Occupancy of a Single Anesthetic Binding Pocket Is Sufficient to Enhance Glycine Receptor Function

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Alcohols and volatile anesthetics enhance the function of inhibitory glycine receptors (GlyRs). This is hypothesized to occur by their binding to a pocket formed between the transmembrane domains of individual α1 GlyR subunits. Because GlyRs are pentameric, it follows that each GlyR contains up to five alcohol/anesthetic binding sites, with one in each subunit. We asked how many subunits per pentamer need be bound by drug in order to enhance receptor-mediated currents. A cysteine mutation was introduced at amino acid serine 267 (S267C) in the transmembrane 2 domain as a tool to block GlyR potentiation by some anesthetic drugs and to provide a means for covalent binding by the small, anesthetic-like thiol reagent propyl methanethiosulfonate. *Xenopus laevis* oocytes were co-injected with various ratios of wild-type (wt) to S267C α1 GlyR cDNAs in order to express heteromeric receptors with a range of wt:mutant subunit stoichiometries. The enhancement of GlyR currents by 200 mM ethanol and 1.5 mM chloroform was positively correlated with the number of wt subunits found in heteromeric receptors. Furthermore, currents from oocytes injected with high ratios of wt to S267C cDNAs (up to 200:1) were significantly and irreversibly enhanced following propyl methanethiosulfonate labeling and washout, demonstrating that drug binding to a single subunit in the receptor pentamer is sufficient to induce enhancement of GlyR currents.

Although volatile anesthetics were long believed to have nonspecific, lipid-based mechanisms of action, accumulating evidence led to a shift in research focus to the study of protein sites of anesthetic action and especially to the effects of these drugs on ion channels (1, 2). Among the most studied molecular targets for these drugs are members of the Cys-loop family of ligand-gated ion channels, including the glycine receptor (GlyR), the primary inhibitory neurotransmitter receptor in the spinal cord and brain stem (3). Studies in rats have shown that GlyRs mediate the immobilizing effects of the anesthetics halothane, isoflurane, and cyclopropane (4). In transgenic mice expressing an alcohol-insensitive mutant GlyR, these receptors mediate, at least in part, the sedating and anesthetic effects of alcohol (5). These *in vivo* data agree with functional studies indicating that pharmacologically relevant concentrations of alcohols and volatile anesthetics potentiate GlyRs in both brain slices and heterologous expression systems (6–9).

Significant advances have been made in the understanding of the molecular mechanisms of alcohol and volatile anesthetic enhancement of GlyR function. The GlyR consists of a pentameric assembly of subunits surrounding a central, anion-conducting pore (10). Each of these subunits contains a large, N-terminal extracellular domain responsible for agonist binding, as well as four transmembrane (TM) domains, of which TM2 lines the channel pore and forms the channel gate (11). Mihic et al. (12) identified two amino acids, serine 267 (Ser-267) in TM2 and alanine 288 (Ala-288) in TM3, that, when mutated, blocked alcohol and anesthetic enhancement of GlyR-mediated currents. Alcohols and anesthetics were hypothesized to potentiate GlyRs by binding in a water-filled pocket formed between TM2 and TM3. A subsequent study supported this hypothesis by showing that thiol reagents could covalently react with cysteine residues at position 267 (S267C) to irreversibly enhance receptor function (13). Thiols, like volatile anesthetics, may enhance GlyR function by occupying space within the water-filled pocket. Interestingly, recent structural data for the nicotinic acetylcholine receptor, another Cys-loop family member, indicates that there is a water-filled crevice located between the transmembrane domains of each receptor subunit (14).

Each of the five subunits in a homomeric GlyR possesses a possible TM2/TM3 anesthetic binding site. In previous studies of binding pocket mutants, the mutant subunits were expressed without wt subunits so that all five subunits in receptors bore the mutation; as a result it was unclear how many subunits had to be bound by anesthetics for their enhancing effects to be observed. In the present study, we tested the hypothesis that occupancy of a single anesthetic binding pocket per receptor pentamer is sufficient to potentiate GlyR currents. By co-injecting different ratios of the S267C mutant cDNA with wild-type α1 GlyR subunit cDNAs in *Xenopus laevis* oocytes, we generated heteromeric receptors containing one to four mutant subunits and then tested the effects of alcohols, anesthetics, and the thiol labeling reagent propyl methanethiosulfonate (PMTS) on these receptors. Our results suggest for the first time that occupancy of even a single alcohol or anesthetic binding pocket in a GlyR pentamer can result in enhancement of GlyR function.

