Glycine and GABA_A Ultra-Sensitive Ethanol Receptors as Novel Tools for Alcohol and Brain Research[§]

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ABSTRACT

A critical obstacle to developing effective medications to prevent and/or treat alcohol use disorders is the lack of specific knowledge regarding the plethora of molecular targets and mechanisms underlying alcohol (ethanol) action in the brain. To identify the role of individual receptor subunits in ethanol-induced behaviors, we developed a novel class of ultra-sensitive ethanol receptors (USERS) that allow activation of a single receptor subunit population sensitized to extremely low ethanol concentrations. USERS were created by mutating as few as four residues in the extracellular loop 2 region of glycine receptors (GlyRs) or γ -aminobutyric acid type A receptors (GABA_ARs), which are implicated in causing many behavioral effects

linked to ethanol abuse. USERS, expressed in *Xenopus* oocytes and tested using two-electrode voltage clamp, demonstrated an increase in ethanol sensitivity of 100-fold over wild-type receptors by significantly decreasing the threshold and increasing the magnitude of ethanol response, without altering general receptor properties including sensitivity to the neurosteroid, allopregnanolone. These profound changes in ethanol sensitivity were observed across multiple subunits of GlyRs and GABA_ARs. Collectively, our studies set the stage for using USER technology in genetically engineered animals as a unique tool to increase understanding of the neurobiological basis of the behavioral effects of ethanol.

Introduction

Alcohol use disorders (AUDs) have a serious impact on global health and economics. In the United States alone, AUDs affect more than 18 million people, cause approximately 100,000 deaths, and cost over \$200 billion annually (Harwood, 2000; Grant et al., 2004; Rehm et al., 2009; Bouchery et al., 2011; Litten

et al., 2012). Unfortunately, the success rate of available drugs has been limited, with approximately 70% of patients relapsing back to heavy drinking within the first year of treatment (Johnson, 2008; Litten et al., 2012). Thus, the development of new pharmacotherapies to treat AUDs is an important endeavor.

A critical barrier to the development of medications to prevent and/or treat AUDs has been the lack of specific

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knowledge about where and how alcohol (ethanol) acts in the brain and the resultant neurochemical cascades leading to behavioral change. This paucity of knowledge largely reflects the physical-chemical mechanism of ethanol action and low potency that requires millimolar concentrations to alter brain function. The resultant lack of a high-affinity structure-activity relationship precludes the classic approach of using specific agonists and antagonists to identify the sites and mechanisms of ethanol action (Deitrich et al., 1989; Little, 1991). This problem is further complicated by the multiple receptor subunit combinations affected by ethanol and the complex acute and chronic mechanisms of ethanol action (Trudell et al., 2014).

Current strategies that knock out (KO) receptor subunits or knock in (KI) mutant ethanol-insensitive receptors have provided important insights (Blednov et al., 2003, 2010; Chandra et al., 2008; Liang et al., 2008; Moore et al., 2010; Trudell et al., 2014). However, these studies involving functional deletion or reduction in receptor sensitivity to ethanol require the use of relatively high ethanol concentrations (10–50 mM) (Borghese et al., 2006; Blednov et al., 2010, 2011) that may affect other native receptor systems and signaling pathways that modulate additional physiologic processes (Chandra et al., 2008; Harris et al., 2008; Liang et al., 2008; Kumar et al., 2009; Howard et al., 2011; Kelm et al., 2011). In comparison, 17 mM ethanol is equivalent to the 0.08% blood ethanol concentration (BEC) legal driving limit in the United States (Wallner et al., 2003; Ogden and Moskowitz, 2004). Moreover, KO studies can be complicated by developmental compensatory responses that can alter the expression levels of different receptor subtypes/families in the genetically modified animals (Brickley et al., 2001; Peng et al., 2002; Homanics et al., 2005; Ponomarev et al., 2006). Thus, the observed changes in ethanol-induced behaviors may be a result of the indirect effects of the interplay among compensatory responses resulting from the gene replacement, thereby complicating the interpretation of results.

