Published in final edited form as:
Anesthesiology. 2011 September ; 115(3): 464–473. doi:10.1097/ALN.0b013e31822550f7.

A single phenylalanine residue in the main intracellular loop ofα1 γ-aminobutyric acid type A and glycine receptors influences their sensitivity to propofol

Gustavo Moraga-Cid, Ph.D[£], **Gonzalo E. Yevenes, Ph.D**[¥], **Günther Schmalzing, M.D**[¥], **Robert W. Peoples, Ph.D**[‡], and **Luis G. Aguayo, Ph.D**[§]
Department of Physiology, Faculty of Biological Sciences, University of Concepcion, Concepcion, Chile

Abstract

Background—The intravenous anaesthetic propofol acts as a positive allosteric modulator of glycine (GlyRs) and γ-aminobutyric acid type A ($GABA_A$Rs) receptors. Although the role of transmembrane residues is recognized, little is known about the involvement of other regions in the modulatory effects of propofol. Therefore, we explored the influence of the large intracellular loop (LIL) in propofol sensitivity of both receptors.

Methods—We screened the LIL of α_1 GlyRs and $\alpha_1\beta_2$ $GABA_A$Rs using alanine replacement. Sensitivity to propofol was studied using patch-clamp recording in HEK293 cells transiently transfected with WT (wild type) or mutant receptors.

Results—Alanine mutation of a conserved phenylalanine residue within the α_1 LIL significantly reduced propofol enhancement in both GlyRs (360±30 vs 75±10%, mean±SEM) and $GABA_A$Rs (361±49% vs 80±23%). Remarkably, propofol-hyposensitive mutant receptors retained their sensitivity to other allosteric modulators such as alcohols, etomidate, trichloroethanol and isoflurane. At the single channel level, the ability of propofol to increase open probability was significantly reduced in both α_1 GlyR (189±36 vs 22±13%) and $\alpha_1\beta_2$ $GABA_A$R (279±29 vs 29±11%) mutant receptors.

Conclusion—In this study, we demonstrate that the LIL of both GlyR and $GABA_A$R has a conserved single phenylalanine residue (F380 and F385, respectively) that influences its sensitivity to propofol. Our results suggest a new role of the LIL in the allosteric modulation of two members of the Cys-loop superfamily. Thus, these data provide new insights into the molecular framework behind the modulation of inhibitory ion channels by propofol.

INTRODUCTION

Glycine and γ-aminobutyric acid receptors (GlyRs and $GABA_A$Rs) mediate fast synaptic inhibition in the central nervous system[1,2]. These receptors are members of the Cys-loop receptor family, which share considerable structural and functional features[3,4]. As pentamers, they assemble around a central pore that transiently opens, allowing the passive

Corresponding author: Dr. Luis G. Aguayo, Department of Physiology, University of Concepción, Barrio Universitario s/n, P.O. Box 160-C, Concepción, Chile., Telephone: 56-41-2203380, Fax: 56-41-2245975, laguayo@udec.cl.
[£]Research Assistant, Department of Physiology, University of Concepcion, Concepción, Chile.
[¥]Research Assistant, Department of Physiology, University of Concepcion, Concepción, Chile.
[§]Professor, Department of Physiology, University of Concepcion, Concepción, Chile.
[¥]Professor, Department of Molecular Pharmacology, Rheinisch-Westfaelische Technische Hochschule (RWTH) Aachen, Germany
[‡]Associate Professor, Department of Biomedical Sciences, Marquette University, Milwaukee, WI, USA.

Presented at the Annual Meeting of the Society for Neuroscience, Washington, DC, Nov 12-19, 2008.

diffusion of anions[3,4]. Similar to other members of the family, their topology consists of an extracellular N-terminal domain, containing the binding site for the agonist, four transmembrane domains (TM1-TM4), of which TM2 is critical for pore formation, and a large intracellular loop connecting TM3 and TM4. To date, molecular cloning has identified five subunits of the GlyR (α_{1-4} and β) and nineteen subunits of the $GABA_AR$, with the GlyR$\alpha_1\beta$ and $GABA_AR$ $\alpha_1\beta_2\gamma_2$ combinations being predominant in the adult mammalian central nervous system[1,2].

$GABA_AR$ and GlyR play important roles in the actions of general anaesthetics, including propofol[5–9], which is widely used in intensive care units[10]. Previous studies indicated that these receptors contain sites important for propofol action[6,11,12]. For instance, residues in the TM domains in α_1 and $\beta_{2/3}$ subunits of the $GABA_AR$ were shown to be important for actions of anaesthetics, including propofol[13–21]. Experiments using a photoreactive analog of etomidate identified two residues (α_1M236 in M1 and β_2M286 in M3) as part of a binding pocket for this anaesthetic[22]. Additionally, based on the capacity of propofol to protect a sulfhydryl-specific reagent from reacting with a substituted cysteine, it was proposed that M286 in M3 served as an anaesthetic binding site in β_2[23]. A more recent study showed that binding of the photoreactive analog of etomidate to this site was either directly or allosterically inhibited by other general anaesthetics, suggesting complex intramolecular interactions[24]. In addition to binding sites in TM2/TM3 of α/β subunits in $GABA_A$ receptors, a tyrosine in TM4 (Y444) was found to influence the action of propofol, but not etomidate, on the receptor[14].

Studies in animal and molecular experimental models have shown that the sites of general anaesthetics on $GABA_AR$ and GlyR are somewhat overlapping for different chemical structures. For example, transgenic mice carrying propofol-insensitive $GABA_AR$ receptors (β_3N265M) also showed resistance to etomidate and exhibited substantial reductions in the modulatory actions of the volatile anaesthetic enflurane[19]. Similarly, it was reported that residues S267 and A288 of α_1 GlyRs[25], which previously had been reported as critical for modulation by alcohols and enflurane[26], also affected propofol sensitivity.

Although these previous studies have predicted that several residues might constitute a propofol binding pocket, the absence of high resolution structures of drug-receptor complexes for eukaryotic receptors has hindered a complete understanding of the molecular mechanisms underlying propofol actions. Molecular analysis based on homology modelling approaches showed that the implicated TM domain residues form a water-filled cavity that might be able to accommodate structurally unrelated molecules[16,27]. However, the structure and characteristics of these putative cavities remain unresolved[18,19,22–24]. For example, in cysteine cross-linking studies, propofol weakly protected the M286 residue, but was not able to protect the β_2N265C residue from modification by p-chloromercuribenzene sulfonate, implying that this residue does not contribute significantly to the binding site[23]. Moreover, the replacement of α_1N265 or β_2M286 with bulky hydrophobic residues promoted changes in channel gating and increased agonist potency, complicating the interpretation regarding the reduced propofol sensitivity[13,28].

All these studies are in agreement with the idea that residues in the TM domains are important for propofol actions in both $GABA_AR$ and GlyRs. However, little is known about the contribution of other receptor regions. In this regard, a very recent study has demonstrated that the LIL of the α_1 GlyR can influence the allosteric effects exerted by ethanol[29,30], which has been proposed to act at a site in the TM domains. Therefore, in the present study we investigated the influence of the LIL on the allosteric action of propofol in two members of the Cys-loop superfamily. Our results identified a single phenylalanine residue, conserved in the α_1 subunit of both $GABA_ARs$ and GlyRs, which affects their

sensitivity to propofol. These results provide novel information about the relevance of the LIL in the allosteric modulation of the Cys-loop superfamily.

