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Manipulations of extracellular Loop 2 in $\alpha 1$ GlyR ultra-sensitive ethanol receptors (USERS) enhance receptor sensitivity to isoflurane, ethanol, and lidocaine, but not propofol

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Abstract

We recently developed Ultra-Sensitive Ethanol Receptors (USERS) as a novel tool for investigation of single receptor subunit populations sensitized to extremely low ethanol concentrations that do not affect other receptors in the nervous system. To this end, we found that mutations within the extracellular Loop 2 region of glycine receptors (GlyRs) and γ -aminobutyric acid type A receptors (GABA_ARs) can significantly increase receptor sensitivity to micro-molar concentrations of ethanol resulting in up to a 100-fold increase in ethanol sensitivity relative to wild type (WT) receptors. The current study investigated: 1) Whether structural manipulations of Loop 2 in $\alpha 1$ GlyRs could similarly increase receptor sensitivity to other anesthetics; and 2) If mutations exclusive to the C-terminal end of Loop 2 are sufficient to impart these changes. We expressed $\alpha 1$ GlyR USERS in *Xenopus* oocytes and tested the effects of three classes of anesthetics, isoflurane (volatile), propofol (intravenous), and lidocaine (local), known to enhance

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glycine-induced chloride currents using two-electrode voltage clamp electrophysiology. Loop 2 mutations produced a significant 10-fold increase in isoflurane and lidocaine sensitivity, but no increase in propofol sensitivity compared to WT $\alpha 1$ GlyRs. Interestingly, we also found that structural manipulations in the C-terminal end of Loop 2 were sufficient and selective for $\alpha 1$ GlyR modulation by ethanol, isoflurane, and lidocaine. These studies are the first to report the extracellular region of $\alpha 1$ GlyRs as a site of lidocaine action. Overall, the findings suggest that Loop 2 of $\alpha 1$ GlyRs is a key region that mediates isoflurane and lidocaine modulation. Moreover, the results identify important amino acids in Loop 2 that regulate isoflurane, lidocaine, and ethanol action. Collectively, these data indicate the commonality of the sites for isoflurane, lidocaine, and ethanol action, and the structural requirements for allosteric modulation on $\alpha 1$ GlyRs within the extracellular Loop 2 region.

Keywords

$\alpha 1$ GlyRs; Loop 2; isoflurane; lidocaine; ethanol; ligand-gated ion channel

Glycine receptors (GlyRs), one of the major inhibitory neurotransmitter receptor systems in the adult mammalian central nervous system (CNS) (Dutertre et al., 2012), are fast-acting ligand-gated ion channels (LGICs) that belong to the Cys-loop superfamily, whose members also include the closely related γ -aminobutyric acid-type A ($GABA_A$), nicotinic acetylcholine (nACh), and 5-hydroxytryptamine₃ (5-HT₃) receptors (Ortells and Lunt, 1995, Xiu et al., 2005). GlyRs mediate inhibitory neurotransmission in the spinal cord, brain stem, cerebral cortex, ventral tegmental area, nucleus accumbens, dorsal raphe, and amygdala, and are considered a site of action for allosteric modulators including, alcohols, general anesthetics (volatile and intravenous), neuroactive steroids, endocannabinoids, divalent cations, and avermectins (Downie et al., 1996, Rajendra et al., 1997, Lynch, 2004, Dutertre et al., 2012, Maguire et al., 2014). $\alpha 1$ subunit-containing GlyRs are widely expressed in the spinal cord and brain stem and have been implicated in playing a role in the immobilizing effects of ethanol and anesthetic action by inhibiting motor responses to noxious stimuli and mediating ethanol-induced loss of righting reflex (Antognini and Schwarz, 1993, Williams et al., 1995, Legendre, 2001, Ye et al., 2009, Aguayo et al., 2014).

A large body of evidence implicates sites within the transmembrane (TM), extracellular (EC), and intracellular (IC) domain of GlyRs and $GABA_A$ Rs as important targets of ethanol and anesthetic action. Site-directed mutagenesis and radioligand binding studies, for example, have identified both inter- and intra-subunit regions within the TM domains of $\alpha 1$ GlyRs, and $\alpha 2$ and $\beta 1$ subunits of $GABA_A$ Rs as critical sites of action for ethanol as well as volatile and general anesthetics (Mihic et al., 1997, Krasowski et al., 1998, Mascia et al., 2000). Current evidence suggests that some of these anesthetics act, in part, via binding at different regions within the TM domain or the central pore region of the receptors (Cummins, 2007). For example, ethanol and volatile anesthetics (isoflurane, and its structural isomer, enflurane) are reported to share overlapping sites of action in the TM2 and TM3 domain that are distinctly different from the site of action of the intravenous anesthetic, propofol (Mascia et al., 1996, Mihic et al., 1997, Krasowski et al., 1998, Krasowski et al., 2001, Nury et al., 2011, Sauguet et al., 2013). Mutations in the TM domain of $GABA_A$ Rs at

positions 270 or 291 in $\alpha 2$, or at positions 265 or 286 in $\beta 2$ rendered these receptors insensitive to isoflurane, but maintained sensitivity to propofol (Krasowski et al., 1998).