Glycine receptors (GlyRs) and γ -aminobutyric acid type A receptors (GABA_ARs) are the primary inhibitory ligand-gated ion channels (LGICs) in the brain that have been implicated in causing many acute and chronic behavioral effects of ethanol, including tolerance, dependence, reward, anxiolysis, motor ataxia, impaired cognition, sedation, and aggression (Deitrich et al., 1989; Grobin et al., 1998; Follesa et al., 2006; Olsen et al., 2007; Lobo and Harris, 2008; Kumar et al., 2009; Dutertre et al., 2012). Prior studies identified extracellular loop 2 of GlyRs and GABA_ARs as a site of ethanol action, and found that structural modifications in this region profoundly influence receptor sensitivity to ethanol (Mascia et al., 1996b; Davies et al., 2003, 2004; Crawford et al., 2007; Perkins et al., 2008). This initial work found that replacing loop 2 of $\alpha 1$ GlyR and $\gamma 2$ GABA_AR subunits with loop 2 of the more ethanol-sensitive δ GABA_ARs significantly increased ethanol sensitivity of the resultant receptor (Perkins et al., 2009), and identified important physical-chemical properties of loop 2 that alter receptor sensitivity to ethanol and agonist (Crawford et al., 2008; Perkins et al., 2012).

The objective of our current study was to further characterize loop 2 as a novel tool that would distinguish the contribution of individual receptor subunits in ethanol action. Thus, we manipulated the physical-chemical characteristics

of loop 2 to develop ultra-sensitive ethanol receptors (USERs) in GlyRs and GABA_ARs that 1) are sensitive to ethanol concentrations lower than those that affect any other receptor system, 2) have wild-type (WT)-like receptor properties, and 3) can be produced across multiple receptor subunits of LGICs. The unique characteristics of USERs provide the rationale for exploiting these receptors in genetically engineered animals to link the role of specific receptor subunits in behaviors mediated by ethanol action without affecting other targets. Ultimately, USERs would increase understanding of the neurologic basis of AUDs.

Materials and Methods

Adult female *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). Gentamicin, 3-aminobenzoic acid ethyl ester, glycine, GABA, ethanol, and collagenase were purchased from Sigma-Aldrich (St. Louis, MO), and allopregnanolone (3 α ,5 α -THP) was purchased from Steraloids (Newport, RI). All chemicals used were of reagent grade. Glycine and GABA stock solutions were prepared from powder and diluted with modified Barth's solution (MBS) containing (in millimolar concentrations) 88 NaCl, 1 KCl, 10 HEPES, 0.82 MgSO₄, 2.4 NaHCO₃, 0.91 CaCl₂, and 0.33 Ca(NO₃)₂, adjusted to pH 7.5 (Davies et al., 2003). Ten millimolar 3 α ,5 α -THP stock solution was prepared from powder and diluted with dimethylsulfoxide (DMSO) and serially diluted with MBS (DMSO $\leq$ 0.05%) immediately prior to testing. Pilot studies found that DMSO at this concentration, with or without agonist, had no appreciable effect on $\alpha 1\beta 2\gamma 2$ GABA_AR currents in WT or mutant receptors.

Mutagenesis and Expression of GlyRs and GABA_ARs in Oocytes

Homologous amino acid sequences of extracellular loop 2 regions of WT $\alpha 1$ and $\alpha 2$ GlyR and $\alpha 1$ and $\gamma 2$ GABA_AR subunits were identified (Table 1). For the purpose of this study, loop 2 is defined as the following amino acid positions: 50–59 in the $\alpha 1$ GlyR subunit, 57–66 in the $\alpha 2$ GlyR subunit, 43–52 in the $\alpha 1$ GABA_AR subunit, and 64–73 in the $\gamma 2$ GABA_AR subunit (Mihic et al., 1997; Perkins et al., 2009). $\alpha 1$ GlyR and $\gamma 2$ GABA_AR USER 1 were developed according to methods described previously (Perkins et al., 2009). In brief, site-directed mutagenesis was performed in $\alpha 1$ GlyR and $\gamma 2$ GABA_AR subunit cDNA so that the resulting receptors' loop 2 regions were identical to the δ GABA_AR loop 2 to produce $\alpha 1$ GlyR and $\gamma 2$ GABA_AR USER 1. Subsequently, we used prior structure activity relationships gleaned from mutational studies in loop 2 to develop second-generation GlyR and GABA_AR USERs. Site-directed mutagenesis was performed by subcloning human $\alpha 1$ and $\alpha 2$ GlyR and $\alpha 1$ and $\gamma 2$ GABA_AR cDNA into mammalian vector pCIS2 or pBK-CMV using the 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 isolated and injected with homomeric $\alpha 1$ or $\alpha 2$ GlyR cDNA (1 ng/32 nl) or $\alpha 1\beta 2\gamma 2$ GABA_AR cDNA (1:1:10 ratio for a total volume of 1 ng of $\alpha 1\beta 2\gamma 2$).

