ANOVA with Sidak's multiple comparisons post-hoc test.

Receptor	Ethanol	n	Hexanol	n
ρ1 WT	↓ −42 ± 1 %	26	↓ −27 ± 3 %	21
ρ1(**T6'Y**)	↑↑ 12 ± 2 % ****	7	↑↑ 4 ± 1 % ****	4
ρ1(**T6'F**)	↑↑ 14 ± 4 % ****	6	↑↑ 13 ± 5 % ***	6
ρ1(**T6'V**)	↓↓ −61 ± 2 % ****	6	↓↓ −96 ± 1 % ****	6
ρ1(**T6'M**)	↓ −35 ± 2 %	4	↓↓ −39 ± 1 % *	6
ρ1 WT	↓ −43 ± 1 %	19	↓ −22 ± 2 %	18
ρ1(**I5'A**)	↓ −38 ± 3 %	7	↓↓ −33 ± 1 % ****	7
ρ1(**I5'V**)	↓ −50 ± 4 %	4	↓ −36 ± 1 %	4
ρ1(**I5'F**)	↓ −40 ± 3 %	4	↓↓ −37 ± 2 % **	4
ρ1(**I5'W**)	↓↓ −57 ± 2 % **	5	↓↓ −52 ± 2 % ****	5

Table 3

Summary of alcohol effects on EC_{20} GABA responses of mutant ρ1 receptors. Symbols: ↑, potentiation; ↓, inhibition; —, no effect. Numbers indicate the percent change of the EC_{20} GABA responses in the presence of alcohol (+, potentiation; −, inhibition). The colored text corresponds to the color of the amino acids in the model representations; WT, wild-type.

Receptor	Ethanol	Propanol	Butanol	Pentanol	Hexanol	Heptanol	Octanol
ρ1 WT	↓ −55%	↓↓ −83%	↓↓ −70%	↓↓ −48%	↓ −23%	— −10%	— −10%
ρ1(T6'Y)	↑ +19%	↑ +43%	↑ +18%	— +12%	— +4%	— −12%	— −11%
ρ1(I15'S)	↓ −27%	↓ −29%	— −3%	— +14%	↑ +22%	↑ +53%	↑ +46%
ρ1(T6'Y,I15'S)	↑ +32%	↑↑ +133%	↑↑ +116%	↑↑ +146%	↑↑ +121%	↑↑ +70%	↑ +17%