

Identification of an Inhibitory Alcohol Binding Site in GABA_A ρ 1 Receptors

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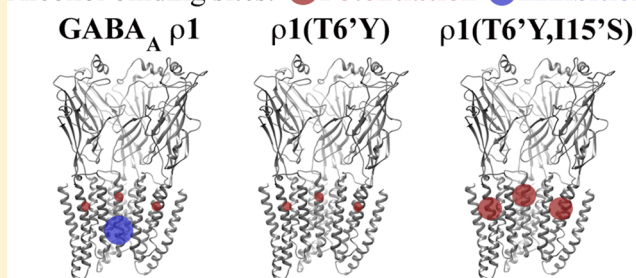
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S Supporting Information

ABSTRACT: Alcohols inhibit γ -aminobutyric acid type A ρ 1 receptor function. After introducing mutations in several positions of the second transmembrane helix in ρ 1, 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 ρ 1 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 ρ 1 receptors [ρ 1(I15'S), ρ 1(T6'Y), and ρ 1(T6'Y,I15'S)] to further characterize the inhibitory alcohol pocket in the wild-type ρ 1 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 ρ 1 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

Alcohol binding sites: ● Potentiation ● Inhibition



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: α 1–6, β 1–3, γ 1–3, δ , ϵ , π , θ and ρ 1–3.¹ 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 ρ 1 and S270 and A291 in α 1 subunits. GABA ρ 1 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 ρ 1 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 ρ 1 expression in nucleus accumbens with ethanol consumption and motor activation was found using BxD recombinant inbred mice [$r=0.77$, ethanol preference¹⁰ and $r=-0.48$, ethanol-induced motor response,¹¹ from genenetwork.org]. Furthermore, single nucleotide polymorphisms (SNPs) in the human genes that encode ρ 1 and ρ 2 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 ρ 1 receptors.¹⁵ Single mutants, ρ 1(I307S) and ρ 1(W328A), and the double mutant ρ 1(I307S,W328A) were potentiated by long-chain alcohols, but

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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 $\alpha 1$ TM2 in $\alpha 1\beta 2\gamma 2$ GABA_A receptors¹⁷ and was described in $\alpha\beta$ and $\alpha\beta\gamma$ 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 GluCl²¹ and a homomeric GABA_A receptor²² 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 $\rho 1$ 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 $\rho 1$ receptors, based on the wild-type GluCl, which has been identified as the best template to model the $\rho 1$ receptor out of five different X-ray crystal structures.²⁵

RESULTS AND DISCUSSION

Our approach to identify the inhibitory ethanol binding site in $\rho 1$ 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 Figure 1. The 6' position is occupied by a threonine in the wild-type receptor. In the model, this residue leans toward the 5' position in TM2 of the neighboring subunit (7.5 Å *C α -C α* distance), which is essentially facing toward 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 *C α -C α* 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 *C α -C α* distance). The leucine in the 3' position (TM2) and L277 (TM1) are both facing toward the interface with the neighboring subunit and proximal to I5' (7.1 and 8.7 Å *C α -C α* distance, respectively). Recently, a crystal structure of human $\alpha 3$ glycine receptor bound to strychnine was described²⁶ and the zebrafish $\alpha 1$ glycine receptor was studied using electron cryo-microscopy.²⁷ In both cases, the