Oocytes were stored in incubation medium [ND96 supplemented with 2 mM sodium pyruvate, 50 mg/ml gentamicin, and 10 ml of heat-inactivated HyClone horse serum (VWR, San Dimas, CA), adjusted to pH 7.5] in Petri dishes (VWR). 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.

ABBREVIATIONS: AUD, alcohol use disorder; BEC, blood ethanol concentration; DMSO, dimethylsulfoxide; DREADD, designer receptors exclusively activated by designer drugs; GABA_AR, γ -aminobutyric acid type A receptor; GlyR, glycine receptor; $I_{\max}$, maximum current; KI, knock in; KO, knock out; LGIC, ligand-gated ion channel; MBS, modified Barth's solution; USER, ultra-sensitive ethanol receptor; WT, wild-type.

TABLE 1

Loop 2 sequence alignment and receptor characteristics for the human WT and $\alpha 1$ and $\alpha 2$ GlyR USERS and $\alpha 1$ and $\gamma 2$ GABA_AR USERS

Loop 2 of $\alpha 1$ and $\alpha 2$ GlyRs spans exon 3 (indicated by solid line) and exon 4 (indicated by dotted line). In WT $\alpha 1$ and $\gamma 2$ GABA_AR USERS, the GABA_AR isoform represented is $\alpha 1\beta 2\gamma 2$. Loop 2 of $\alpha 1$ and $\gamma 2$ GABA_AR USER subunits spans exon 3. GlyR and GABA_AR EC₅₀, Hill slope, and $I_{\max}$ are presented as the mean $\pm$ S.E.M. from at least 4–23 oocytes. *t* Tests revealed no significant differences in $I_{\max}$ and Hill slope between GlyR and GABA_AR WT and USERS. There were no statistically significant differences in the EC₅₀ values of $\alpha 1$ GlyR USER 3, $\alpha 2$ GlyR USER 1, and $\alpha 1$ GABA_AR USER 1 compared with those of respective WT receptors. EC₅₀ values of $\alpha 1$ GlyR USERS 1 and 2 and $\gamma 2$ GABA_AR USERS 1–4 were significantly reduced compared with those of respective WT receptors. EC₅₀ values of $\alpha 1$ GlyR USER 3, $\alpha 2$ GlyR USER 1, and $\alpha 1$ GABA_AR USER 1 were not significantly different from respective WT receptors.