The structural homology among Cys-loop receptors implies that the sites of ethanol and anesthetic action identified in GABA_ARs may also correlate to GlyRs. Isoflurane and propofol enhance glycine-induced chloride currents of GlyRs, suggesting their involvement in the actions of these anesthetics (Downie et al., 1996, Krasowski and Harrison, 1999). Prior studies found that a point mutation at position 52 in the EC Loop 2 region of $\alpha 1$ GlyRs altered ethanol sensitivity (Davies et al., 2004, Crawford et al., 2007, Crawford et al., 2008, Perkins et al., 2008) but did not affect propofol sensitivity (Mascia et al., 1996). These differences have also been reported in GABA_ARs (Bali and Akabas, 2004). In addition, recent studies have characterized position 380 in the large IC loop between TM 3 and TM 4 of $\alpha 1$ GlyRs as an important site for propofol action, but not for other anesthetics (alcohols, etomidate, trichloroethanol and isoflurane) (Moraga-Cid et al., 2011). Furthermore, lidocaine, a local anesthetic, potentiates GlyRs, implicating their involvement in lidocaine action (Hara and Sata, 2007). Recent studies report that lidocaine-induced potentiation of GlyR currents was abolished when the serine S267 in the TM domain of $\alpha 1$ GlyRs, a previously reported target site for ethanol (Mihic et al., 1997), was mutated (Hara and Sata, 2007). Taken together, these studies indicate that ethanol and certain anesthetics have overlapping sites of action on $\alpha 1$ GlyRs that are distinct from those of propofol.

Interestingly, previous studies indicate that GABA_ARs containing the δ subunit are especially sensitive to potentiation by intoxicating (10-20 mM) concentrations of ethanol (Wallner et al., 2003). With this in mind, we studied the differences in the amino acid sequences between GABA_ARs containing the δ subunit and GlyR $\alpha 1$ subunits and found major differences within the Loop 2 regions of the EC domain of these two receptors. We therefore conducted a series of investigations that focused on the EC domain of $\alpha 1$ GlyRs. Notably, we identified EC Loop 2 (positions 50-59) as a critical site of ethanol action that mediates ethanol sensitivity of GlyRs and GABA_ARs (Crawford et al., 2007, Olsen et al., 2007, Crawford et al., 2008, Perkins et al., 2008, Perkins et al., 2009, Perkins et al., 2012, Naito et al., 2014).

Over the course of these aforementioned investigations, we found that manipulation of the structural features of EC Loop 2 conferred a significant increase in ethanol sensitivity of up to 100-fold as compared to homomeric recombinant WT $\alpha 1$ GlyRs and resulted in the discovery of Ultra-Sensitive Ethanol Receptors (USERS) (Perkins et al., 2009, Naito et al., 2014). When expressed in oocytes and mammalian cells *in vitro*, USERS respond to extremely low micro-molar ethanol concentrations – concentrations too low to affect native receptors. Remarkably, despite this significant increase in ethanol sensitivity, these USERS featured minimal changes relative to WT $\alpha 1$ GlyRs in general receptor characteristics including the maximum current amplitude ($I_{\max}$), Hill slope, and agonist sensitivity measured by the half maximal effective concentration (EC_{50}). In addition, similar Loop 2 manipulations also produced USERS in homomeric $\alpha 2$ GlyRs and heteromeric $\alpha 1$ and $\gamma 2$ subunits of $\alpha 1 \beta 2 \gamma 2$ GABA_ARs (Naito et al., 2014). Interestingly, reversion of the residues in the C-terminal region of Loop 2 in both $\alpha 1$ and $\alpha 2$ GlyR USERS (positions 55-59) back to WT, significantly increased ethanol sensitivity by reducing the threshold and increasing the

magnitude of response, without altering agonist EC₅₀ sensitivity relative to WT $\alpha 1$ GlyRs (Naito et al., 2014). Structural manipulation of the C-terminal region of Loop 2 in $\gamma 2$ GABA_ARs eliminated ethanol sensitivity; thus implicating this region as an important regulator of ethanol sensitivity (Naito et al., 2014). Overall, these findings demonstrate the importance of the EC Loop 2 region of ethanol action across multiple subunits of GlyRs and GABA_ARs and could be useful in elucidating the role that these receptor subunits play in mediating the behavioral manifestations of ethanol.

While USERS are ultra-sensitive to the effects of the general anesthetic, ethanol, their selectivity to other anesthetics has not been investigated. The present study tested the hypothesis that structural manipulations of Loop 2, particularly the C-terminal end, of human $\alpha 1$ GlyRs are important for transducing the potentiating effects of three classes of anesthetics – volatile, intravenous, and local. Based on prior studies that identify common sites among ethanol and isoflurane, we expect that the Loop 2 manipulations that increased ethanol sensitivity of $\alpha 1$ GlyR USERS would similarly increase receptor sensitivity to the volatile anesthetic, isoflurane, but would not significantly alter the effects of the intravenous anesthetic, propofol. Similarly, since earlier studies have demonstrated that an ethanol site in the TM domain of $\alpha 1$ GlyRs modulated the effects of the local anesthetic, lidocaine, we tested the effects of this drug in $\alpha 1$ GlyR USERS to elucidate its sites of action on $\alpha 1$ GlyRs. We investigated the potentiating effects of isoflurane, lidocaine, and propofol on $\alpha 1$ GlyR USERS using two-electrode voltage clamp electrophysiology.

A long-term goal of our ongoing USER investigations is the creation of knock-in mice that would allow us to evaluate the effectiveness of the Loop 2 mutations *in vivo*. Expression of Loop 2 in GlyRs is controlled by two exons: exon 3 for the N-terminal end and exon 4 for the C-terminal end. Since mutations exclusively contained within a single exon would greatly simplify the development of knock-in mice, we developed a new $\alpha 1$ GlyR USER (i.e., USER 4) to investigate whether the C-terminal region of Loop 2 is sufficient to impart changes in $\alpha 1$ GlyR sensitivity to ethanol and other anesthetics. Overall, the findings supported the hypothesis and provide additional insight into the structure-activity relationship of anesthetics on $\alpha 1$ GlyRs.