**MATERIALS AND METHODS**

Two-electrode voltage clamp experiments were conducted on *X. laevis* oocytes expressing α1 GlyR subunits. Frogs (*Xenopus Express*) were maintained and oocytes harvested and isolated as described previously (15). A cDNA encoding the wild-type (wt) α1 GlyR subunit was previously subcloned into the pBK-CMV expression vector (12). Using the QuikChange mutagenesis kit (Stratagene), the codon for serine 267 was mutated to a cysteine codon (S267C) or a methionine codon (S267M) and the codon for proline 250 was mutated to a threonine codon (P250T). cDNA concentrations and purities were ascertained through UV absorption spectra on a DU 640 spectrophotometer (Beckman). Wild-type, S267C, S267M, and P250T cDNAs were injected into the
nuclei of isolated oocytes, either individually to express homomeric GlyRs or in a range of wt:S267C, S267M:S267C, or P250T:S267C cDNA ratios so as to promote the expression of heteromeric GlyRs. Assuming that wt and S267C subunits are equally likely to assemble into receptor pentamers, the predicted distribution of receptor compositions can be described by the formula shown in Equation 1

\[ P = \frac{100 \times p_{wt}^X \times p_{S267C}^{(5-X)} \times 5!}{X!(5-X)!} \]  

(Eq. 1)

where \( P \) represents the percentage of receptors containing \( x \) wt subunits and \((5-x)\) S267C subunits, \( p_{wt} \) represents the fraction of wt cDNAs injected, and \( p_{S267C} \) represents the fraction of S267C cDNAs injected. Calculated receptor composition percentage distributions for the cDNA ratios tested are shown in Table 1.

Recordings were made 1–7 days after cDNA injection. Oocytes were perfused with modified Barth’s solution, impaled with two electrodes filled with 3 M KCl, and voltage clamped at −70 mV. Glycine and drug solutions were prepared in modified Barth’s solution at the indicated concentrations and were applied through the bath perfusion. At the beginning of each experiment, maximal GlyR currents were determined by a 30-s application of 10 mM glycine. All subsequent glycine and glycine + drug solutions were applied for 45 s and were followed by 10-min modified Barth’s solution washouts to ensure receptor recovery from desensitization. In most experiments, the concentration of glycine producing roughly a 10% maximal effect (EC_{10}) was determined next, and the oocyte was considered suitable for recording if the EC_{10} current had lower resistances (3–6 MΩ) similar to single-channel recordings except that the patch electrodes described previously (17). Outside-out macropatch recordings were conducted as single-channel recordings from outside-out patches were conducted as cals, and PMTS (Toronto Research Chemicals). All chemicals were obtained from Sigma-Aldrich except for isoflurane, enflurane, and halothane on glycine-gated currents in oocytes expressing either homomeric wt α1 GlyRs or homomeric S267C α1 GlyRs. As expected, all these compounds produced significant potentiation of wt GlyR currents when co-applied with EC_{10} glycine (Fig. 1, A–C). In oocytes expressing the mutant receptor, the S267C mutation blocked GlyR potentiation by 200 mM ethanol and changed the strong potentiation effect of 1.5 mM chloroform into a slight inhibition (Fig. 1, A and B). S267C had no significant effect on receptor potentiation by 25 mM decanol, 0.6 mM isoflurane, 1.2 mM enflurane, and 0.5 mM halothane (Fig. 1, A–C).