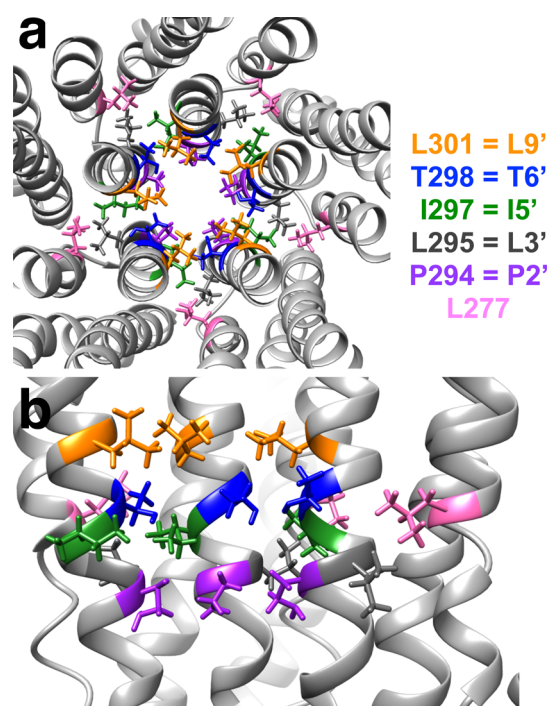


Figure 1. GABA_A $\rho 1$ receptor homology model based on GluCl (PDB ID: 3RHW). The second TM helix of each subunit lines the pore of the channel. The amino acids that were mutated are shown as color-coded sticks. All amino acids in the TM2 at the top of panel (A) and the center of panel (B) are shown 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.

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 $\rho 1$ 6' mutant receptors can be found in Supporting Information Figure 1, and the corresponding parameters are in Table 1. Only the T6'F and T6'V mutations significantly changed the GABA sensitivity of the $\rho 1$ receptors.

Increasing the residue volume in the 6' position drastically changed the alcohol effect on GABA-mediated currents in $\rho 1$ receptors (representative tracings can be found in Figure 2). Instead of inhibition, significant potentiation by both ethanol and hexanol was observed in mutants T6'Y and T6'F (Figure 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 (Figure 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 (Figure 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 (Figure 3e). In the molecular model of this mutant, the small 6' valine residues are lining the pore (Figure 3f), similar to the 6' threonine residues (Figure 1a). Replacing T6' with a methionine residue produced small or no changes in alcohol effects compared with wild-type receptors (Figure 3g). The M6' residues also line the pore in the molecular model (Figure 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 $\rho 1$ (T6'Y), we tested

Table 1. GABA Concentration–Response Curve Parameters^a

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'V,I25'S)	0.33 (0.29 to 0.37)****	2.2 ± 0.2	6

^aEC₅₀ 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 ± SEM; n is the number of oocytes per receptor. *p < 0.05, ***p < 0.001, ****p < 0.0001 versus WT for log EC₅₀ based on nonlinear regression analysis. The colored text corresponds to the color of the amino acids in the model representations; WT, wild-type.

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 EC₂₀ 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 (r² = 0.79, p < 0.05, Supporting Information (SI) Figure 2a), but it did not reach significance for hexanol (r² = 0.50, p = 0.18, SI Figure 2b). In contrast, there was no correlation between the alcohol effect and the hydrophobicity (r² = 0.24, SI Figure 2c and r² = 0.36, SI Figure 2d). The ethanol effect had no linear correlation with the GABA EC₅₀ for any of the 6' mutants (r² = 0.52, SI Figure 2d), but the hexanol effect was correlated with the GABA EC₅₀ (r² = 0.81, p = 0.03, SI Figure 2f). However, this correlation is not meaningful, as it

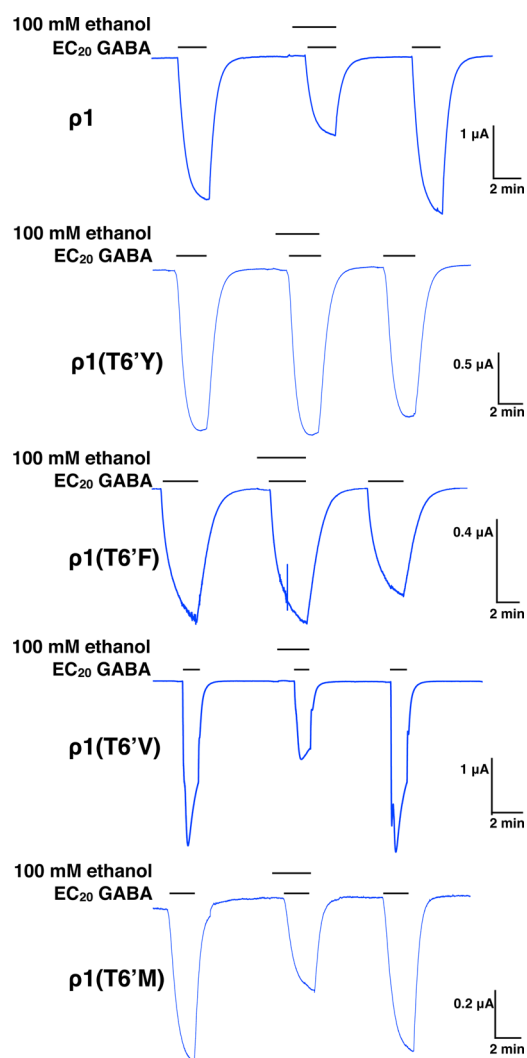


Figure 2. Representative tracings of GABA responses in the presence of ethanol in ρ1T6' mutants expressed in *Xenopus laevis* oocytes.