Experimental Procedures

Materials

Stage V or VI *Xenopus* oocytes were purchased commercially from EcoCyte Bioscience (Austin, TX). Gentamycin, 3-aminobenzoic acid ethyl ester, glycine, and propofol were purchased from Sigma (St. Louis, MO). Isoflurane (USP grade) was purchased from Abbott Laboratories (North Chicago, IL), lidocaine was purchased from Tocris Bio-Techne (Minneapolis, MN), and ethanol was purchased from Decon Labs, Inc. (King of Prussia, PA). Glycine stock solutions were prepared from powder and diluted with Modified Barth's Solution (MBS) containing (in mM) 88 NaCl, 1 KCl, 10 HEPES, 0.82 MgSO₄, 2.4 NaHCO₃, 0.91 CaCl₂, and 0.33 Ca(NO₃)₂, adjusted to a final pH of 7.5 (Davies et al., 2003). 1 mM stock solutions of isoflurane, lidocaine, and propofol were prepared in DMSO and serially diluted with buffer (DMSO = 0.1%) immediately prior to testing. Pilot studies found

that DMSO at this concentration, with or without glycine, had no appreciable effect on $\alpha 1$ GlyR currents in WT and USERS.

Mutagenesis and Expression of $\alpha 1$ GlyR in Oocytes

Amino acid sequences of the Loop 2 regions of human $\alpha 1$ GlyR wild type (WT) and USERS are shown in Table 1. Loop 2 is defined as positions 50-59 in the $\alpha 1$ GlyR subunit (Mihic et al., 1997, Perkins et al., 2009), and spans exon 3 (positions 50-56) and exon 4 (positions 57-59) (Table 1). $\alpha 1$ GlyR USERS were developed according to methods described previously (Perkins et al., 2009). Briefly, we replaced the Loop 2 region of human $\alpha 1$ GlyRs with that of the ethanol-sensitive Loop 2 of δ GABA_ARs using site-directed mutagenesis to produce $\alpha 1$ GlyR USER 1 (Table 1). Using Loop 2 of $\alpha 1$ GlyR USER 1 as a template, we reverted specific non-conserved amino acid residues back to WT $\alpha 1$ GlyR residues to generate USERS 2, 3 (Naito et al., 2014) and 4. $\alpha 1$ GlyR USER 4 represents Loop 2 mutations of USER 1 at positions 57 and 59 in exon 4 only (Table 1). Site-directed mutagenesis was performed by subcloning human $\alpha 1$ GlyR into mammalian vector pCIS2 or pBK-CMV using Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and verified by partial sequencing (DNA Core Facility, University of Southern California) as described previously (Davies et al., 2003). Stage V or VI *Xenopus* oocytes were injected with $\alpha 1$ GlyR WT or USER cDNA (1 ng/32 nl). Oocytes were stored in incubation medium [ND96 supplemented with 2 mM sodium pyruvate, 50 mg/ml of gentamycin and 10 ml heat inactivated HyClone® horse serum (VWR, San Dimas, CA), adjusted to pH 7.5] in Petri dishes (VWR, San Dimas, CA). All solutions were sterilized by passage through 0.22- μ m filters. Injected oocytes were stored at 18°C and used in electrophysiological experiments 24 - 48 hours after injection for a period of 1 week.

Whole Cell Two-Electrode Voltage Clamp Recordings

Two-electrode voltage clamp recording was performed using techniques according to those previously reported (Davies et al., 2003, Perkins et al., 2009). Briefly, oocytes were voltage clamped at a membrane potential of -70 mV using oocyte clamp OC-725C (Warner Instruments; Hamden, CT) and the oocyte recording chamber was continuously perfused with MBS $\pm$ anesthetic (isoflurane, lidocaine, or propofol), ethanol and/or glycine using a Dynamax peristaltic pump (Rainin Inst Co., Emeryville, CA) at 3 ml/min using an 18-gauge polyethylene tube (Becton Dickinson, Sparks, MD) and resultant currents were recorded.

Anesthetic Concentration Responses—Concentration response curves for isoflurane, lidocaine, and propofol were generated with oocytes expressing $\alpha 1$ GlyR WT or USERS by measuring the Cl⁻ currents elicited by agonist concentrations producing 2% of the maximal effect (EC₂) $\pm$ 5%. EC₂ glycine concentrations were applied until Cl⁻ currents were within 10% of each other. We studied EC₂ concentrations because GlyR responses are quite sensitive to potentiation at this concentration, whereas, they are much less sensitive at EC₅₀ glycine concentrations and potentiation is not observed at I_{max} concentrations. EC₂ was used as a control pre- and post-anesthetic application. Once stable, oocytes were tested for potentiation by anesthetics. Oocytes were pre-incubated with either isoflurane, lidocaine, or propofol for 60 s followed by co-application of the tested anesthetic and glycine for 30 s at a perfusion rate of 3 ml/min (Davies et al., 2004). Washout periods (5 - 15 min depending on

anesthetic concentration tested) were allowed between glycine and anesthetic applications to ensure complete receptor re-sensitization. WT and USER responses were measured across anesthetic concentrations ranging 1 - 400 μ M for isoflurane and 0.1 - 50 μ M for propofol. Holding currents were not significantly affected during pre-incubation with isoflurane, lidocaine, or propofol alone, i.e. in the absence of glycine. As indicated in previous studies, the final bath concentration of isoflurane is approximately 50% of the prepared concentration due to losses during delivery (Krasowski and Harrison, 2000; Lopreato et al., 2003). To maintain the intended concentration of isoflurane, solutions were prepared immediately prior to application. Furthermore, to minimize loss of isoflurane due to volatilization, containers were sealed with parafilm during perfusion. The anesthetic concentrations tested roughly correspond to physiologically relevant concentrations that cause anesthesia (immobility) in mammals (the human MAC for isoflurane is 260 - 320 μ M; and EC₅₀ concentrations for lidocaine, and propofol are 1 -1.9 μ M, and 0.4 μ M, respectively) (Franks and Lieb, 1994, Downie et al., 1996, Krasowski and Harrison, 1999, Miller, 2010). Although studies have reported direct activation of GlyRs by volatile and intravenous anesthetics, these effects typically occur at concentrations beyond clinical relevance and have been observed to a greater extent in GABA_ARs (Krasowski and Harrison, 1999). Therefore, this effect was not studied in detail.