USER	Loop 2 Sequence	EC ₅₀ μM	Hill Slope	$I_{\max}$ $n\text{A}$
$\alpha 1$ GlyR WT	50 SIAETTM ---- DYR	92.3 $\pm$ 8.6	2.3 $\pm$ 0.3	16,658 $\pm$ 923
$\alpha 2$ GlyR WT	57 SVTETTM ---- DYR	115.0 $\pm$ 21.0	1.9 $\pm$ 0.4	16,658 $\pm$ 923
$\alpha 1$ GlyR USER 1 S50H, A52S, T54A, T55N, D57E, R59T	57 HISEANMEYT	22.9 $\pm$ 4.1*	1.8 $\pm$ 0.1	13,186 $\pm$ 761
$\alpha 1$ GlyR USER 2 S50H, T54A, T55N, D57E, R59T	50 HIAEANMEYT	47.3 $\pm$ 8.0*	2.3 $\pm$ 0.2	17,710 $\pm$ 1290
$\alpha 1$ GlyR USER 3 S50H, A52S, T54A, T55N	50 HISEANMDYR	80.7 $\pm$ 9.8	1.6 $\pm$ 0.1	13,467 $\pm$ 854
$\alpha 2$ GlyR USER 1 S57H, V58I, T59S, T61A, T62N	57 HISEANMDYR	79.8 $\pm$ 16.0	1.6 $\pm$ 0.2	13,467 $\pm$ 854
$\alpha 1\beta 2\gamma 2$ GABA _A R WT				
$\alpha 1$ GABA _A R WT	43 PVSDHDM ---- EY ---- T	264.0 $\pm$ 23.0	1.1 $\pm$ 0.0	5558 $\pm$ 952
$\gamma 2$ GABA _A R WT	64 PVNAINMEYT			
$\alpha 1$ GABA _A R USER 1 P43H, V44I, D46E, H47A, D48N	43 HISEANMEYT	301.0 $\pm$ 15.0	1.0 $\pm$ 0.0	3146 $\pm$ 258
$\gamma 2$ GABA _A R USER 1 P64H, V65I, N66S, A67E, I68A	64 HISEANMEYT	31.0 $\pm$ 6.0*	1.6 $\pm$ 0.2	5018 $\pm$ 1121
$\gamma 2$ GABA _A R USER 2 P64H, V65I, A67E, I68A	64 HINEANMEYT	31.0 $\pm$ 4.0*	1.1 $\pm$ 0.1	5637 $\pm$ 1275
$\gamma 2$ GABA _A R USER 3 P64H, V65I, N66S, I68A	64 HISAANMEYT	83.0 $\pm$ 7.0*	1.2 $\pm$ 0.4	5402 $\pm$ 2396
$\gamma 2$ GABA _A R USER 4 P64H, V65I, N66S, A67E, I68A, E71D, T73R	64 HISEANMDYR	39.0 $\pm$ 12.0*	1.0 $\pm$ 0.1	5291 $\pm$ 1202

**P* < 0.05 compared to respective WT receptors.

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). In brief, 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 $\pm$ ethanol and/or agonist using a Dynamax peristaltic pump (Rainin Instrument Co., Emeryville, CA) at 3 ml/min using an 18-gauge polyethylene tube (Becton Dickinson, Sparks, MD), and resultant currents were recorded.

Application of Agonist. GlyR or GABA_AR WT and USERS were exposed to $1 \mu\text{M}$ to 10 mM glycine or GABA for 30 seconds at a rate of 3 ml/min, with 5- to 15-minute washout periods between applications to ensure complete receptor resensitization.

Application of Ethanol. Potentiation of Cl^- currents by ethanol is difficult to measure since the degree of ethanol potentiation is decreased and probability of receptor desensitization is increased when using agonist concentrations close to EC₅₀ (Mihic et al., 1994a,b; Mascia et al., 1996b). In general, most studies involving mutagenesis of GlyRs and GABA_AR often result in changes in EC₅₀ for agonists, whereas the maximum current remains unchanged (Mascia et al., 1996a,b; Davies et al., 2004). However, in these mutant receptors, the agonist concentrations to achieve EC₂ to EC₁₀ are not significantly different from that of WT. Therefore, agonist concentrations producing 2% of the maximal effect (EC₂) were used. EC₂ $\pm$ 5% agonist concentrations were applied until Cl^- currents were stable, i.e., within 10% of each other. EC₂ was used as a control pre- and postethanol treatment. Once stable, oocytes were tested for ethanol potentiation. Oocytes were preincubated with ethanol for 60 seconds followed by coapplication of ethanol and agonist for 30 seconds at a perfusion rate of 3 ml/min (Davies et al., 2004). Washout periods (5–15 minutes depending on ethanol concentration tested) were allowed between agonist and drug applications to ensure complete receptor resensitization. WT and mutant receptor responses were measured across ethanol concentrations ranging 0.025–50 mM for $\alpha 1$ GlyRs and 0.1–50 mM for $\alpha 2$ GlyRs. $\alpha 1$ and $\gamma 2$ GABA_AR USER responses were measured across ethanol concentrations ranging 0.1–50 mM, and were compared with $\alpha 1\beta 2\gamma 2$ GABA_AR WT. Holding currents were not significantly affected during preincubation with ethanol, i.e., in the absence of agonist.