We hypothesized that varying the number of mutant subunits/GlyR would reveal a graded correlation between receptor composition and the degree of receptor potentiation by ethanol and chloroform. To express heteromeric GlyRs, oocytes were injected with mixtures of wt and S267C cDNAs prepared in ratios of 4:1, 1:1, and 1:4 (wt:S267C). Given the reasonable assumptions (addressed under “Discussion”) that wt and S267C cDNAs yield receptor subunits equally well and that the S267C mutation has little or no effect on pentamer assembly, these cDNA combinations should give rise to receptors with an average of one, two to three, and four mutant subunits, respectively. Table 1 provides calculated distributions of receptor subunit compositions for each of the cDNA ratios tested. In these oocytes, receptor enhancement by 200 mM ethanol and 1.5 mM chloroform decreased as the proportion of S267C subunits increased (Fig. 1D). For ethanol, GlyR potentiation was no longer apparent when an average of two-three of the receptor subunits contained mutant binding pockets, whereas for chloroform, potentiation was evident until an average of four subunits bore the mutant binding pocket. As with the homomeric receptors, potentiation of the heteromeric receptors by 25 mM decanol, 0.6 mM isoflurane, 1.2 mM enflurane, and 0.5 mM halothane did not depend on receptor subunit makeup, indicating that the heteromeric receptors retain the ability to be potentiated by alcohols and anesthetics (Fig. 1D).

To more directly assess how many binding pockets must be occupied to induce receptor potentiation, we covalently modified the anesthetic binding pocket. PMTS is a small, uncharged molecule that reacts with the thiol group of cysteine residues to attach a propyl group through a disulfide bond. It was previously demonstrated that perfusion of homomeric S267C mutant GlyRs with PMTS results in labeling of the mutant cysteine residue and permanent potentiation of receptor function after PMTS washout (13). However, before using this approach to examine binding pocket occupancy, we showed that 5 mM PMTS enhances GlyR function like alcohols or anesthetics when directly co-applied to wt and S267C GlyRs with an EC_{10} concentration of glycine (Fig. 2). After a 10-min washout, the wt response to EC_{10} glycine was slightly decreased from baseline, indicating that PMTS interacts reversibly with these receptors. In contrast, the S267C response remained significantly potentiated after PMTS washout, indicating that PMTS covalently labels the mutant cysteine residue in S267C receptors such that the binding pocket remains permanently occupied by the propyl group attached to the mutant cysteine.

We next asked whether PMTS labeling of a single mutant cysteine residue in receptor heteromers containing four wt subunits and one S267C subunit would lead to receptor enhancement. To express receptors containing one mutant subunit, oocytes were injected with cDNAs in the ratio of 30:1 wt:S267C. Based on the calculated values shown in
Table 1, this ratio should give rise to a pool of receptors in which 85% are homomeric wt and the remaining 15% are heteromeric. Of the heteromeric receptors, 94% were calculated to contain only one mutant subunit and the remaining 6% to contain two S267C subunits. For each oocyte, an EC10 concentration of glycine was established, 50 μM PMTS was perfused for 220 s, and the response to EC10 glycine was measured 5 min later (Fig. 3, A and B). Currents from oocytes injected with the 30:1 ratio of wt:S267C cDNAs were significantly potentiated following PMTS application and washout, as were currents from oocytes injected only with S267C GlyR cDNAs. In contrast, currents from wt cDNA-injected oocytes were not enhanced by the treatment.

Because PMTS labeling of the 30:1 wt:S267C-injected oocytes yielded a sizable potentiation, we hypothesized that we could further decrease the proportion of S267C cDNAs injected and still observe a significant potentiation of GlyR function after PMTS labeling. By reducing the proportion of S267C subunits in the overall subunit pool, the chances of more than one mutant subunit assembling into a receptor pentamer would be minimized. To this end, oocytes were injected with increasing ratios of wt:S267C subunit cDNAs (50:1, 100:1, and 200:1), and, as above, the response to EC10 glycine was determined before and after 50 μM PMTS application. Confirming the hypothesis, potentiation was observed for each cDNA ratio, and the averaged data revealed a linear correlation between the predicted percentage of heteromeric receptors relative to total receptors and the degree of receptor enhancement (Fig. 3C). This correlation strongly supports the overall hypothesis that occupancy of a single alcohol and anesthetic binding pocket/receptor pentamer is sufficient to induce receptor potentiation.