Ethanol Concentration Response—Electrophysiological recordings were conducted according to the methods described above for anesthetic application.

Data Analysis

Data for each experiment were obtained from a sample size (n) of 6-10 oocytes. Results are expressed as mean $\pm$ SEM. Graphs and statistical analyses were generated by Prism (GraphPad Software Inc., San Diego, CA). The percent potentiation of α 1 GlyR currents were determined by calculating the percent change in glycine EC₂-induced Cl⁻ currents in the presence of anesthetic at each concentration tested. The threshold for anesthetic sensitivity was determined by comparing the α 1 GlyR USER percent potentiation at the lowest concentration against WT percent potentiation. EC₅₀ values were approximated using a non-linear, exponential, least squares (ordinary) fit analysis with a 95% confidence interval. Statistical significance was determined using one-way repeated measures analysis of variance (ANOVA), Bonferroni *post hoc* analysis, and student's t test. Statistical significance was defined as **p* < 0.05.

Results

1. α 1 GlyR USERS 1 and 2 have increased sensitivity to isoflurane

1.1. α 1 GlyR WT—We tested the effects of isoflurane on WT α 1 GlyRs at concentrations ranging 1 - 400 μ M. These concentrations encompass clinical relevance based on published minimum alveolar concentration (MAC) values of inhaled anesthetics that cause immobility in 50% of mammals (Krasowski and Harrison, 2000). We found that isoflurane produced concentration-dependent potentiation of EC₂ glycine-induced Cl⁻ currents in WT α 1 GlyRs with significant effects starting at 100 μ M isoflurane (Fig. 1). There were no significant effects of isoflurane on these receptors at concentrations below 100 μ M. Representative

glycine-activated Cl^- current tracings are shown in Fig. 2. There was minimal direct activation by isoflurane at concentrations $\geq 100 \mu\text{M}$ (Fig. 2), however these effects were not statistically significant ($< 1\%$ of maximal GlyR currents, $p > 0.05$). EC_{50} values are presented in Table 2.

1.2. $\alpha 1$ GlyR USER 1—We tested the effects of isoflurane on $\alpha 1$ GlyR USERS to establish whether the sites responsible for increasing receptor sensitivity to ethanol would similarly increase sensitivity to isoflurane. In agreement with our recent ethanol findings (Naito et al., 2014), isoflurane produced concentration-dependent potentiation of EC_2 glycine-induced currents in $\alpha 1$ GlyR USER 1 starting at $10 \mu\text{M}$ (Figs. 1 and 2). The left shift in the isoflurane concentration response relative to WT $\alpha 1$ GlyRs (Fig. 1) is corroborated by a significant reduction in EC_{50} compared to WT (Table 2). There was a significant reduction in the threshold for isoflurane sensitivity from $100 \mu\text{M}$ in WT $\alpha 1$ GlyRs to $10 \mu\text{M}$ in $\alpha 1$ GlyR USER 1. The magnitude of glycine response to isoflurane significantly increased in a dose-dependent manner in $\alpha 1$ GlyR USER 1 relative to WT $\alpha 1$ GlyRs (Fig 1).

1.3. $\alpha 1$ GlyR USER 2—Isoflurane produced concentration-dependent potentiation of EC_2 glycine-induced currents in $\alpha 1$ GlyR USER 2 starting at $10 \mu\text{M}$ (Fig. 1), significantly reducing the threshold for isoflurane sensitivity 10-fold from $100 \mu\text{M}$ in WT $\alpha 1$ GlyRs. There was a significant decrease in the EC_{50} of $\alpha 1$ GlyR USER 2 compared to WT (Table 2). At concentrations $\geq 10 \mu\text{M}$, the magnitude of isoflurane potentiation significantly increased in $\alpha 1$ GlyR USER 2 relative to WT (Fig. 1). However, the magnitude of isoflurane potentiation was lower than those produced by $\alpha 1$ GlyR USER 1 across all tested concentrations (Fig. 1).

1.4. $\alpha 1$ GlyR USER 3—Isoflurane produced concentration-dependent potentiation of EC_2 glycine-induced currents starting at $100 \mu\text{M}$ in $\alpha 1$ GlyR USER 3, indicating no change in the threshold for isoflurane sensitivity compared to WT $\alpha 1$ GlyRs (Fig. 1). While there was a degree of isoflurane-induced potentiation at $10 \mu\text{M}$, these responses were not significant ($p = 0.1$). In addition, there was no significant difference in the EC_{50} of $\alpha 1$ GlyR USER 3 compared to WT (Table 2). The magnitude of glycine response to isoflurane did not differ significantly compared to WT $\alpha 1$ GlyR (Fig. 1) except at $400 \mu\text{M}$, and was lower than that of other $\alpha 1$ GlyR USERS.

2. $\alpha 1$ GlyR USERS 1 and 2 have increased sensitivity to lidocaine

2.1. $\alpha 1$ GlyR WT—We tested the effects of lidocaine on WT $\alpha 1$ GlyRs at concentrations ranging $0.1 - 100 \mu\text{M}$. The tested concentrations were based on clinical data, in which plasma toxicity in humans is found at concentrations as low as 4.5 mg/kg (Miller, 2010). Lidocaine potentiated EC_2 glycine-induced Cl^- currents in WT $\alpha 1$ GlyRs with significant effects starting at $1 \mu\text{M}$ (Fig. 3). There were no significant effects of lidocaine on these receptors at concentrations below $1 \mu\text{M}$.