Materials and Methods

Complementary DNA constructs

Mutations were inserted using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) in constructs encoding the human GlyR α_1 subunit subcloned in the pCI vector (Promega, Madison, WI) and the rat α_1 and β_2 $GABA_A$Rs subunits subcloned in the pRK5 vector (Clontech, Mountain View, CA). All mutations were confirmed by full sequencing. The GlyRs and $GABA_A$R amino acids were numbered according to their position in the mature protein sequence.

Cell culture and transfection

HEK293 cells (CRL-1573; American Type Culture Collection, Manassas, VA) were cultured using standard methods. For the GlyR experiments, HEK293 cells were cotransfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with the α_1 GlyR plus the pGreenLantern plasmid (Invitrogen) codifying the green fluorescent protein (ratio 1:1; 2 μg of DNA for each plasmid). Expression of green fluorescence protein was used as a marker of positively transfected HEK293 cells and recordings were made after 18–36 hrs. In some experiments in which $GABA_A$R $\alpha_1\beta_2\gamma_2$ subunits were expressed, HEK293 cells were cotransfected using Lipofectamine 2000 (Invitrogen) with the α_1 and β_2 subunits subcloned in the pRK5 vector and the γ_2 subunit subcloned in the vector internal ribosome entry site 2-enhanced green fluorescent protein (pIRES2-EGFP, Clontech) using a cotransfection ratio for $\alpha_1\beta_2\gamma_2$ of 1:2:5. To express the $\alpha_1\beta_2$ subunit combination, cells were cotransfected with the α_1 subunit subcloned in pRK5 and the β_2 subunit subcloned in the pIRES2-EGFP, using the ratio 1:2 respectively.

Electrophysiology

Whole-cell recordings were performed as previously described[29,30]. A holding potential of −60 mV was used. Patch electrodes were filled with (in mM): 140 CsCl, 10 2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 10 HEPES (pH 7.4), 4 $MgCl_2$, 2 Adenosine-5′-triphosphate and 0.5 Guanosine-5′-triphosphate. The external solution contained (in mM): 150 NaCl, 5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4), and 10 glucose. The amplitude of the GABA (γ-aminobutyric acid) or glycine current was assayed using a brief (1–2 s) pulse of GABA or glycine every 60 seconds. The modulation of the GABA or glycine current by propofol (2,6 diisopropylphenol; Sigma-Aldrich, St Louis, MO) was assayed using a pulse of glycine (EC_{10}) or GABA (EC_{10}) co-applied with propofol for each receptor studied, without any pre-application. The EC_{10} and EC_{50} values were obtained from concentration-response curves for GABA (1–1000 μM) and glycine (1–100 μM) and the response was normalized to saturating concentrations of the agonist (100%). In all the experiments, a brief pulse of 1 mM of GABA or glycine was performed at the end of the recording period to verify that the concentration used corresponded to the actual EC_{10} in each cell. Cells that displayed responses < EC_5 or > EC_{15} were discarded. The methodology for single-channel recordings in the outside-out configuration has been previously published[30,31]. Briefly, patch pipettes were coated with R6101 elastomer (Dow-Corning, Midland, MI) and had tip resistances of 7–15 megaohms following fire polishing. Cells were voltage clamped at −60 mV for GlyRs and −100 mV for $GABA_A$Rs and the data were filtered (5 kHz low-pass 8-pole Bessel) and acquired at 50 kHz using pClamp software (Molecular Devices, Sunnyvale, CA). Etomidate, ethanol, butanol and trichloroethanol were purchased from Sigma-Aldrich. Isoflurane was purchased from Baxter (Baxter International Inc. Deerfield, IL). Agonist and allosteric drug solutions were applied to cells using a

stepper motor-driven rapid solution exchanger (Warner Instrument Corp. Hamden, CT). Cells were maintained in extracellular medium containing (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, 10 glucose; pH 7.4. The intracellular recording solution contained (in mM): 140 CsCl, 2 MgATP, 10 2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid and 10 HEPES; pH 7.2.

Data analysis

Whole-cell data analysis was performed using OriginPro 7.0 (OriginLab, Northampton, MA). Non-linear regression analysis was used to fit concentration-response curves for glycine or GABA responses. Determination of significant differences between control and drug treatment groups were performed using one-way ANOVA or paired Student's t tests followed by the Bonferroni *post hoc* test. Concentration-response curves were generated by fitting the data to the Hill equation: $I = I_{max}/(1 + EC_{50}/[A]^n)$, where I is the current, I_{max} is the maximum current, [A] is the agonist concentration and n is the Hill coefficient. All results are expressed as mean ± SEM; values of $P \leq 0.05$ were considered statistically significant. Data from single-channel recordings were idealized using the segmentation K-means algorithm in the QUB software suite (The Research Foundation State University of New York, Buffalo, NY). Dwell time histograms were fitted with three or four exponential components using Clampfit software (Molecular Devices), and mean open times were obtained from the proportionally-weighted averages of the individual components. Values for open probability were calculated from idealized records. All-points amplitude histograms were generated and fitted with Gaussian functions using ClampFit.

RESULTS

A conserved phenylalanine residue is important for the sensitivity of GlyRs and $GABA_A$ to propofol

We tested the propofol sensitivity in a series of truncated GlyRs in which a large extension of the LIL was deleted. Three truncated forms of the GlyR, referred to in this study as Δ326–384, Δ326–355 and Δ355–382, were constructed and examined (Figure 1A). When the major portion of the LIL was deleted in Δ326–384, the potentiation of the glycine-activated current by propofol (30 μM) was significantly reduced from 363±33% to 70±12% (Figure 1B,C). In contrast, the sensitivity of the Δ326–355 mutant (358±48%) was similar to WT (tested at EC_{10}). Sensitivity of the Δ355–384 mutant to propofol was attenuated to 71±13% (Figure 1C), which is consistent with the view that propofol effects on GlyRs are influenced by residues located in the C terminal region. In order to identify critical amino acids involved in propofol modulation within the G326–Q384 sequence, a sequential series of substitutions of the WT amino acids with alanine and concentration-response curves for potentiation of the glycine current by propofol (1–100 μM) were constructed. These analyses showed that with the exception of the mutant ^{376}MRKLF^{380} →^{376}AAAAA^{380} (376–380A), all the other mutants retained their normal sensitivity to propofol (Figure 2A). For example, the sensitivity to 30 μM propofol was reduced to 65±14% in the 376–380A mutant (open circles, n=18) as compared to 360±30% in WT (closed circles, n=22). Additional single alanine substitutions showed that only the replacement of the phenylalanine residue (F380A) was able to affect the propofol modulation of GlyRs (75±10%, Figure 2B,C).

Given the functional and structural homology of the $GABA_A$Rs with the GlyRs[1,2], we hypothesized that the action of propofol on the $GABA_A$R could similarly be influenced by residues located within the LIL. Because residues of both α and β subunits have been implicated in the sensitivity of $GABA_A$Rs to propofol[17], we deleted a homologous intracellular sequence near the TM4 domain in both the α_1 and β_2 subunits (Figure 3A). The

γ_2 subunit, however, does not appear to be required for the potentiation of GABA-evoked currents by propofol[32]. To further confirm these results, we tested the sensitivity of $GABA_AR$ $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ combinations and did not find any significant differences (345% potentiation for $\alpha_1\beta_2\gamma_2$ and 340% potentiation for $\alpha_1\beta_2$). Based on these data, we used $GABA_ARs$ composed of $\alpha_1\beta_2$ subunits in our subsequent experiments. Deletion of the sequence between positions R354 and S388 in the α_1 subunit ($\alpha_1\Delta354–388\beta_2$) significantly reduced the potentiation of $GABA_AR$ by propofol (74±18%, n=14) (Figure 3B,C). However, when the homologous deletion in the β_2 subunit was examined, the sensitivity to propofol was unaltered (361±49%, n=8) (Figure 3C). Therefore, we carried out alanine scanning of the C terminal region of the LIL of the α_1 subunit. We found that propofol produced equivalent modulation of all mutants, with the exception of ^{384}TFNSV^{388} $\rightarrow$ ^{384}AAAAA^{388} (384–388A), which was potentiated by only 98±23% (Figure 4A, open circles). Consequently, we next determined which amino acids in this region were involved. Similar to the α_1 GlyR, there is a conserved phenylalanine residue (F385) in the homologous position in the α_1 $GABA_AR$, which when mutated caused a significant reduction in propofol potentiation (80±23%) (Figure 4B,C). In contrast, mutations at flanking residues had no effect on the potentiation by propofol (Figure 4C). Thus, these data allow us to conclude that a conserved phenylalanine residue, in both the GlyR and $GABA_AR$ α_1 subunits, is critically important for the allosteric modulation exerted by propofol.