Because drug binding to one subunit/receptor pentamer was sufficient to enhance receptor currents, we tested the hypothesis that the remaining subunits not binding drug could modify the enhancing effects of the drug bound to the first subunit. To accomplish this, we utilized a serine to methionine mutation at position 267 (S267M) that cannot covalently react with PMTS. S267M GlyRs do not display potentiation by most alcohols and volatile anesthetics when expressed homomerically (data not shown). Oocytes were injected with 100:1 and 30:1 ratios of S267M:S267C or wt:S267C GlyR subunit cDNAs, and current responses to EC10 glycine were determined before and after application of 50 μM PMTS (Fig. 4A). At both the 100:1 and 30:1 cDNA ratios, the S267M mutation significantly reduced the enhancing effects of PMTS when compared with currents from oocytes injected with wt:S267C cDNAs at the same ratios (Fig. 4B). These results suggest that anesthetic enhancement of GlyR function can be affected by subunits in the pentamer that themselves are not directly interacting with anesthetic.

To assess whether the S267C mutation significantly alters GlyR expression or function, we examined homomeric and heteromeric GlyRs in two assays. First, currents elicited from oocytes upon application of a maximal concentration of glycine (10 mM) were determined. The magnitudes of these responses provide a measure of the expression level and function of receptors (17). There was a small but significant

![Figure 1](image-url)
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FIGURE 2. PMTS acts like an alcohol or anesthetic on wt and S267C receptors. A, current tracings of representative wt and S267C homomeric GlyR responses to the application of 5 mM PMTS + EC₁₀ glycine and to EC₁₀ glycine 10 min later. B, PMTS enhanced wt and S267C GlyR currents when co-applied with glycine. After a 10-min washout, wt GlyR currents were slightly lower than before the PMTS application, whereas S267C GlyR currents remained enhanced due to covalent labeling of the mutant cysteine residue by PMTS. Data are mean ± S.E. of 5–6 oocytes. *, p < 0.05, t-test versus baseline.

FIGURE 3. Occupancy of a single binding site by PMTS induces receptor potentiation. A, representative tracings of currents elicited by EC₁₀ glycine 5 min before and 5 min after a 220-s application of 50 μM PMTS. Oocytes were injected with wt, S267C, or a 30:1 ratio of wt:S267C α1 GlyR cDNAs. B, PMTS application led to irreversible enhancement of currents from oocytes injected with a 30:1 ratio of wt:S267C cDNAs or with S267C cDNAs alone. Currents from oocytes injected with only wt cDNAs were not affected by 50 μM PMTS after the 5-min washout. C, oocytes were injected with 50:1, 100:1, and 200:1 ratios of wt:S267C cDNAs or with wt cDNAs alone. The change from baseline after PMTS labeling is reported versus the percentage of receptors predicted to contain at least one S267C subunit. Data are mean ± S.E. of 5–14 oocytes. *, p < 0.05, Tukey’s test versus wt (B), t-test with Bonferroni correction versus wt (C).

difference (<30%) between the maximal currents elicited by homomeric wt and S267C receptors (Fig. 5A). Additionally, maximal currents from oocytes injected with the wt:S267C cDNA ratios examined in this report did not differ significantly from wt or S267C homomeric receptors. Because the magnitude of maximal currents depends on the unitary conductance, activation and desensitization kinetics, and cell surface expression levels of receptors, we characterized wt and S267C receptor function in recordings from human embryonic kidney 293 cell macropatches and single-channel patches to determine whether the S267C mutation specifically affects any of these properties. Rapid perfusion macropatch studies showed that the activation time constants were <1 ms for both wt and S267C homomeric receptors and that the receptors did not differ in their rates of desensitization in response to a 1-s pulse of 10 mM glycine (data not shown). The single-channel conductance of wt α1 GlyR in response to application of 10 μM glycine was 103 pS, whereas for homomeric S267C α1 GlyR it was 54 pS. The observed difference in maximally evoked currents from wt and S267C homomers seen in oocytes (Fig. 5A) can likely be explained by the lower conductance of S267C receptors. Thus, if channel kinetics do not differ between wt and S267C receptors (i.e. individual wt and S267C receptors behave almost identically) and unitary conductance can be accounted for, the whole-oocyte maximal currents should be reflective of channel numbers on the cell surface. It is therefore likely that the ratios of subunit cDNAs injected accurately reflect the protein levels of subunits found on the cell surface.