2.2. $\alpha 1$ GlyR USER 1—Lidocaine potentiated EC_2 glycine-induced currents in $\alpha 1$ GlyR USER 1 starting at $0.1 \mu\text{M}$ (Fig. 3), significantly reducing the threshold for lidocaine sensitivity from $1 \mu\text{M}$ in WT $\alpha 1$ GlyRs to $0.1 \mu\text{M}$ in $\alpha 1$ GlyR USER 1. The pattern of

glycine response to lidocaine followed an inverted-U profile with a peak response at 1 μ M (Fig. 3). Similar to our previous ethanol findings (Naito et al., 2014) and our current isoflurane findings, α 1 GlyR USER 1 demonstrated increased sensitivity to lidocaine with respect to threshold and magnitude of response (up to 100 μ M) relative to WT α 1 GlyRs.

2.3. α 1 GlyR USER 2—Lidocaine potentiated EC₂ glycine-induced currents in α 1 GlyR USER 2 starting at 0.1 μ M (Fig. 3), significantly reducing the threshold for lidocaine sensitivity 10-fold from 1 μ M in WT α 1 GlyRs. At concentrations below 0.1 μ M, there were no significant effects of lidocaine on these USERS. The magnitude of lidocaine potentiation was significantly greater than WT α 1 GlyRs at 0.1 and 1 μ M. However, the magnitude of lidocaine potentiation did not significantly differ from that of WT at lidocaine concentrations $\geq$ 10 μ M.

2.4. α 1 GlyR USER 3—Lidocaine produced concentration-dependent potentiation of EC₂ glycine-induced currents starting at 1 μ M in α 1 GlyR USER 3 (Fig. 3), indicating no change in the threshold for lidocaine sensitivity compared to WT α 1 GlyRs. The magnitude of glycine response to lidocaine across all tested concentrations also did not differ significantly compared to WT α 1 GlyRs (Fig. 3), and was lower than that of other α 1 GlyR USERS.

3. α 1 GlyR USERS exhibit no significant changes in propofol sensitivity compared to WT

We tested a range of clinically relevant propofol concentrations ranging from 0.1 - 50 μ M. While propofol appeared to inhibit EC₂ glycine-induced Cl⁻ currents in α 1 GlyR WT and USERS 1, 2 and 3 at 0.1 μ M (Fig. 4), these effects were not statistically significant. Propofol potentiated WT α 1 GlyR currents starting at 0.5 μ M in a concentration-dependent manner (Fig. 4). Representative tracings are shown in Fig. 5. In the current study, no significant effects of direct receptor activation by propofol were observed across tested concentrations. There were no significant changes in the threshold for propofol sensitivity relative to WT for all α 1 GlyR USERS. The magnitude of glycine response to propofol in α 1 GlyR USER 1 did not significantly differ from that of WT across all concentrations tested (Fig. 4). However, in α 1 GlyR USERS 2 and 3, the magnitude of glycine response to propofol increased significantly from that of WT α 1 GlyRs and USER 1 at concentrations $\geq$ 50 μ M (Fig. 4). EC₅₀ values indicate no significant differences among α 1 GlyR WT and all USERS (Table 2).

4. Loop 2 mutations exclusive to exon 4 of α 1 GlyRs are sufficient to increase ethanol, isoflurane and lidocaine sensitivity

The Loop 2 region of α 1 GlyRs spans both exons 3 and 4 (Table 1). Although Loop 2 mutations spanning exons 3 and 4 of α 1 GlyR USERS 1 and 2 significantly increased isoflurane and lidocaine sensitivity, Loop 2 mutations in the N-terminal region, exclusive to exon 3, in α 1 GlyR USER 3 did not significantly alter sensitivity to these anesthetic agents with regards to the threshold and magnitude of response relative to WT α 1 GlyRs (Fig. 1). Thus, we tested whether mutations exclusive to exon 4 in the C-terminal end of Loop 2 (Table 1) are sufficient to alter ethanol and anesthetic sensitivity in α 1 GlyR USER 4.

As illustrated in Fig. 6A, ethanol significantly potentiated EC₂ glycine-induced currents starting at 0.25 mM. The magnitude of response of α 1 GlyR USER 4 in response to 0.25 mM ethanol did not significantly differ from the response of WT α 1 GlyRs exposed to 50 mM ethanol (Fig. 6A). In addition, isoflurane produced concentration-dependent potentiation of EC₂ glycine-induced currents starting at 10 μ M (Fig. 6B), indicating a decrease in the threshold for isoflurane sensitivity relative to WT α 1 GlyRs. Furthermore, EC₅₀ significantly decreased in α 1 GlyR USER 4, compared to WT (Table 2). Thus, α 1 GlyR USER 4 exhibited a similar increase in isoflurane sensitivity as that seen in α 1 GlyR USERS 1 and 2 with regards to the threshold, but not the extent of the magnitude of response. Although there was no incremental increase in the magnitude of isoflurane potentiation relative to α 1 GlyR USERS 1 and 2, the responses remained significantly greater than that of WT α 1 GlyRs at 10 μ M and 100 μ M isoflurane (Figs. 1 and 6B).

Lidocaine produced concentration-dependent potentiation of EC₂ glycine-induced currents in α 1 GlyR USER 4 starting at 0.1 μ M (Fig. 6C). Similar to α 1 GlyR USERS 1 and 2, the threshold for lidocaine sensitivity was reduced 10-fold relative to WT α 1 GlyRs. At concentrations below 0.1 μ M, there were no significant effects of lidocaine on these receptors. While the magnitude of lidocaine potentiation increased in α 1 GlyR USER 4 relative to WT, these results were not significantly greater than those produced by USER 1 (Figs. 3 and 6C). In agreement with our initial findings with propofol, the threshold for propofol sensitivity, the EC₅₀ (Table 2), as well as the magnitude of propofol response in α 1 GlyR USER 4 did not significantly differ from that of WT α 1 GlyRs and other α 1 GlyR USERS (Fig. 6D).