Replacement of a phenylalanine residue within the LIL selectively reduced the sensitivity to propofol but not to other allosteric modulators

Suggested putative sites of propofol action in the TM domains in both GlyRs and $GABA_ARs$ were often associated with the effects of other structurally unrelated molecules. These observations suggest that these sites might be related to more than one allosteric modulator. To address this issue, we tested the sensitivity of the propofol-resistant mutants that we identified to alcohols, neurosteroids and other intravenous or volatile anaesthetics. Contrary to the idea that there is a common molecular site for pharmacological modulators[16–19, 26,33,34], our electrophysiological data show that the sensitivity of the mutant receptors to modulation by other allosteric regulators was unaffected (Figure 5A,B). For instance, the volatile anaesthetic isoflurane potentiated both the GlyR WT (183±19%, n=8) and the F380A mutant (186±18%, n=8) to a similar degree (Figure 5A). Notably, GABA-evoked currents in the $GABA_A\alpha_1F385A\beta_2$ propofol-hyposensitive receptor were potentiated by the intravenous anaesthetic etomidate (265±32%), which has been suggested to share a common or overlapping binding site[22,23] (Figure 5C). Likewise, isoflurane was able to potentiate both WT and F385A mutant receptors in a similar manner (Figure 5D). Altogether, contrary to the previously reported molecular site for propofol[16–18,33,34], our data suggest that mutation of a conserved phenylalanine residue within the LIL affects only the sensitivity to propofol, but does not alter the sensitivity to other allosteric modulators.

Propofol effects on the single-channel activity of the propofol-hyposensitive GlyRs and $GABA_ARs$

We next examined the effects of propofol on single-channel currents in WT and mutant GlyR and $GABA_AR$ using the outside-out configuration. For GlyRs, the results showed that both the WT and F380A mutant exhibited similar channel gating and conductance levels (Figure 6A–C) (Table 1). Interestingly, the channel conductance and open probability were not modified by the introduction of the F380 mutation. When propofol (1 μM) was applied to a WT GlyR, it produced a large enhancement in channel open probability (189±36% above control, n=5) (Figure 6B). In agreement with the results obtained in whole-cell recordings, the application of propofol to membrane patches containing an F380A mutant channel did not increase channel activity (22±13% above control, n=5, Figure 6B). Similar to GlyRs, the analysis of the $GABA_AR$ $\alpha_1F385A\beta_2$ mutant receptor showed that

conductance and open probability were not changed (Table 1). However, the $GABA_AR$ $\alpha_1\beta_2$ WT was strongly enhanced by propofol (Figure 6D–F), whereas the $GABA_AR$ $\alpha_1F385A\beta_2$ mutant did not show any significant potentiation (Figure 6E). Thus, these results demonstrate that mutations in intracellular sites did not cause noticeable effects in either GlyR or $GABA_AR$ channel function, but specifically altered the sensitivity to propofol of these receptors.

DISCUSSION

In the present study, we provide evidence supporting a new role of the LIL for propofol actions in two members of the Cys-loop superfamily. The data show that mutation of a phenylalanine residue, which is conserved in both $GABA_AR$ and GlyRs, significantly reduced their sensitivity to propofol. Single-channel recordings showed that kinetic parameters of wild type and mutant receptors were very similar, suggesting that the reduction in propofol sensitivity was not due to changes in ion channel properties.

Previous studies showed that $GABA_A$ and GlyRs carrying mutations that affected the sensitivity to allosteric modulators displayed altered gating properties[13,28,35,36]. For instance, mutant $GABA_A$ and GlyRs with reduced propofol, etomidate and general anesthetic sensitivity showed significant changes in agonist potency and channel gating[13,28,35,36]. Additionally, the mutant (S267Q in GlyRs)[37] that showed reduced sensitivity to ethanol and general anaesthetics also displayed reduced channel gating activity[36]. Thus, one can argue that the impaired effects of several allosteric modulators in these mutant receptors were due to changes in gating mechanisms[13,28,35–37]. In contrast, we found that mutation of F380 in α_1 GlyRs and F385 in α_1 $GABA_A$ subunits strongly reduced the allosteric modulation exerted by propofol without noticeable changes in the channel properties. At the single-channel level, we found that low concentrations of glycine or GABA elicited single-channel currents with conductances and mean open times similar to those previously published[37,38].

In agreement with previous studies[39], we found that propofol increased the open probability, without changes in mean open time, of GlyR and $GABA_ARs$. The absence of an effect of propofol on open time suggests that it does not affect the channel closing rate. Thus, the increased open probability observed in this study is either attributable to an increase in burst duration, which is in agreement with a study in $GABA_ARs$[40], or to an increase in opening frequency. Further studies will be required to distinguish between these possibilities.

Taken together, our results suggest that the mutation of the conserved phenylalanine residue generates propofol-hyposensitive $GABA_A$ and GlyRs through a mechanism that does not involve changes in ion channel gating. Therefore, our results are consistent with the hypothesis that the impaired propofol sensitivity is due to an alteration in allosteric mechanisms rather than to impaired ion channel function.

Regarding the subunits involved in propofol effects on $GABA_A$ receptors, previous reports have suggested that mutation of residues in the β subunit were sufficient to abolish the sensitivity to propofol of αβ heteropentamers[17,34]. In contrast, our results showed that mutation of the F385 residue in the α_1 subunit strongly reduced the sensitivity of $GABA_A\alpha_1\beta_2$ receptors to propofol. In agreement with our finding, it was demonstrated that $GABA_A$ receptors containing the α_6 subunit were four-fold less sensitive to propofol than those with α_1, independent of the presence of β and γ subunits[41]. Interestingly, sequence alignment of the LIL showed that while the α_1 subunit has the phenylalanine residue, the α_6 subunit has an isoleucine residue in the homologous position. Thus, our results are

consistent with the hypothesis that the presence or absence of this phenylalanine residue within the LIL influences propofol sensitivity.