Application of Allopregnanolone (3 α ,5 α -THP). To investigate the effects of endogenous neurosteroids on USERS, we tested two physiologically relevant concentrations of 3 α ,5 α -THP (20 and 100 nM) on $\alpha 1\beta 2\gamma 2$ GABA_AR and $\alpha 1$ and $\gamma 2$ GABA_AR USERS. The concentrations of 3 α ,5 α -THP were based on findings describing 3 α ,5 α -THP levels in the temporal cortex of postmortem brain tissue in cognitively intact patients ranging 5.644–32.11 ng/g (18–100 nM) (Naylor et al., 2010) and the highest concentration of serum 3 α ,5 α -THP levels in pregnant women during the third trimester reported at 50 ng/ml (157 nM) (Luisi et al., 2000). Electrophysiological recordings were conducted according to the methods described earlier for ethanol application.

Molecular Modeling

Homology models of $\alpha 1\beta 2\gamma 2$ GABA_AR with mutations in loop 2 of the $\gamma 2$ subunit were built by concatenating the primary sequence of GABA_AR in the order $\gamma/\alpha/\beta/\alpha/\beta$. This sequence was aligned with the five subunits of the glutamate-gated chloride channel (GluCl; PDB ID 3RHW) (Hibbs and Gouaux, 2011). As shown in Table 1, mutations in the sequence of $\gamma 2$ loop 2 were performed to correspond to $\gamma 2$ GABA_AR USERS 1 and 4. The models were built with the Modeler Module of Discovery Studio 3.5 (Accelrys, San Diego, CA) and then optimized as described previously (Perkins et al., 2012).

Data Analysis

Data for each experiment were obtained from 4–23 oocytes from at least three different frogs. Sample size *n* refers to the number of oocytes tested. Results are expressed as the mean $\pm$ S.E.M. Where no error bars are shown, bars are smaller than the symbols. Nonlinear regression analysis was used to analyze EC₅₀, Hill slope, and maximum current ($I_{\max}$) for the agonist concentration-response curves. The thresholds for ethanol sensitivity for GlyR and GABA_AR WT and USERS were determined by comparing the percent change in agonist EC₂-induced Cl^- currents in the presence and absence of ethanol across all tested ethanol concentrations using Student's *t* test. Statistical significance was defined as *P* < 0.05.

Results

The structure activity relationships identified by previous mutational studies in loop 2 (Crawford et al., 2008; Perkins et al.,

2009, 2012) served as the basis for selecting mutations in the homologous regions of $\alpha 1$ and $\alpha 2$ GlyRs and $\alpha 1$ and $\gamma 2$ GABA_ARs to develop USERS as described later. Loop 2 sequence alignments of USER subunits are shown in Table 1. Representative tracings for the WT and mutant $\alpha 1$ GlyRs are exhibited in Fig. 1.

Manipulation of Loop 2 Results in GlyR and GABA_AR USERS That Are Sensitive to Ultra-Low Ethanol Concentrations (< 1 mM)

$\alpha 1$ GlyR WT. Ethanol produced concentration-dependent potentiation of glycine-induced Cl[−] currents in $\alpha 1$ GlyR WT

with significant effects starting at 30 mM ethanol (Fig. 2A; Table 2). There were no significant effects of ethanol on these WT receptors at concentrations below 30 mM.

$\alpha 1$ GlyR USER 1 (First-Generation $\alpha 1$ GlyR USER). Earlier studies reported that the loop 2 mutant, $\alpha 1$ GlyR USER 1 had increased ethanol sensitivity compared with WT receptors at 1–30 mM ethanol (Perkins et al., 2009). To establish the ethanol threshold in these receptors, we expanded the ethanol concentration range to 0.025–50 mM. At ultra-low concentrations (ultra-low ethanol concentrations refer to concentrations