heavily depends on the GABA EC₅₀ 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 EC₅₀) 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 S' mutant receptors are shown in SI Figure 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 (Figure 4a, c, e, and g). In contrast, almost all substitutions significantly increased the hexanol inhibition (Figure 4a, c, e,

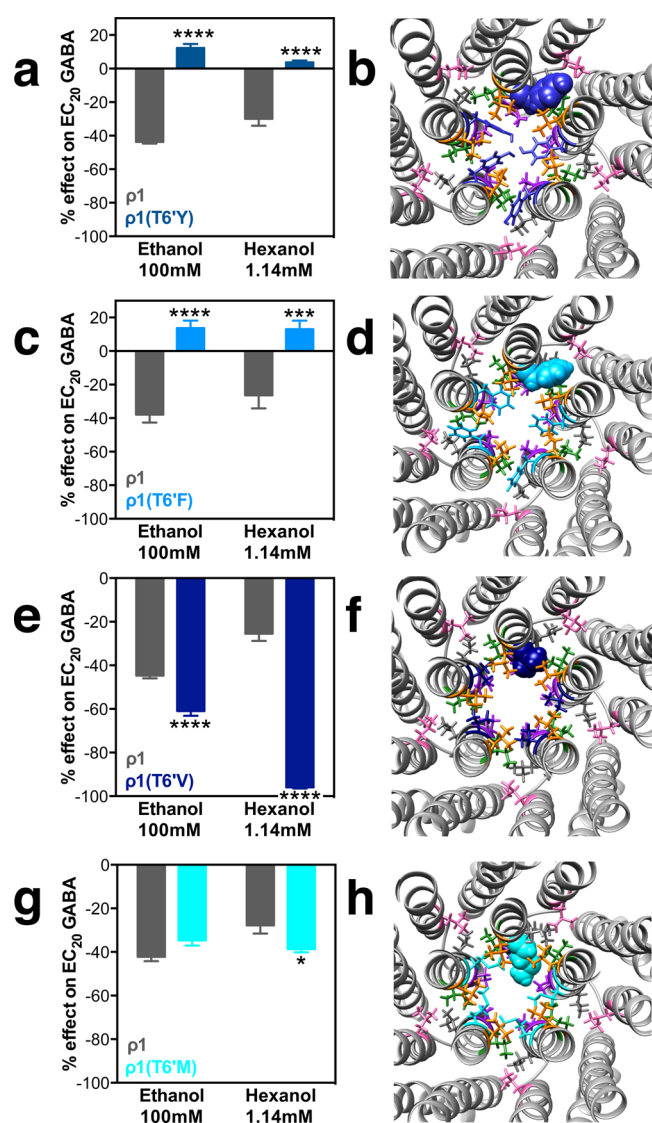


Figure 3. $\rho 1T6'$ mutants: alcohol effects and molecular models. Left panels show ethanol (100 mM) and hexanol (1.14 mM) effects on EC_{20} GABA responses. Right panels are molecular models of the pore and TM domains of the corresponding $\rho 1$ mutant, viewed from the plane between the extracellular and the TM domains. The graph bars and the corresponding amino acid in the model are the same blue color. (a, b) T6'Y; (c, d) T6'F; (e, f) T6'V; (g, h) T6'M. Bars represent means \pm SEM, $n = 4$ –9. Two-way ANOVA, Sidak's multiple comparisons test: $p < 0.05$, $***p \leq 0.001$, $****p \leq 0.0001$.

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 (Figure 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 ($r^2 = 0.45$, SI Figure 4a) or hexanol ($r^2 = 0.35$, SI Figure 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

Table 2. Summary of Alcohol Effects on EC_{20} GABA Responses of Mutant P1 Receptors^a

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

^aSymbols: ↑, 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ 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.