Several studies have suggested that residues in TM regions of $GABA_A$Rs and GlyRs can form a water-filled cavity capable of binding propofol[15–20]. In addition, these residues also influence the receptor sensitivity to several structurally unrelated molecules[20–26]. However, studies addressing the specificity of the residues involved in anaesthetic actions have yielded conflicting results. While a single residue (Y444W) within the $GABA_AR$ β_2 subunit was shown to be important for the action of propofol[14], a recent report showed that this mutation also reduced potentiation by menthol[33], suggesting a lack of specificity. Furthermore, it was found that residues in TM2 and TM3 of GlyRs and the homologous residues in the $GABA_AR$ reduced the sensitivity to alcohols and volatile anaesthetics without changes in the sensitivity to propofol[26]. However, it was recently shown that the same mutations affected the sensitivity to propofol[25]. Our electrophysiological results, in contrast to both of these studies, demonstrated that both GlyRs and $GABA_AR$ mutant receptors conserved their sensitivity to other allosteric modulators. For instance, the sensitivity to etomidate, previously suggested to share a binding site with propofol in $GABA_A$Rs[22–24], was not altered in the $GABA_A\alpha_1$ F385A mutant. Altogether, our findings suggest that the intracellular phenylalanine residue is a determinant of propofol sensitivity in both GlyRs and $GABA_A$Rs, but does not appear to affect the sensitivity to any other allosteric modulator.

At present, high resolution molecular features for the intracellular region connecting TM3 and TM4 in eukaryotic Cys-loop ion channels are not available and only initial structural assessments can be obtained from homology modelling. Previous studies in the 5-hydroxytryptamine$_{3A}$ receptor have suggested that this region might be structured as an alpha-helix (termed membrane-associated stretch)[42,43]. In agreement with this, a previous model of α_1 GlyRs generated using the *Torpedo* nicotinic acetylcholine receptor as a template predicted an alpha-helical structure in this region[29,30]. Even though our results can only suggest that the phenylalanine residue forms a binding site, we speculate that propofol is accommodated by antiparallel helices and stabilized by hydrophobic interactions with two phenylalanine residues nearby. This agrees with data obtained in crystallized proteins in complex with propofol[44,45] that showed that binding pockets are formed by the arrangement of two helices, and that the putative cavity was lined by basic amino acids and hydrophobic residues, which provide the necessary environment to accommodate the anaesthetic molecule.

In conclusion, we have identified a conserved phenylalanine residue localized in the LIL, which influences the propofol sensitivity of GlyRs and $GABA_A$Rs. These results provide the first evidence indicating that the LIL plays a role in anaesthetic effects on inhibitory Cys-loop ion channels. Thus, these data provide new insights into the molecular mechanism of modulation of inhibitory ion channels by propofol, and will contribute to the understanding of the complex molecular framework underlying the modulation of central nervous system activity by general anaesthetics.

Acknowledgments

Supported by grants AA151-01 (L.G.A.) from the National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland, USA and FONDECYT 1060368 Chilean Council of Science and Technology, Santiago, Chile (L.G.A.)

The authors thank Lauren Aguayo, B.S. (Technician, Department of Physiology, Faculty of Biological Sciences, University of Concepcion, Concepcion, Chile) for technical assistance and help with the GlyR constructions.

References

1. Lynch JW. Molecular structure and function of the glycine receptor chloride channel. Physiol Rev. 2004; 84:1051–95. [PubMed: 15383648]
2. Michels G, Moss S. $GABA_A$ receptors: Properties and trafficking. Crit. Rev. Biochem. Mol. Biol. 2007; 42:3–14.
3. Keramidas A, Moorhouse AJ, Schofield PR, Barry PH. Ligand-gated ion channels: Mechanisms underlying ion selectivity. Prog Biophys Mol Biol. 2004; 86:161–204. [PubMed: 15288758]
4. Connolly CN, Wafford KA. The Cys-loop superfamily of ligand-gated ion channels: The impact of receptor structure on function. Biochem Soc Trans. 2004; 32:529–34. [PubMed: 15157178]
5. Rudolph U, Antkowiak B. Molecular and neuronal substrates for general anaesthetics. Nat Rev Neurosci. 2004; 5:709–20. [PubMed: 15322529]
6. Pistis M, Belelli D, Peters JA, Lambert JJ. The interaction of general anaesthetics with recombinant $GABA_A$ and glycine receptors expressed in *Xenopus laevis* oocytes: A comparative study. Br. J Pharmacol. 1997; 122:1707–19.
7. Hara M, Kai Y, Ikemoto Y. Enhancement by propofol of the gamma-aminobutyric acid A response in dissociated hippocampal pyramidal neurons of the rat. Anesthesiology. 1994; 81:988–94. [PubMed: 7943850]
8. O'Shea SM, Becker L, Weiher H, Betz H, Laube B. Propofol restores the function of "hyperekplexic" mutant glycine receptors in Xenopus oocytes and mice. J Neurosci. 2004; 24:2322–7. [PubMed: 14999083]
9. Sebel LE, Richardson JE, Singh SP, Bell SV, Jenkins A. Additive effects of sevoflurane and propofol on gamma-aminobutyric acid receptor function. Anesthesiology. 2006; 104:1176–83. [PubMed: 16732088]
10. Trapani G, Altomare C, Sanna E, Biggio G, Liso G. Propofol in Anesthesia: Mechanism of Action, Structure-Activity Relationships, and Drug Delivery. Curr Med Chem. 2000; 7:249–71. [PubMed: 10637364]
11. Daniels S, Roberts RJ. Post-synaptic inhibitory mechanisms of anaesthesia; Glycine receptors. Toxicol Lett. 1998; 100–101:71–6.
12. Nguyen HT, Li KY, daGraca RL, Delphin E, Xiong M, Ye JH. Behavior and cellular evidence for propofol-induced hypnosis involving brain glycine receptors. Anesthesiology. 2009; 110:326–32. [PubMed: 19194159]
13. Chang CS, Olcese R, Olsen RW. A Single M1 Residue in the β_2 Subunit Alters Channel Gating of $GABA_A$ Receptor in Anesthetic Modulation and Direct Activation. J Biol. Chem. 2003; 278:42821–8.
14. Richardson JE, Garcia PS, O'Toole KK, Derry JM, Bell SV, Jenkins A. A Conserved Tyrosine in the β2 Subunit M4 Segment Is a Determinant of γ-Aminobutyric Acid Type A Receptor Sensitivity to Propofol. Anesthesiology. 2007; 10:412–8. [PubMed: 17721243]
15. Rudolph U, Moler H. Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Annu. Rev. Pharmacol. Toxicol. 2004; 44:475–98.
16. Campagna-Slater V, Weaver DF. Anaesthetic binding sites for etomidate and propofol on a $GABA_A$ receptor model. Neurosci Lett. 2007; 418:28–33. [PubMed: 17412502]
17. Krasowski MD, Koltchine VV, Rick CE, Ye Q, Finn SE, Harrison NL. Propofol and other intravenous anesthetics have sites of action on the γ-aminobutyric acid type A receptor distinct from that for isoflurane. Mol Pharmacol. 1998; 53:530–8. [PubMed: 9495821]
18. Siegwart R, Jurd R, Rudolph U. Molecular determinants for the action of general anesthetics at recombinant alpha(2)beta(3)gamma(2)gamma-aminobutyric acid(A) receptors. J Neurochem. 2002; 80:140–8. [PubMed: 11796752]
19. Jurd R, Arras M, Lambert S, Drexler S, Siegwart R, Crestani F, Zaug M, Vogt K, Ledermann B, Antkoviak B, Rudolph U. General anesthetics actions *in vivo* strongly attenuated by a point mutation in the $GABA_A$ receptor β3 subunit. FASEB J. 2003; 17:250–2. [PubMed: 12475885]
20. Franks NP, Lieb WR. Anaesthetics set their sites on ion channels. Nature. 1997; 389:334–5. [PubMed: 9311772]