Discussion

The present study tested the hypothesis that structural manipulations of EC Loop 2 that affect ethanol sensitivity of human α 1 GlyR USERS would also affect sensitivity to the volatile anesthetic, isoflurane, but would not significantly alter the effects of the intravenous anesthetic, propofol. We also tested the effects of lidocaine on these USERS to understand the possible sites and mechanisms of this local anesthetic on α 1 GlyRs. This was accomplished by characterizing the selectivity of α 1 GlyR USERS to volatile (isoflurane), local (lidocaine) and intravenous (propofol) anesthetics. In agreement with earlier findings, ethanol, isoflurane, and lidocaine sites of action do not appear to overlap with those of propofol action on α 1 GlyRs, as indicated by the lack of change in the threshold for propofol sensitivity of α 1 GlyR USERS with respect to WT α 1 GlyRs (Figs. 1, 3, 4 and 6). These findings suggest that the sites of propofol action in α 1 GlyRs are distinct from those of ethanol and isoflurane, reinforcing similar findings reported in the TM domain of GABA_ARs (Krasowski et al., 1998; Bali et al., 2004). Overall, our findings confirm that Loop 2 plays an important role in mediating ethanol, isoflurane, and lidocaine action by decreasing the threshold for sensitivity and increasing the magnitude of α 1 GlyR response to these agents. Furthermore, we identified the C-terminal region of Loop 2 as a key site responsible for the enhancement of GlyR function by ethanol, isoflurane, and lidocaine.

The Loop 2 mutations spanning both exons 3 and 4 (positions 50 – 59) in α 1 GlyR USERS 1 and 2 that significantly increased ethanol sensitivity in prior studies (Naito et al., 2014), also

increased isoflurane and lidocaine sensitivity relative to WT $\alpha 1$ GlyRs by demonstrating a 10-fold decrease in threshold (Figs. 1 and 3). In contrast, Loop 2 mutations that are exclusive to the N-terminal region (exon 3) in $\alpha 1$ GlyR USER 3 shared similar characteristics in the threshold and magnitude of potentiation to WT $\alpha 1$ GlyRs in response to isoflurane and lidocaine (Figs. 1 and 3). Interestingly, the same mutations in exon 3 of $\alpha 1$ GlyR USER 3 imparted ultra-sensitivity to ethanol and normalized the agonist sensitivity back to WT levels (Naito et al., 2014). These findings suggest that certain regions within Loop 2 may differentially regulate allosteric modulation of $\alpha 1$ GlyRs. In fact, merely two point mutations, D57E and R59T, at the C-terminal end of Loop 2 in exon 4 of $\alpha 1$ GlyR USER 4 were sufficient to significantly increase receptor sensitivity to ethanol, isoflurane, and lidocaine (Figs. 6A, B, C). Furthermore, the difference between maintaining the WT residues at positions 57 and 59 of the C-terminal end of Loop 2 (USER 3), and mutating this region (USER 4), seems to control isoflurane and lidocaine enhancement of $\alpha 1$ GlyRs. Altogether, these results suggest that structural changes at the C-terminal end of Loop 2 appear to be a key determinant that is both sufficient, and importantly, selective to increase ethanol, isoflurane, and lidocaine sensitivity of $\alpha 1$ GlyRs. Furthermore, this study is the first to report an extracellular site of action for lidocaine on $\alpha 1$ GlyRs.

Interestingly, there were no significant differences in the threshold for propofol sensitivity between WT and USER $\alpha 1$ GlyRs (Fig. 3). While propofol enhanced the magnitude of potentiation of $\alpha 1$ GlyR USERS at concentrations $\geq 0.5 \mu\text{M}$ relative to WT, there were no significant differences in the magnitude of propofol-induced potentiation of $\alpha 1$ GlyR USERS compared to WT at clinically relevant concentrations between 0.5 and 10 μM (Fig 3). These findings indicate that the mutations in Loop 2 that increase ethanol, isoflurane, and/or lidocaine sensitivity in $\alpha 1$ GlyRs do not influence propofol sensitivity, and further reinforce findings by Krasowski and colleagues that the sites in the $\alpha 2$ or $\beta 1$ subunits of GABA_ARs that abolish isoflurane potentiation do not affect propofol potentiation (Krasowski et al., 1998). More importantly, these results highlight the selective nature of Loop 2 mutations to increase $\alpha 1$ GlyR sensitivity to certain anesthetic agents, but not all. Recent studies have reported propofol sites at the interface between the EC and TM 2 domains, specifically between adjacent TM 2 helices in the $\beta 3$ subunit of GABA_ARs (Franks, 2015). Our current findings suggest that EC Loop 2 is not a critical region for propofol action and modulation of $\alpha 1$ GlyRs.

The distinguishing features that account for the increase in sensitivity to isoflurane and lidocaine in $\alpha 1$ GlyR USERS 1, 2, and 4 (relative to WT and USER 3) appear to be attributed to the differences between the C-terminal regions of Loop 2. As reported earlier, the charged residue arginine R59 at the C-terminal end of Loop 2 increases the propensity to form salt-bridge interactions with N-terminal residues across Loop 2, for instance, with the histidine H50 in $\alpha 1$ GlyR USER 3 (Naito 2014). Thus, the introduction of charged residues at the C-terminal end may contribute to conformational changes to the structure of Loop 2, preventing accessibility of allosteric modulators from acting at this site. Further studies should explore the gating properties as a result of these Loop 2 mutations on USERS to determine the functional changes induced in $\alpha 1$ GlyR USERS.

Through $\alpha 1$ GlyR USER 4, we demonstrate that mutations within a single exon create receptors with high sensitivity to several anesthetic molecules. These findings could potentially provide a discrete DNA sequence for searching databases for other receptors that might share high sensitivity to alcohol and anesthetics. This goal is important because, so far, only GABA_ARs containing delta subunits have exhibited sensitivity to alcohol in the range of mild intoxication (18 mM) (Olsen et al., 2007, Wallner et al., 2003). It is possible that there are undiscovered receptors that share the high alcohol sensitivity of USERS; these receptors might help explain the constellation of effects on coordination, balance, fear conditioning, and memory that corresponds to mild intoxication.