($r^2 = 0.41$, SI Figure 4c) but a positive linear correlation was established for hexanol ($r^2 = 0.85$, $p < 0.05$, SI Figure 4d). In addition, there was no correlation between the alcohol effects and the GABA EC_{50} for each of the 5' mutants ($r^2 = 0.34$, SI Figure 4e and $r^2 = 0.00$, SI Figure 4f).

The GABA concentration–response curves for the neighboring mutations in $\rho 1$ receptors are shown in SI Figure 5, and the corresponding parameters can be found in Table 1. All the mutations modified the $\rho 1$ receptor GABA sensitivity.

Some of the amino acids near the 5' and 6' positions also affected alcohol action on $\rho 1$ 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 open²⁸), but the effect of hexanol was not changed (Figure 5a). In the molecular model, all 9'F residues were lining the pore (Figure 5b). The L3'F mutant showed a decrease both in ethanol and hexanol inhibition (Figure 5c). All 3'F residues were located in the center of the four α -helix bundle within a single subunit (Figure 5d). Lastly, there were two mutants that did not change either ethanol or hexanol inhibition: $\rho 1(L277F)$ and P2'G (Figure 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, Figure 1a) and the mutant (phenylalanine, Figure 5f). The P2'G mutant lacks a side chain, but it remains lining the pore (Figure 5h).

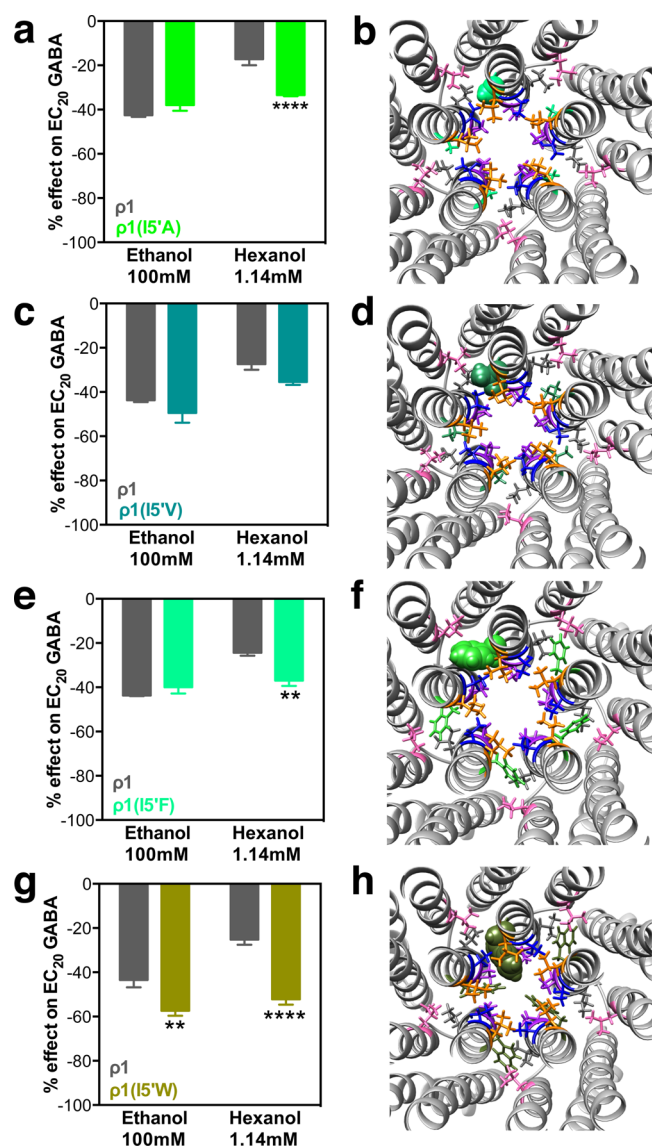


Figure 4. $\rho 1I5'$ mutants: alcohol effects and molecular models. Left panels show ethanol (100 mM) and hexanol (1.14 mM) effects on EC₂₀ GABA responses. Right panels are molecular models of the pore and TM domains of the corresponding $\rho 1$ mutant, viewed from the plane between the extracellular and the TM domains. The graph bars and the corresponding amino acid in the model are the same green color. (a, b) I5'A; (c, d), I5'V; (e, f), I5'F; (g, h), I5'W. Bars represent means \pm SEM, $n = 4-9$. Two-way ANOVA, Sidak's multiple comparisons test: * $p \leq 0.01$, **** $p \leq 0.0001$.