Second, glycine concentration-response data were obtained from oocytes injected with wt or S267C cDNAs or a 30:1 mixture of wt:S267C cDNAs (Fig. 5B). Fits of the Hill function indicate that the S267C mutation had only a small effect on glycine sensitivity in both heteromeric and homomeric mutant receptors. To further test whether the S267C mutation might somehow increase subunit expression levels, we adapted an approach developed by Breitinger and Becker (19). These investigators identified a GlyR α1 mutation (P250T) that results in receptors with significantly right-shifted glycine responses. When they mixed various ratios of wt to P250T cDNAs, they found that the glycine EC₅₀ of receptors increased as the proportion of P250T cDNAs transfected increased. We reasoned that if this P250T mutant were co-injected with the S267C mutant, which shows no evidence of a right shift, and if the S267C mutant were expressed at a higher level than we predicted, then one would expect P250T:S267C heteromers to display greater glycine sensitivity than P250T homomers. This was not the case (Fig. 5B).

DISCUSSION

Recent studies suggest that alcohols and anesthetics modulate GlyR function by binding to pockets formed between the transmembrane domains of individual GlyR subunits. Because GlyRs are pentameric,
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FIGURE 4. Receptor enhancement by PMTS labeling of one S267C subunit is decreased when the remaining four subunits are mutated from wt to S267M. A, representative tracings of currents elicited by EC\textsubscript{10} glycine 5 min before and 5 min after a 220-s application of 30 \mu M PMTS. Oocytes were injected with 100:1 and 30:1 ratios of wt:S267C or S267M:S267C wt GlyR cDNAs. B, the S267M mutation led to significantly less enhancement of receptor currents after PMTS labeling of S267M:S267C oocytes when compared with the PMTS enhancement of wt:S267C oocytes. Data are mean \pm S.E. of 6–8 oocytes. *, p < 0.05, t-test versus wt:S267C.

FIGURE 5. The S267C mutation does not significantly alter normal receptor function according to two measures. A, maximal currents, as determined by 30-s perfusion with 10 mM glycine, were largely unaffected by the S267C mutation. B, glycine concentration-response data from oocytes injected with wt, S267C, or P250T GlyR cDNAs alone or with a 30:1 wt:S267C ratio or 200:1 P250T:S267C ratio of cDNAs. Data were fit with the Hill function. The resulting EC\textsubscript{50} values were 230 \pm 16 \mu M (wt), 180 \pm 12 \mu M (30:1 wt:S267C), 200 \pm 19 \mu M (S267C), 840 \pm 65 \mu M (P250T), and 1430 \pm 140 \mu M (200:1 P250T:S267C). The Hill coefficients (n\textsubscript{H}) were 1.60 \pm 0.15 (wt), 1.64 \pm 0.15 (30:1 wt:S267C), 1.12 \pm 0.10 (S267C), 1.34 \pm 0.11 (P250T), and 1.08 \pm 0.09 (200:1 P250T:S267C). A two-way analysis of variance revealed that the P250T mutation significantly (p < 0.05) shifted glycine concentration-response curves to the right. No significant difference was detected between homomeric P250T and 200:1 P250T:S267C heteromeric receptors. Data are mean \pm S.E. of 8–93 oocytes (A) or 5 oocytes (B). *, p < 0.05, Tukey’s test versus wt.

Each GlyR necessarily contains five binding pockets. In the present study, we asked how many of these binding pockets must be occupied by drug in order to enhance GlyR currents. To examine this problem, GlyR subunits containing the S267C binding pocket mutation were co-expressed with wild-type subunits in various ratios to generate heteromeric receptors containing different numbers of mutant binding pockets. The properties of these mutant binding pockets were then used to investigate the modulatory effects of alcohols, anesthetics, and the thiol reagent PMTS on these receptors.