In agreement with earlier studies, the *in vivo* expression of USERS in genetically engineered animals may provide a novel tool for investigation of single receptor subunit populations sensitized to extremely low anesthetic concentrations that do not affect other receptors in the nervous system. While the current study tested $\alpha 1$ GlyR USER sensitivity to anesthetics in the presence of EC₂ agonist concentrations, a recent study reported that the effects of ethanol potentiation (30 mM) diminish in the presence of higher agonist concentrations (EC₅₀) in $\alpha 4\beta 1\delta$ GABA_ARs (Bowen et al., 2015), suggesting the possibility that the effects of USER potentiation may be diminished *in vivo*. However, a recent investigation reported a glycine-dependent mechanism of regulating neuronal excitability in the orbitofrontal cortex (OFC) by ethanol exposure, providing the first line of evidence that ethanol-induced potentiation of GlyRs are the primary mediators of inhibitory projections in the OFC (Badanich et al., 2013). Importantly, while both GABA_ARs and GlyRs are expressed in OFC neurons, GABA_ARs were not sensitive to behaviorally relevant concentrations of ethanol. Thus, the inhibitory projections induced by ethanol in the OFC are glycine-dependent and not regulated through GABA_AR activity (Badanich et al., 2013). Furthermore, application of the selective GlyR antagonist, strychnine, did not significantly alter tonic glycine-mediated currents; thus implicating the existence of extrasynaptic GlyRs, and GlyR transporters, that maintain low levels of extracellular glycine (Badanich et al., 2013). Additional work reported similar findings of extrasynaptic GlyRs in the dorsal raphe nucleus that largely govern ethanol-induced tonic inhibition (Maguire et al., 2014). In light of these findings, GlyR USERS have the potential to significantly affect excitability and neurochemical cascades in certain brain regions in the presence of low anesthetic concentrations. Thus, testing these USERS *in vivo* would provide unambiguous results about the role of individual receptor subunits in transducing the effects of anesthetics without concerns about collateral effects on other nervous systems that often confound these studies.

Conclusions

Collectively, our results demonstrate that the Loop 2 region of $\alpha 1$ GlyRs is a site of ethanol, isoflurane, and lidocaine action. Moreover, we demonstrate that subtle structural manipulations (D57E, R59T) at the C-terminal end of Loop 2 are sufficient to significantly increase ethanol, isoflurane, and lidocaine sensitivity in $\alpha 1$ GlyR USER 4. Our results also add to the body of literature that sites of ethanol, isoflurane, and lidocaine action do not overlap with those of propofol. Importantly, these findings demonstrate the selectivity of $\alpha 1$ GlyR USERS in that increases in receptor sensitivity are not solely limited to ethanol, but also apply to isoflurane and lidocaine. The common structural homology among the Cys-

loop superfamily of receptors implies that similar increases in anesthetic sensitivity via Loop 2 may also apply across multiple receptors and receptor subunits. Moreover, the advantage of harboring mutations to a single exon in Loop 2 may promote genetic incorporation to develop genetically engineered USER knock-in animals. Ultimately, USERS allow activation of specific receptor subunits at concentrations that do not affect WT receptors, and could potentially provide a valuable tool to understand the functional role of specific receptors in mediating the behavioral effects of anesthetic action.

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Abbreviations

GlyR	glycine receptor
GABA_AR	γ-aminobutyric acid type A receptor
nACh	nicotinic acetylcholine
5HT₃	5-hydroxytryptamine ₃
I_{max}	maximum current
CNS	central nervous system
EC₅₀	half-maximal effective concentration
EC₂	effective concentration at 2% of maximal response
WT	wild type
AUD	alcohol use disorder
USER	ultra-sensitive ethanol receptor
TM	transmembrane
EC	extracellular
IC	intracellular
MAC	minimum alveolar concentration
SEM	standard error of the mean
ANOVA	analysis of variance
OFC	orbitofrontal cortex

- Loop 2 of $\alpha 1$ GlyRs is a critical site of ethanol, isoflurane, and lidocaine action
- USRs have up to a 10-fold increase in sensitivity to isoflurane and lidocaine
- Sites of ethanol, isoflurane, and lidocaine action do not overlap with propofol
- Loop 2 C-terminus regulates GlyR sensitivity to ethanol, isoflurane, and lidocaine

Figure 1. α1 GlyR USERS 1 and 2 have increased sensitivity to isoflurane compared to WT
 Isoflurane-induced potentiation of glycine EC₂-activated Cl⁻ currents in *Xenopus* oocytes expressing α1 GlyR USERS are shown. Values for isoflurane potentiation are presented as percentage of glycine EC₂ control. The glycine EC₂ concentrations utilized ranged from 5 - 10 μM in USERS and 18 - 25 μM for WT. The threshold for isoflurane sensitivity in α1 GlyR USERS 1 and 2 was 10 μM, and 100 μM for α1 GlyR WT and USER 3. Significant increases in the magnitude of isoflurane response for α1 GlyR USERS compared to WT are denoted by * $p < 0.05$. There were no significant differences in the threshold for isoflurane sensitivity in α1 GlyR USER 3 relative to WT. Each data point represents the mean $\pm$ SEM from 6 - 9 oocytes.