21. Franks NP. General anaesthesia: From molecular targets to neuronal pathways of sleep and arousal. Nat Rev Neurosci. 2008; 9:370–86. [PubMed: 18425091]
22. Li GD, Chiara DC, Sawyer GW, Husain SS, Olsen RW, Cohen JB. Identification of a $GABA_A$ receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog. J Neurosci. 2006; 26:11599–605. [PubMed: 17093081]
23. Bali M, Akabas MH. Defining the propofol binding site location on the $GABA_A$ receptor. Mol. Pharmacol. 2004; 65:68–76.
24. Li GD, Chiara DC, Cohen JB, Olsen RW. Numerous classes of general anesthetics inhibit etomidate binding to gamma-aminobutyric acid type A (GABAA) receptors. J Biol Chem. 2010; 285:8615–20. [PubMed: 20083606]
25. Ahrens J, Leuwer M, Stachura S, Krampfl K, Belelli D, Lambert JJ, Haeseler G. A transmembrane residue influences the interaction of propofol with the strychnine-sensitive glycine alpha1 and alpha1beta receptor. Anesth Analg. 2008; 107:1875–83. [PubMed: 19020133]
26. Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL. Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. Nature. 1997; 389:385–9. [PubMed: 9311780]
27. Bertaccini EJ, Wallner B, Trudell JR, Lindahl E. Modeling anesthetic binding sites within the glycine alpha one receptor based on prokaryotic ion channel templates: The problem with TM4. J Chem Inf Model. 2010; 50:2248–55. [PubMed: 21117677]
28. Stewart D, Desai R, Cheng Q, Liu A, Forman SA. Tryptophan mutations at azietomidate photo-incorporation sites on α1 or β2 subunits enhance $GABA_A$ receptor gating and reduce etomidate modulation. Mol. Pharmacol. 2008; 74:1687–95.
29. Yevenes GE, Moraga-Cid G, Guzmán L, Haeger S, Oliveira L, Olate J, Schmalzing G, Aguayo LG. Molecular determinants for G protein βγ modulation of ionotropic glycine receptors. J Biol Chem. 2006; 281:39300–7. [PubMed: 17040914]
30. Yevenes GE, Moraga-Cid G, Peopples RW, Schmalzing G, Aguayo LG. A selective Gβγ-linked intracellular mechanism for modulation of a ligand-gated ion channel by ethanol. Proc Nat Acad Sci. 2008; 105:20523–8. [PubMed: 19074265]
31. Ren H, Honse Y, Karp BJ, Lipsky RH, Peoples RW. A site in the fourth membrane associated domain of the NMDA receptor regulates desensitization and ion channel gating. J Biol Chem. 2003; 278:276–83. [PubMed: 12414797]
32. Jones MV, Harrison NL, Pritchett DB, Hales TG. Modulation of the $GABA_A$ receptor by propofol is independent of the gamma subunit. J Pharmacol. Exp. Ther. 1995; 274:962–8.
33. Watt EE, Betts BA, Kotey FO, Humbert DJ, Griffith TN, Kelly EW, Veneskey KC, Gill N, Rowan KC, Jenkins A, Hall AC. Menthol shares general anesthetic activity and sites of action on the GABA(A) receptor with the intravenous agent propofol. Eur J Pharmacol. 2008; 590:120–6. [PubMed: 18593637]
34. Krasowski MD, Nishikawa K, Nikolaeva N, Lin A, Harrison N. Methionine 286 in transmembrane domain 3 of the GABAA receptor β subunit controls a binding cavity for propofol and other alkylphenol general anesthetics. Neuropharmacol. 2001; 41:952–64.
35. Lobo I, Mascia P, Trudell J, Harris A. Channel Gating of the Glycine Receptor Changes Accessibility to Residues Implicated in Receptor Potentiation by Alcohols and Anesthetics. 2004; 279:33919–27.
36. Findlay GS, Phelan R, Roberts MT, Homanics GE, Bergeson SE, Lopreato GF, Mihic SJ, Blednov YA, Harris RA. Glycine receptor knock-in mice and hyperekplexia-like phenotypes: Comparisons with the null mutant. J Neurosci. 2003; 23:8051–9. [PubMed: 12954867]
37. Burzomato V, Groot-Kormelink PJ, Sivilotti LG, Beato M. Stoichiometry of recombinant heteromeric glycine receptors revealed by a pore-lining region point mutation. Receptors Channels. 2003; 9:353–61. [PubMed: 14698963]
38. Beato M, Groot-Kormelink PJ, Colquhoun D, Sivilotti LG. The activation mechanism of alpha1 homomeric glycine receptors. J Neurosci. 2004; 24:895–906. [PubMed: 14749434]
39. Kitamura A, Sato R, Marszalec W, Yeh JZ, Ogawa R, Narahashi T. Halothane and propofol modulation of gamma-aminobutyric acid A receptor single-channel currents. Anesth Analg. 2004; 99:409–15. [PubMed: 15271715]

40. O'Shea S, Williams C, Jenkins A. Inverse Effects on Gating and Modulation Caused by a Mutation in the M2-M3 Linker of the GABAA Receptor γ Subunit. Mol Pharmacol. 2009; 76:641–51. [PubMed: 19553237]

41. Krasowski MD, O'Shea SM, Rick CE, Whiting PJ, Hadingham KL, Czajkowski C, Harrison NL. Alpha subunit isoform influences $GABA_A$ receptor modulation by propofol. Neuropharmacol. 1997; 36:941–9.

42. Barnes NM, Hales TG, Lummis SC, Peters JA. The 5-HT3 receptor: The relationship between structure and function. Neuropharmacol. 2009; 56:273–84.

43. Peters JA, Cooper MA, Carland JE, Livesey MR, Hales TG, Lambert JJ. Novel structural determinants of single channel conductance and ion selectivity in 5-hydroxytryptamine type 3 and nicotinic acetylcholine receptors. J Physiol. 2010; 588:587–96. [PubMed: 19933751]

44. Vedula LS, Brannigan G, Economou NJ, Xi J, Hall MA, Liu R, Rossi MJ, Dailey WP, Grasty KC, Klein ML, Eckenhoff RG, Loll PJ. A unitary anesthetic binding site at high resolution. J Biol Chem. 2009; 275:38731–8.

45. Bhattacharya AA, Curry S, Franks NP. Binding of the general anesthetics propofol and halothane to human serum albumin: High resolution crystal structures. J Biol Chem. 2009; 284:24176–84. [PubMed: 19605349]

MS #201004021 Finalbox summary

What we already know about this topic

- Propofol positively modulates receptors for the inhibitory transmitters GABA and glycine, but the molecular mechanisms involved are unclear.

What this article tells us that is new

- A single homologous residue in the large M3-M4 intracellular loops of the alpha subunits of GABAa and glycine receptors modulates the action of propofol but not of other general anesthetics.