In homomeric mutant receptors, the S267C mutation blocked GlyR potentiation by 1.5 mM chloroform and 200 mM ethanol but did not block potentiation by 25 \mu M decanol, 1.2 mM enflurane, 0.5 mM halothane, and 0.6 mM isoflurane. These data indicate that S267C blocks the enhancing effects of the relatively small ligands chloroform and ethanol but not those of larger ligands. This may be because the higher volumes of larger anesthetics cause them to interact with more residues in the binding pocket than do ethanol and chloroform, thereby allowing them to avoid the effects of the mutant residue in the binding pocket. It should be noted that the concentration of ethanol used in this study (200 mM) represents an anesthetic concentration and is much higher than the ~17 mM blood alcohol concentration that defines the legal limit for alcohol intoxication. However, regulatory proteins, neuromodulators, and/or receptor phosphorylation may make the GlyR more sensitive to ethanol at concentrations closer to the legal limit (20, 21). When oocytes were injected with 4:1, 1:1, and 1:4 ratios of wt:S267C cDNAs, chloroform and ethanol potentiation of receptor currents decreased in a graded fashion as the proportion of S267C subunits increased. These data suggest that receptor currents could still be enhanced even when chloroform or ethanol could not productively interact with all possible binding pockets in the receptors. The low affinities of alcohols and anesthetics prevented us from using these drugs to more precisely determine the minimal number of binding pockets that needed to be occupied for enhancement of receptor function. Covalent labeling by PMTS, a molecule structurally related to alcohols and anesthetics that enhanced receptor currents just as an alcohol or anesthetic would, allowed us to circumvent this problem.

We reasoned that co-injecting oocytes with a high ratio of wt:S267C GlyR cDNAs would strongly favor the formation of homomeric wt GlyRs. A small percentage of receptors would be assembled into heteromers containing four wt subunits and one mutant subunit, and fewer still would contain multiple S267C subunits. In oocytes injected with a 30:1 ratio of wt:S267C cDNAs, 50 \mu M PMTS application, followed by a 5-min washout, resulted in >150% enhancement of GlyR currents. Extending this approach, we increased the ratio of wt:S267C cDNAs injected to 50:1, 100:1, and 200:1. Each of these cDNA ratios yielded receptors that were significantly and irreversibly potentiated by PMTS. At the 200:1 ratio, 97.5% of receptors were calculated to be composed exclusively of wt subunits, whereas the remaining receptors contained only a single S267C subunit and four wt subunits. Because PMTS had no permanent enhancing effect on homomeric wt GlyRs, the observed enhancement of receptor currents could only be attributed to PMTS occupancy of the single mutant binding pocket in heteromeric receptors containing one S267C subunit. Importantly, the degree of potentiation in these receptors was linearly correlated with the cDNA ratio tested, confirming our hypothesis that fewer S267C subunits were expressed when less S267C cDNA was injected.

Application of PMTS resulted in marked potentiation of GlyR function in receptors containing S267C subunits. For example, after injection of a 100:1 ratio of wt:S267C cDNAs, one would expect 5% of expressed receptors to contain a single S267C subunit and the remain-
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Anesthetic binding sites for halothane were identified in human serum albumin (24), two binding sites for bromoform in luciferase (25), and there is a single alcohol binding site in the Drosophila odorant-binding protein LUSH (26). Interestingly, in the case of LUSH it was found that the binding of a single alcohol molecule dramatically increased the conformational stability of the protein (26). In the case of the glycine receptor, whose alcohol binding pocket shares a motif with the LUSH alcohol binding pocket, there is therefore some precedent for the idea that the interaction of even a single anesthetic molecule at position 267 is sufficient to affect GlyR conformation and, by extension, GlyR function. This effect is perhaps due to the close proximity of Ser-267 to both the channel gate in TM2 and the TM2/TM3 loop that transduces agonist binding information to the gate (27). Our data do not indicate whether binding of additional drug molecules to a GlyR can lead to additional potentiation of receptor currents. It seems likely that this is the case. Single-channel studies indicate that binding of a single glycine molecule to one agonist binding site on a GlyR can lead to channel opening, whereas binding of additional glycine molecules increases both the likelihood and duration of opening events (28, 29). Thus, one binding event is sufficient to modify activity, but multiple binding events may do so more effectively. This conforms to allosteric theory, which posits that, due to the symmetry of receptors, conformational changes in one receptor subunit will induce concerted changes in the remaining subunits (30, 31).

Using the substituted cysteine accessibility method with homomeric cysteine mutants, Lobo et al. (22) recently found that the GlyR alcohol and anesthetic binding pocket undergoes a conformational change upon receptor gating to the open state. This suggests that alcohol or anesthetic occupancy of the binding pocket promotes the open state by favoring an enlarged pocket conformation. Based on these studies and our current results, we propose that the binding of one alcohol or anesthetic molecule to the TM2/TM3 binding pocket of a single GlyR subunit can induce receptor-wide conformational changes that lead to enhancement of channel gating.

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