Figure 2. Representative tracings of $\alpha 1$ GlyR USERS in response to isoflurane

Two-electrode voltage clamp electrophysiology tracings of homomeric $\alpha 1$ GlyR WT and $\alpha 1$ GlyR USERS expressed in *Xenopus* oocytes in response to isoflurane. (A) $\alpha 1$ GlyR WT in response to 100 μ M isoflurane; (B) $\alpha 1$ GlyR USER 1 in response to 10 μ M isoflurane; (C) $\alpha 1$ GlyR USER 2 in response to 10 μ M isoflurane; and (D) $\alpha 1$ GlyR USER 3 in response to 10 μ M isoflurane. Effects of isoflurane were tested with EC₂ glycine (5 - 25 μ M) and the membrane potential was -70 mV.

Figure 3. α1 GlyR USERS 1 and 2 have increased sensitivity to lidocaine compared to WT
Lidocaine-induced potentiation of glycine EC₂-activated Cl⁻ currents in *Xenopus* oocytes expressing α1 GlyR USERS are shown. Values for lidocaine potentiation are presented as percentage of glycine EC₂ control. The glycine EC₂ concentrations utilized ranged from 5 - 10 μM in USERS and 18 - 25 μM for WT. The threshold for lidocaine sensitivity for α1 GlyR USERS 1 and 2 was 0.1 μM, and 1 μM for α1 GlyR WT and USER 3. Significant increases in the magnitude of lidocaine response for α1 GlyR USERS compared to WT are denoted by * $p < 0.05$. Each data point represents the mean $\pm$ SEM from at least 6 - 9 oocytes.

Figure 4. $\alpha 1$ GlyR USERS show no changes to propofol sensitivity compared to WT

Propofol-induced potentiation of glycine EC₂-activated Cl⁻ currents in *Xenopus* oocytes expressing $\alpha 1$ GlyR USERS are shown. Values for propofol potentiation are presented as percentage of glycine EC₂ control. There were no significant changes in the threshold for propofol sensitivity between $\alpha 1$ GlyR USERS and WT (0.5 μM). However, there was a significant increase in the magnitude of propofol response in $\alpha 1$ GlyR USERS 2 and 3 compared to WT at 50 μM (denoted by * $p < 0.05$). Each data point represents the mean $\pm$ SEM from at least 6 - 9 oocytes.

Figure 5. Representative tracings of $\alpha 1$ GlyR USERS in response to propofol

Two-electrode voltage clamp electrophysiology tracings of homomeric $\alpha 1$ GlyR WT and $\alpha 1$ GlyR USERS expressed in *Xenopus* oocytes in response to propofol. (A) $\alpha 1$ GlyR WT in response to 0.5 μ M propofol; (B) $\alpha 1$ GlyR USER 1 in response to 0.5 μ M propofol; (C) $\alpha 1$ GlyR USER 2 in response to 0.5 μ M propofol; and (D) $\alpha 1$ GlyR USER 3 in response to 0.5 μ M propofol. Effects of propofol were tested with EC₂ glycine (5 - 25 μ M) and the membrane potential was -70 mV.

Figure 6. $\alpha 1$ GlyR USER 4 demonstrates increased sensitivity to ethanol, isoflurane, and lidocaine, but not propofol

$\alpha 1$ GlyR WT and USER 4 glycine EC₂-activated Cl⁻ currents in response to (A) 0.25 mM and 50 mM ethanol; (B) isoflurane; (C) lidocaine; (D) propofol. The threshold for ethanol sensitivity was reduced from 50 mM in $\alpha 1$ GlyR WT to 0.25 mM in $\alpha 1$ GlyR USER 4. The threshold for isoflurane sensitivity was reduced from 100 μ M in WT to 10 μ M in $\alpha 1$ GlyR USER 4. The threshold for lidocaine sensitivity was reduced from 1 μ M in WT to 0.1 μ M in $\alpha 1$ GlyR USER 4. There were no significant changes to the threshold for propofol sensitivity and the magnitude of propofol response in $\alpha 1$ GlyR USER 4 relative to WT. Each data point represents the mean $\pm$ SEM from at least 6 - 9 oocytes.

TABLE 1

Loop 2 sequence alignment and mutations for the human WT and $\alpha 1$ GlyR USERS.

$\alpha 1$ GlyR	Loop 2 Sequence	Mutations
WT	50 <u>SIAETTM</u> DYR	
USER 1	50 HISEAN MEYT	S50H, A52S, T54A, T55N, D57E, R59T
USER 2	50 HIAEAN MEYT	S50H, T54A, T55N, D57E, R59T
USER 3	50 HISEAN MDYR	S50H, A52S, T54A, T55N
USER 4	50 <u>SIAETTM</u> EYT	D57E, R59T

Loop 2 of $\alpha 1$ GlyR spans exon 3 (indicated by solid line) and exon 4 (indicated by dotted line). Mutations present in each USER are indicated in bold. Loop 2 of USER 1 represents the Loop 2 sequence of δ GABA_ARs. [A: Alanine, D: Aspartate, E: Glutamate, H: Histidine, I: Isoleucine, M: Methionine, N: Asparagine, R: Arginine, S: Serine, T: Threonine, Y: Tyrosine].

TABLE 2

Approximate EC₅₀ values for isoflurane and propofol in WT and $\alpha 1$ GlyR USERS.

$\alpha 1$ GlyR	Isoflurane EC ₅₀ (uM)	Propofol EC ₅₀ (uM)
WT	228.3 $\pm$ 21.5	19.3 $\pm$ 6.3
USER 1	78.3 $\pm$ 13.9*	17.1 $\pm$ 14.2
USER 2	174.2 $\pm$ 15.7*	10.6 $\pm$ 15.5
USER 3	242.4 $\pm$ 11.6	15.2 $\pm$ 17.3
USER 4	89.4 $\pm$ 9.7*	15.1 $\pm$ 8.2

EC₅₀ values $\pm$ standard error of the mean (SEM) were approximated using standard curve-fitting analysis for isoflurane and propofol.

* Represent statistically significant differences in EC₅₀ values compared to WT $\alpha 1$ GlyRs.