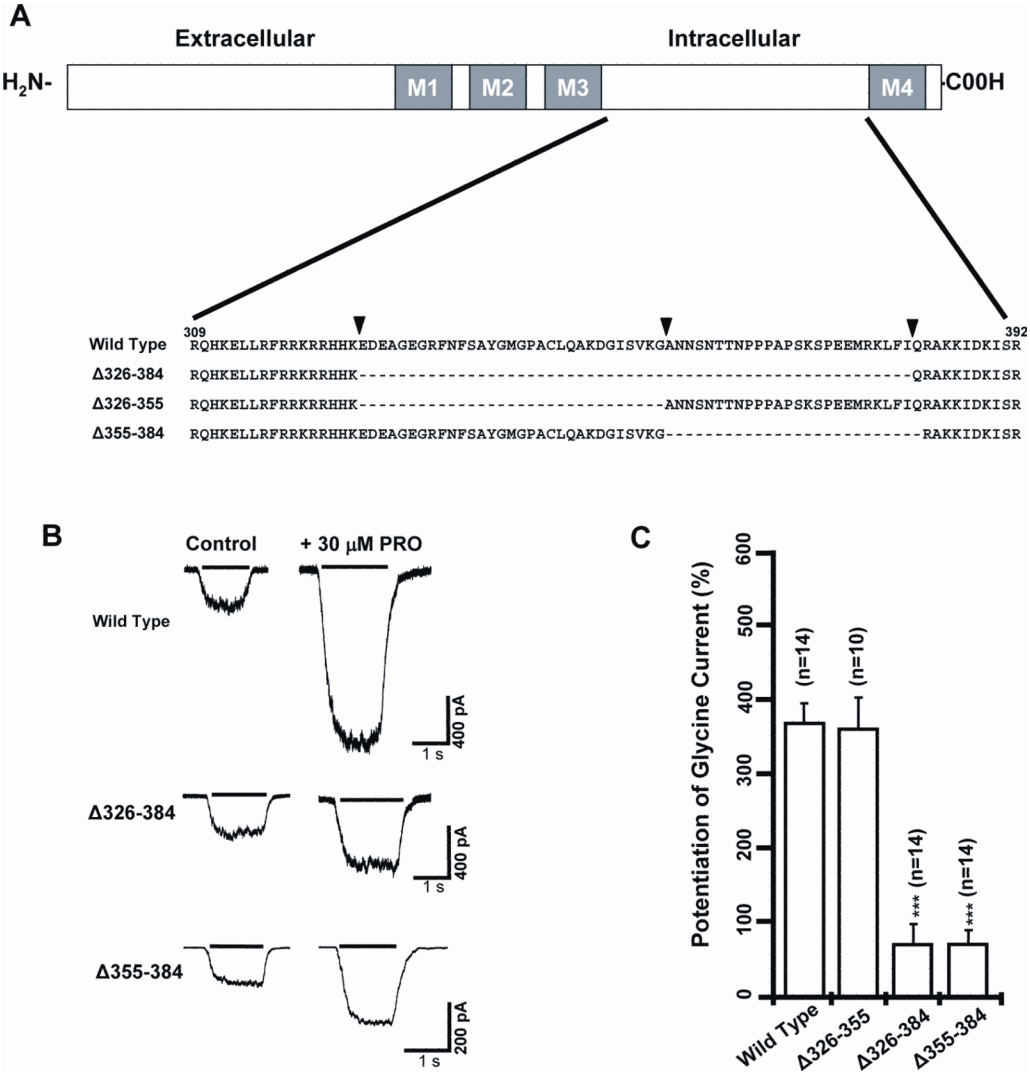

Figure 1. Deletion of the segment between residues E326 and A384 in the large intracellular loop (LIL) of the α_1 glycine receptor (GlyRs) reduced its sensitivity to propofol

A, Schematic representation of the GlyR subunit topology. The entire sequence of the LIL for human α1 GlyRs is shown. Three functional mutants were generated for α_1 GlyR by deletion of different regions of the LIL. The arrows indicate the deleted segments and truncated sequences are illustrated by the dashed line. The numbers indicate the positions in the mature polypeptide. **B,** Glycine-activated (EC_{10}) current in wild type GlyRs was enhanced by propofol (PRO, 30 μM). In contrast, the sensitivity to propofol was significantly reduced in the Δ326–384 and Δ355–384 truncated GlyRs, whereas the sensitivity of the Δ326–355 mutant did not change **C.** The graph shows that the sensitivity to propofol was significantly reduced when the segment Δ355–384 was deleted (one-way ANOVA with Bonferonni's post test). The bars represent the mean ±S.E.M. The asterisks denote a significance of P<0.001 (***).

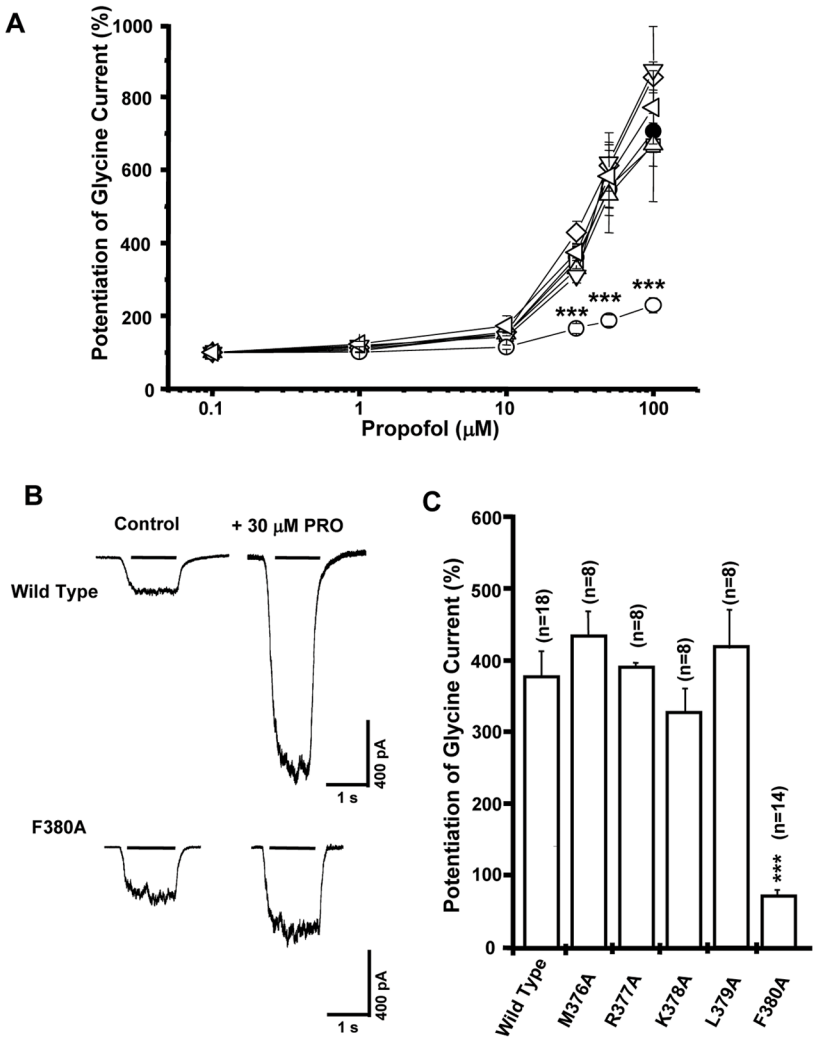

Figure 2. Replacement of the F380 residue by alanine in the large intracellular loop (LIL) of α_1 GlyRs reduced the sensitivity to propofol

A, Graph summarizes the percentage of potentiation in the wild type (black circles) and several alanine-scanning substitution mutants between residues G355 and R392. Five residues were replaced systematically in each mutant. The 376–380A mutant (white circles) was less sensitive to propofol. **B,** Glycine-evoked currents were enhanced by propofol (PRO) in the wild type receptor only, whereas the F380A mutant was less sensitive. **C** The plot summarizes the sensitivity to propofol (30 μM) in the wild type and five mutant GlyR α_1 subunits between residues M376 and F380. The bars represent the mean±S.E.M. The asterisks denote a significance of $P<0.001$ (***).