In summary, neighboring amino acids also participate in alcohol action sites, and mutation of $\rho 1L9'$ decreased ethanol but not hexanol inhibition. Mutation of $\rho 1I5'$ 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' [$\rho 1(L277F)$ in TM1]. Several of the residues studied (2', 6', and 9') line the pore of the channel in the $\rho 1$ receptor, and had been shown to have an important role in receptor function and pharmacology. For instance, residues at the 6' and 9' positions

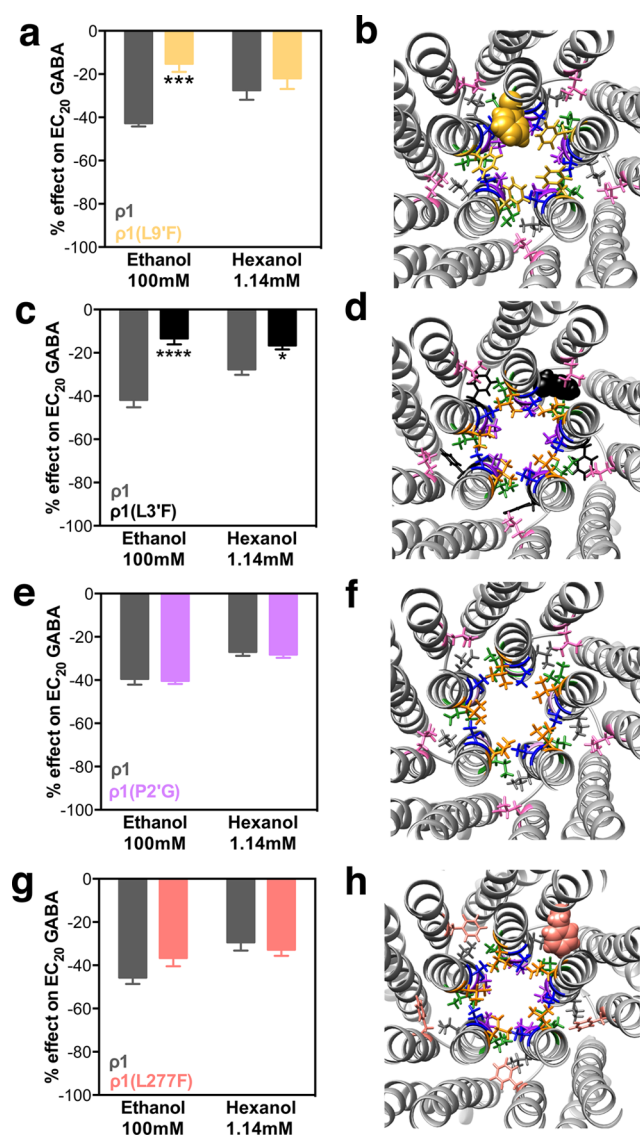


Figure 5. Neighboring $\rho 1$ mutants in TM2 and TM1: alcohol effects and molecular models. Left panels show ethanol (100 mM) and hexanol (1.14 mM) effects on EC₂₀ GABA responses. Right panels are molecular models of the pore and TM domains of the corresponding $\rho 1$ mutant, viewed from the plane between the extracellular and the TM domains. The graph bars and the corresponding amino acid in the model are the same color. (a, b), L9'F; (c, d), L3'F; (e, f), P2'G; (g, h), L277F. Bars represent means \pm SEM, $n = 3-5$. Two-way ANOVA, Sidak's multiple comparisons test: * $p < 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

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 $\rho 1$ 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 nonspecific 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 $\rho 1$ 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 $\rho 1$ 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' (Figure 6a and b show T6' and I15' in the wild-type $\rho 1$,