Fig. 1. Loop 2 mutations in $\alpha 1$ GlyRs produce ultra-sensitive ethanol receptors that are sensitive to ≤ 0.5 mM ethanol. Two-electrode voltage clamp electrophysiology tracings of homomeric $\alpha 1$ GlyR WT and $\alpha 1$ GlyR USERS expressed in *Xenopus* oocytes. (A) $\alpha 1$ GlyR WT in response to 0.5 and 50 mM ethanol. (B) $\alpha 1$ GlyR USER 1 in response to 0.5 mM ethanol. (C) $\alpha 1$ GlyR USER 2 in response to 0.5 mM ethanol. (D) $\alpha 1$ GlyR USER 3 in response to 0.1 mM ethanol. Effects of ethanol were tested with EC₂ glycine (5–25 μ M).

Fig. 2. (A) $\alpha 1$ GlyR USERS have increased ethanol sensitivity and bimodal response. Ethanol-induced potentiation of glycine EC₂-activated Cl⁻ currents in *Xenopus* oocytes expressing $\alpha 1$ GlyR USERS exhibited a bimodal response to ethanol (denoted by black curves). The first curve followed an inverse U pattern that extended from 0.025 to 3 mM ethanol, and the second curve extended across higher concentrations beginning at 3 mM ethanol with concentration-dependent increases in potentiation until 50 mM. Values for ethanol potentiation are presented as the percentage of glycine EC₂ control. The glycine EC₂ concentrations used ranged from 5 to 10 μ M in USERS and 18 to 25 μ M for WT. There was a significant decrease in the threshold for ethanol sensitivity and increase in the magnitude of ethanol response in $\alpha 1$ GlyR USERS compared with WT. Each data point represents the mean $\pm$ S.E.M. from at least 4–9 oocytes. (B) Manipulation of loop 2 restores the agonist concentration response for $\alpha 1$ GlyR USER 3. Glycine-induced Cl⁻ currents were normalized to the maximal current activated by a saturating concentration of glycine (1000–10,000 μ M). The curves represent nonlinear regression analysis of the glycine concentration responses in $\alpha 1$ GlyR USERS and WT. There was a significant left shift in the agonist concentration-response curve for $\alpha 1$ GlyR USERS 1 and 2 relative to WT. The agonist concentration-response curve for $\alpha 1$ GlyR USER 3 was fully restored to WT. Each data point represents the mean $\pm$ S.E.M. from at least 4–23 oocytes.

less than 1 mM; low ethanol concentrations refer to 1–3 mM), ethanol produced a concentration-dependent effect with significant potentiation starting at 0.075 mM. Thus, the threshold for ethanol sensitivity in $\alpha 1$ GlyR USER 1 was significantly reduced to 0.075 from 30 mM in $\alpha 1$ GlyR WT (Fig. 2A; Table 2). At higher concentrations (high ethanol concentrations refer to concentrations greater than 3 mM), ethanol produced a concentration-dependent effect with significant potentiation in $\alpha 1$ GlyR USER 1 beginning at 3 mM (Fig. 2A; Table 2).

Notably, the ethanol concentration response demonstrated a bimodal pattern (Fig. 2A). The first curve followed an inverse U pattern ranging 0.025–1 mM, and the second curve started at 3 mM, with concentration-dependent increases in potentiation until 30 mM ethanol, where the response appeared to plateau.

$\alpha 1$ GlyR USER 2. We and others previously reported that a single point mutation at position 52 in WT $\alpha 1$ GlyRs from alanine to serine (A52S) significantly reduced the sensitivity of the receptor to ethanol (Mascia et al., 1996b; Davies et al., 2004). Interestingly, the $\alpha 1$ GlyR USER 1 includes the same

alanine to serine mutation at position 52. We therefore tested whether reverting serine at position 52 in $\alpha 1$ GlyR USER 1 to the WT alanine would further increase ethanol sensitivity of this new mutant receptor ($\alpha 1$ GlyR USER 2; Table 1).

