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MANIPULATIONS OF EXTRACELLULAR LOOP 2 IN α1 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 ($GABA_A$Rs) 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 α1 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 α1 GlyR USERs in *Xenopus* oocytes and tested the effects of three classes of anesthetics, isoflurane (volatile), propofol (intravenous), and lidocaine (local), known to enhance 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 α1 GlyRs. Interestingly, we also found that structural manipulations in the C-terminal end of Loop 2 were sufficient and selective for α1 GlyR modulation by ethanol, isoflurane, and lidocaine. These studies are the first to report the extracellular region of α1 GlyRs as a site of lidocaine action. Overall, the findings suggest that Loop 2 of α1 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 α1 GlyRs within the extracellular Loop 2 region.

Key words: α1 GlyRs, Loop 2, isoflurane, lidocaine, ethanol, ligand-gated ion channel.

INTRODUCTION

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-hydroxytryptamine$_3$ (5-HT_3) 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). α1 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

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Abbreviations: EC, extracellular; EC_2, effective concentration at 2% of maximal response; EC_{50}, half-maximal effective concentration; $GABA_A$R, γ-aminobutyric acid type A receptor; GlyR, glycine receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC, intracellular; I_{max}, maximum current; MAC, minimum alveolar concentration; OFC, orbitofrontal cortex; SEM, standard error of the mean; TM, transmembrane; USER, ultra-sensitive ethanol receptor; WT, wild type.

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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 α1 GlyRs, and α2 and β1 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, 2001; Nury et al., 2011; Sauguet et al., 2013). Mutations in the TM domain of $GABA_A$Rs at positions 270 or 291 in α2, or at positions 265 or 286 in β2 rendered these receptors insensitive to isoflurane, while preserving receptor sensitivity to propofol (Krasowski et al., 1998). Thus, these findings suggest that the sites of ethanol and isoflurane action on $GABA_A$Rs differs from those of propofol action.

The structural homology among Cys-loop receptors implies that the sites of ethanol and anesthetic action identified in $GABA_A$Rs 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 α1 GlyRs altered ethanol sensitivity (Davies et al., 2004; Crawford et al., 2007, 2008; Perkins et al., 2008), but did not affect propofol sensitivity (Mascia et al., 1996). These differences have also been reported in $GABA_A$Rs (Bali and Akabas, 2004). In addition, recent studies have characterized position 380 in the large IC loop between TM 3 and TM 4 of α1 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 α1 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 α1 GlyRs that are distinct from those of propofol.

Interestingly, previous studies indicate that $GABA_A$Rs 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 $GABA_A$Rs containing the δ subunit and GlyR α1 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 α1 GlyRs. Notably, we identified EC Loop 2 (positions 50–59) as a critical site of ethanol action that mediates ethanol sensitivity of GlyRs and $GABA_A$Rs (Crawford et al., 2007, 2008; Olsen et al., 2007; Perkins et al., 2008, 2009, 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 α1 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 α1 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 α2 GlyRs and heteromeric α1 and γ2 subunits of α1β2γ2 $GABA_A$Rs (Naito et al., 2014). Interestingly, reversion of the residues in the C-terminal region of Loop 2 in both α1 and α2 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 EC_{50} sensitivity relative to WT α1 GlyRs (Naito et al., 2014). Structural manipulation of the C-terminal region of Loop 2 in γ2 $GABA_A$Rs 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 $GABA_A$Rs 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 α1 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 α1 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