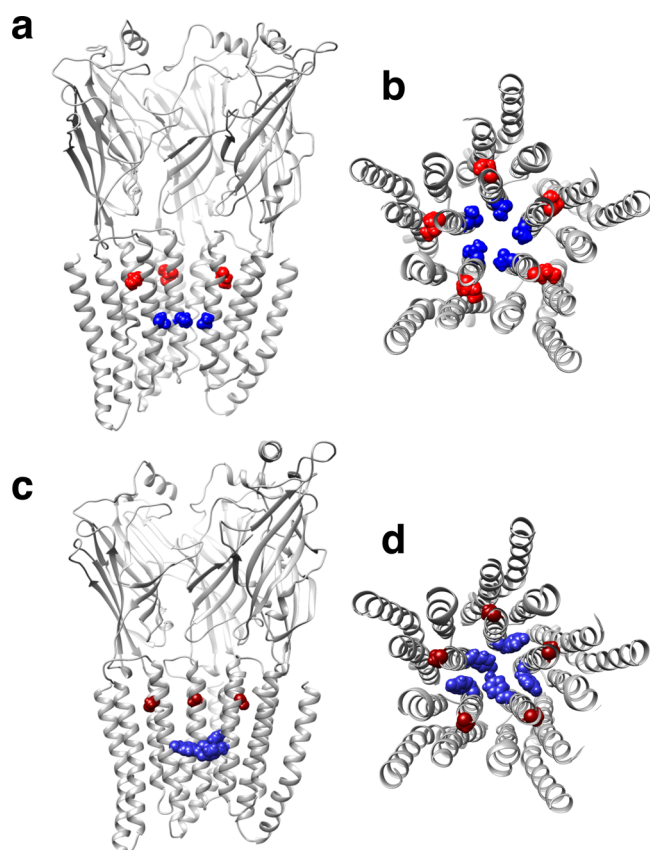


Figure 6. 6' (blue) and 15' (red) positions in molecular models of the $\rho 1$ receptor. View of three subunits from the pore for wild-type $\rho 1$ (a) and $\rho 1$ (T6'Y,I15'S) (c). Extracellular view of the pore from the plane between the extracellular and the TM domains for wild-type $\rho 1$ (b) and $\rho 1$ (T6'Y,I15'S) (d).

and Figure 6c and d show Y6' and S15' in the $\rho 1$ double mutant). Four different $\rho 1$ 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 SI Figure 6, and the corresponding parameters in Table 1.

The alcohol effect on EC₂₀ GABA responses was measured in these four receptors (Figure 7). The wild-type GABA_A $\rho 1$ receptor was inhibited by alcohols containing up to six carbons. The $\rho 1$ (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 $\rho 1$ (T6'Y) mutant showed moderate potentiation by ethanol through pentanol; hexanol had no effect, and heptanol and octanol produced small inhibition. The double mutant $\rho 1$ (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 studies¹⁶ have shown that if the residue in the 15' position is large (e.g., isoleucine in the wild-type), all alcohols inhibit the $\rho 1$ receptor; in contrast, if the residue is small (e.g., serine, as in the $\alpha 1$ GABA_A and Gly receptor subunits), ethanol inhibits but longer chain alcohols potentiate the GABA responses in the $\rho 1$ mutant. Considering those results together

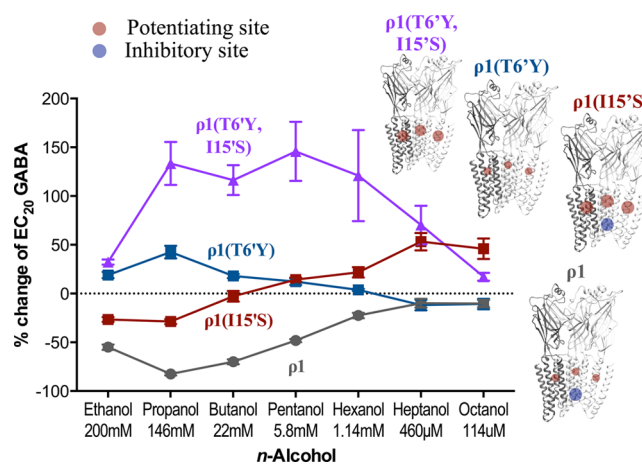


Figure 7. Alcohol effect on single mutants, $\rho 1$ (T6'Y) and $\rho 1$ (I15'S), and double mutant $\rho 1$ (T6'Y,I15'S). The circles in the molecular models serve only as a visual representation of the approximate position and relative size of the potentiating (red circle) and inhibitory (blue circle) alcohol sites.