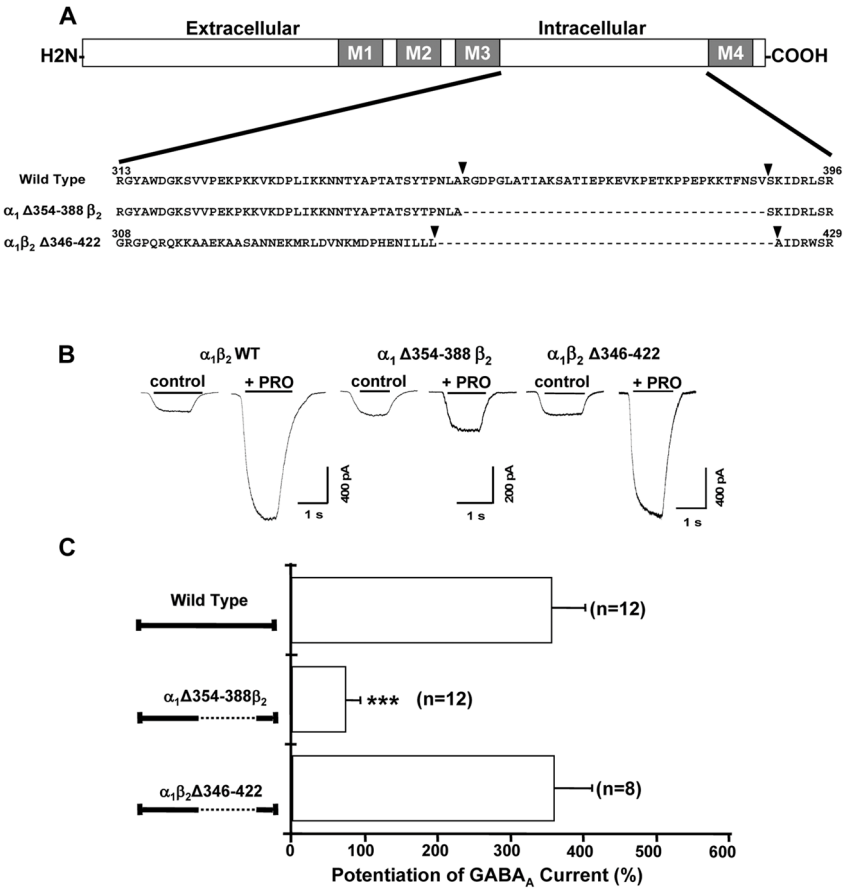

Figure 3. Deletion of a region of the large intracellular loop (LIL) in the γ-Amino Butyric Acid Type A Receptor ($GABA_AR$)α_1 subunit reduced its sensitivity to propofol

A, Schematic representation of the $GABA_AR$ subunit. Functional $GABA_AR$ mutants were generated by deletion of homologous regions of the α_1 and β_2 subunits. **B,** GABA-activated currents in wild type receptors are consistently potentiated by propofol (PRO). In contrast, propofol effects in α_1 Δ354–388 were significantly attenuated, whereas the sensitivity of β_2Δ346–422 mutant was similar to the wild type receptor. **C,** The graph summarizes the percentage of potentiation in the wild type and truncated forms of the $GABA_AR$s by propofol. The bars represent the mean±S.E.M. The asterisks denote a significance of $P<0.001$ (***).

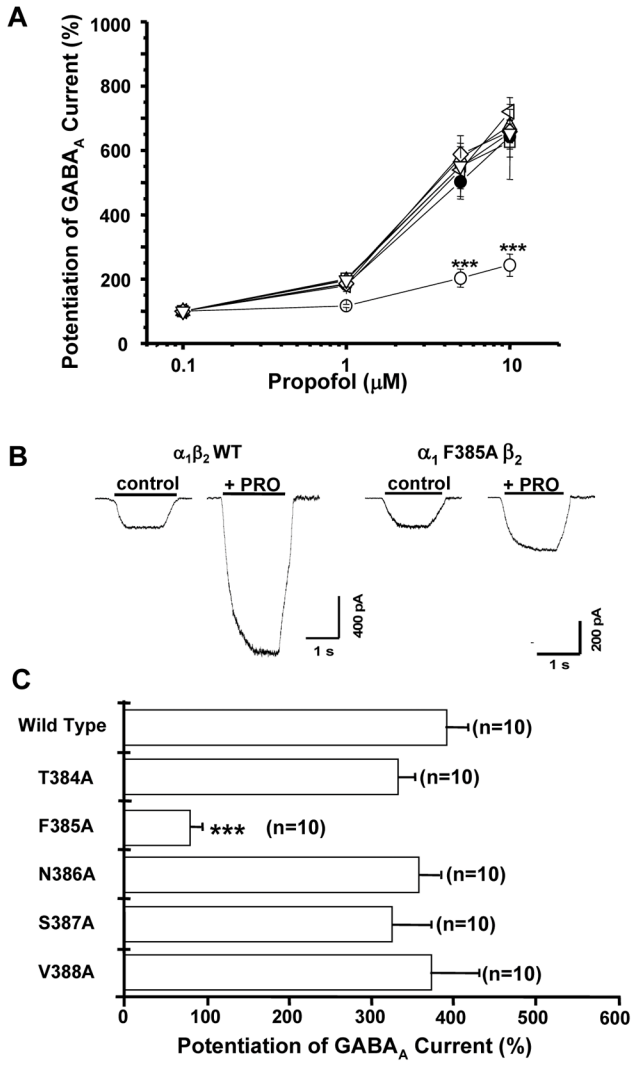

Figure 4. A conserved phenylalanine residue in γ-Amino Butyric Acid Type A Receptor (GABA$_A$R)α$_1$ subunit is important for sensitivity to propofol

A, Graph summarizes the percentage of potentiation in the wild type (black circles) and several alanine-scanning substitution mutants between residues R354 and V388. Five residues were replaced systematically each time. Only the 384–388A mutant (white circles) was significantly less sensitive to propofol. **B**, GABA-evoked currents were enhanced by propofol (PRO) in the wild type (WT) receptor, but not in the F385A mutant. **C,** The graph summarizes the sensitivity to propofol (30 μM) in the wild type and five mutant GABA$_A$R α$_1$ subunits between T384 and V388. The F385A mutant was less sensitive to propofol. The bars represent the mean±S.E.M. The asterisks denote a significance of P<0.001 (***).

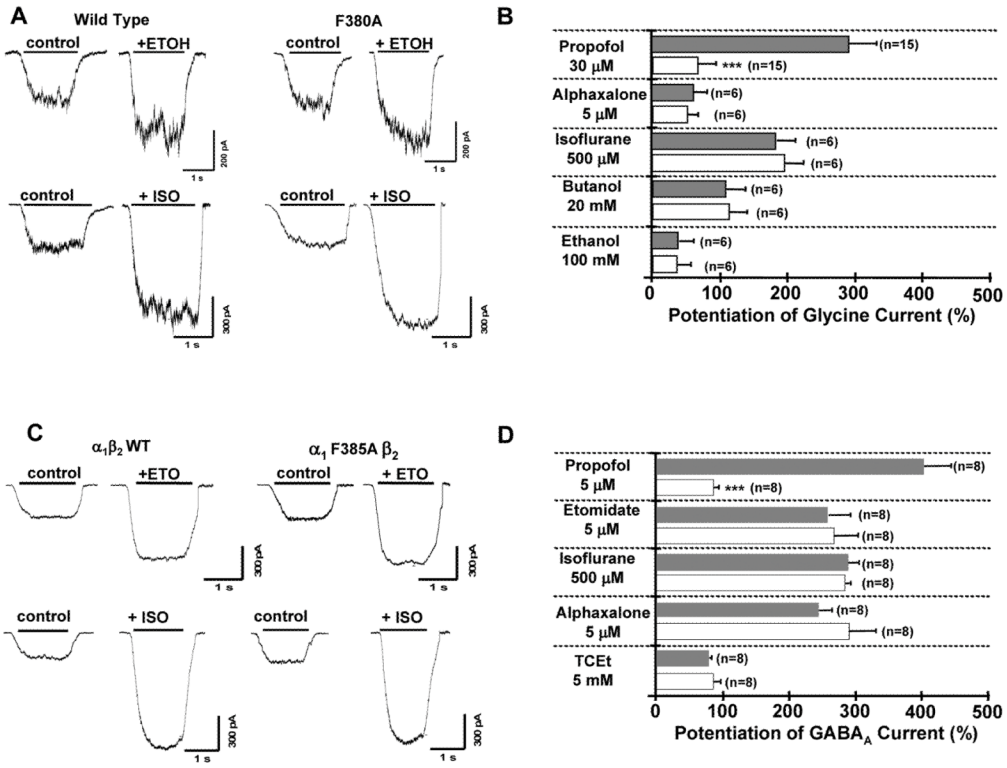

Figure 5. Propofol-hyposensitive receptors retain normal sensitivity to other positive allosteric modulators