Table 3. Summary of Alcohol Effects on EC₂₀ GABA Responses of Mutant $\rho 1$ Receptors^a

Receptor	Ethanol	Propanol	Butanol	Pentanol	Hexanol	Heptanol	Octanol
$\rho 1$ WT	↓ -55%	↓↓ -83%	↓↓ -70%	↓↓ -48%	↓ -23%	—	—
$\rho 1$ (T6'Y)	↑ +19%	↑ +43%	↑ +18%	—	—	—	—
$\rho 1$ (I15'S)	↓ -27%	↓ -29%	—	—	↑ +22%	↑ +53%	↑ +46%
$\rho 1$ (T6'Y,I15'S)	↑ +32%	↑↑ +133%	↑↑ +116%	↑↑ +146%	↑↑ +121%	↑↑ +70%	↑ +17%

^aSymbols: ↑, potentiation; ↓, inhibition; —, no effect. Numbers indicate the percent change of the EC₂₀ 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.

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 $\rho 1$ 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 $\rho 1$ (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 $\rho 1$ (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 $\rho 1(T6'Y, I15'S)$. 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, and so forth; 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 fluorophores⁴³ and cross-linking 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 Cocrystallization 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 $\rho 1$ 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 $\rho 1$ subunits that either lack ethanol inhibition or display ethanol potentiation, enabling behavioral studies of the role of ethanol on $\rho 1$ function in vivo.

METHODS

Clones. Mutations in the $\rho 1$ cDNAs were made through site-directed mutagenesis using QuikChange (Agilent Technologies, Santa Clara, CA).

Transcription and Oocyte Injection. The in vitro transcription of wild-type and mutant $\rho 1$ subunits was performed using mMessage

mMachine (Life Technologies, Grand Island, NY). 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 $\rho 1$ 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 $\rho 1$ 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 CaCl₂, 1 mM MgCl₂, 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 μ M), and responses were expressed as percentages of the maximal current.

Alcohol Application Protocol. Alcohols were first preapplied for 1 min and then coapplied with GABA. The application sequence for the wild-type and mutants $\rho 1$ receptors was as follows: maximal GABA (to obtain maximal response, EC₁₀₀), EC₂₀ GABA, EC₂₀ GABA, preapplication of ethanol (100 mM) immediately followed by a coapplication with EC₂₀ GABA, EC₂₀ GABA, preapplication of hexanol (1.14 mM) immediately followed by a coapplication with EC₂₀ GABA, EC₂₀ 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 EC₅ to assess alcohol effects, as the alcohol effect is larger in the presence of lower EC values. However, we used EC₂₀ 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). Pooled data are represented as mean \pm SEM. 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:

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

where I/I_{\max} is the fraction of the maximally obtained GABA response, EC₅₀ (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 EC₅₀ GABA (determined in this study, Table 1).

Molecular Models. Homology models of $\rho 1$ 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 3RHW²¹). The Modeler module of Discovery Studio 4.1 (DS 4.1Biovia, San Diego, CA) 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/Å² and optimized the resulting models to a gradient of 0.001 kcal/(mol \times 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

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscemneur-0.5b00246.

Figure 1: GABA concentration–response curves for $\rho 1T6'$ mutants. Figure 2: Linear correlation between alcohol effects on $\rho 1T6'$ mutants and several variables. Figure 3: GABA concentration–response curves for $\rho 1I5'$ mutants. Figure 4: Linear correlation between alcohol effects on $\rho 1I5'$ mutants and several variables. Figure 5: GABA concentration–response curves for neighboring $\rho 1$ mutants in TM2 and TM1. Figure 6: GABA concentration–response curves for $\rho 1T6'Y$ and/or $I15'S$ mutants. (PDF)

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Author Contributions

R.A.H., J.R.T., and C.M.B. contributed to the study conception and design. C.M.B., C.I.R., U.S.L., and M.A.C. carried out the experimental procedures. J.R.T. and E.J.B. carried out the molecular modeling; C.M.B., E.J.B., R.A.H., and J.R.T. contributed to the interpretation of data and writing of the manuscript.

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Notes

The authors declare no competing financial interest.

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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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