A, Glycine-evoked currents were enhanced by ethanol (ETOH, 100 mM) and isoflurane (ISO, 500 μM) in the F380A mutant. **B,** Potentiation of the glycine-evoked current in wild type and F380A GlyRs by positive allosteric modulators. **C,** GABA-evoked currents were enhanced by etomidate (ETO, 5 μM) and isoflurane (ISO, 500 μM) in the F385A mutant. **D,** Potentiation of theγ-Amino Butyric Acid Type A Receptor ($GABA_AR$) wild type (WT) and F385A mutant by propofol, etomidate, isoflurane, alphaxalone and trichloroethanol (TCEt). WT values are shown in gray, whereas the mutant responses are shown in white. The bars represent the mean±S.E.M. The asterisks denote a significance of $P<0.001$ (***).

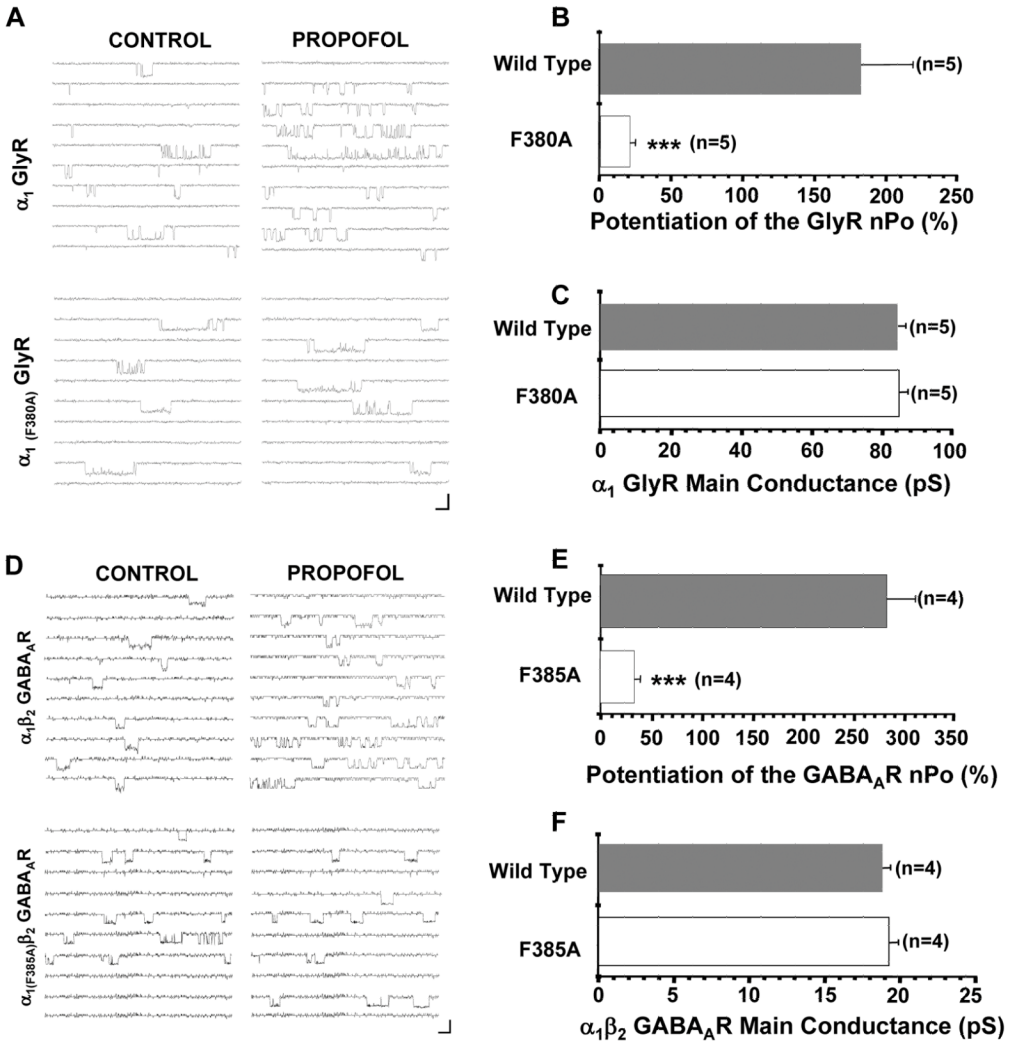

Figure 6. Effects of propofol on single-channel function in WT and mutated glycine receptor (GlyRs) and γ-Amino Butyric Acid Type A Receptor ($GABA_AR$)

A, Single-channel activity recorded in wild type and F380A GlyRs (calibration bar; 5 pA, 10 ms). **B,** Percentage of the open probability (nPo) potentiation by propofol in the wild type and F380A GlyRs. **C,** Main conductance of the wild type and F380A GlyRs. **D,** Single-channel activity recorded in the wild type and F385A $GABA_AR$ (calibration bar; 2 pA, 10 ms) **E,** Percentage of nPo potentiation in the wild type and F385A $GABA_ARs$ by propofol. **F,** Main conductance of the wild type and F385A $GABA_A$ receptor. The bars represent the mean±S.E.M. The asterisks denote a significance of P<0.001 (***).

Table 1

Kinetics parameters of wild type and mutant GlyRs and $GABA_A$Rs in absence or presence of propofol.

				-GlyRs-					
	Glycine 1 μM				**Gycine 1 μM + Propofol 1 μM**				
Condition	**MOT (ms)**	**MST (ms)**	**Main γ (pS)**	**nPo**	**MOT (ms)**	**MST (ms)**	**Main γ (pS)**	**nPo**	**n**
Wild Type	1.15±0.03	25.2±4	92±3	0.048	1.07±0.08	18.9±4	91±3	0.148	4
F380A	1.35±0.05	26.1±3	90±2	0.053	1.28±0.05	22.7±3	90±2	0.048	4
				-$GABA_A$Rs-					
	GABA 1 μM				**GABA 1 μM + Propofol 1 μM**				
Condition	MOT (ms)	MST (ms)	Main γ (pS)	nPo	MOT (ms)	MST (ms)	Main γ (pS)	nPo	n
Wild Type	1.03±0.03	18.3±0.04	19±0.8	0.25	1.14±0.08	17.9±0.4	19±0.5	0.68	4
F385A	1.18±0.04	17.7±0.03	19±0.6	0.31	1.13±0.05	18.2±0.03	20±0.7	0.39	4

$GABA_A$RS: γ-aminobutiric acid receptor type A; GABA:γ-aminobutiric acid; GlyRs: Glycine receptors; MOT: Mean Open Time; MST: Mean Shut Time; nPo: Open Probability; γ: Conductance