The resultant $\alpha 1$ GlyR USER 2 showed an increase in ethanol sensitivity compared with $\alpha 1$ GlyR WT by decreasing the ethanol threshold and increasing the magnitude of ethanol response (Fig. 2A; Table 2). Although significant ethanol sensitivity relative to WT was retained, the mutations in $\alpha 1$ GlyR USER 2 did not cause an incremental increase in ethanol sensitivity relative to $\alpha 1$ GlyR USER 1 with respect to both ethanol threshold (0.075 and 3 mM, respectively, for the ultra-low and high concentration ranges) and the magnitude of ethanol response. The ethanol concentration response followed a bimodal pattern for $\alpha 1$ GlyR USER 2 similar to that of $\alpha 1$ GlyR USER 1.

$\alpha 1\beta 2\gamma 2$ GABA_AR WT (GABA_AR WT). Ethanol produced concentration-dependent potentiation of GABA-induced Cl⁻ currents in GABA_AR WT with significant effects starting at 50 mM ethanol (Fig. 3A; Table 3). There were no significant effects of ethanol on these WT receptors at concentrations below 50 mM.

$\gamma 2$ GABA_AR USER 1 (First-Generation $\gamma 2$ GABA_AR USER). Earlier studies reported that loop 2 mutant $\gamma 2$ GABA_AR USER 1 had increased ethanol sensitivity compared with GABA_AR WT in response to 0.25–50 mM ethanol (Perkins et al., 2009). In the current study, we identified a lower ethanol threshold in $\gamma 2$ GABA_AR USER 1 at 0.25 mM, compared with the previously reported 0.5 mM (Table 3) (Perkins et al., 2009). At ultra-low concentrations, ethanol produced a concentration-dependent effect with significant potentiation starting at 0.25 mM. Thus, the threshold for ethanol sensitivity in $\gamma 2$ GABA_AR USER 1 was reduced to 0.25 mM from 50 mM in GABA_AR WT (Table 3). As seen with all GlyR USERS, the ethanol concentration response demonstrated a bimodal trend, with the first curve following an inverse U pattern ranging 0.1–1 mM, and a second curve beginning at 3 mM with a concentration-dependent increase in ethanol response (Fig. 3A).

$\gamma 2$ GABA_AR USER 2. The reversion at position 52 from serine to the WT alanine in $\alpha 1$ GlyR USER 2 increased ethanol sensitivity relative to $\alpha 1$ GlyR WT. We used a similar approach

TABLE 2

Threshold for ethanol sensitivity in $\alpha 1$ and $\alpha 2$ GlyR USERS is bimodal and lower than WT

$\alpha 1$ And $\alpha 2$ GlyR USERS exhibited a bimodal pattern in response to ethanol. Two threshold concentrations are denoted for USERS to represent the bimodal effect at ultra-low and higher (>3 mM) ethanol concentrations. The threshold for ethanol sensitivity was reduced from 30 mM ethanol in $\alpha 1$ GlyR WT to 0.075 mM in $\alpha 1$ GlyR USERS 1 and 2, and 0.05 mM in $\alpha 1$ GlyR USER 3. At higher ethanol concentrations, $\alpha 1$ GlyR USERS 1 and 2 exhibited a threshold of 3 mM ethanol, whereas $\alpha 1$ GlyR USER 3 exhibited a threshold of 10 mM ethanol. In $\alpha 2$ GlyR USERS, thresholds were significantly reduced from 50 mM ethanol in $\alpha 2$ GlyR WT to 0.25 and 10 mM in $\alpha 2$ GlyR USER 1. Each data point represents the mean $\pm$ S.E.M. from at least four to sixteen oocytes. All threshold values were statistically significant with $P < 0.05$.

	Lower Ethanol Threshold	Upper Ethanol Threshold
	mM	
$\alpha 1$ GlyR WT	n/a	30
$\alpha 1$ GlyR USER 1	0.075	3
$\alpha 1$ GlyR USER 2	0.075	3
$\alpha 1$ GlyR USER 3	0.05	10
$\alpha 2$ GlyR WT	n/a	50
$\alpha 2$ GlyR USER 1	0.25	10

n/a, not applicable.

Fig. 3. (A) $\gamma 2$ GABA_AR USERS 1, 2, and 3 have increased ethanol sensitivity and bimodal response. Ethanol-induced potentiation of GABA EC₂-activated Cl⁻ currents in *Xenopus* oocytes expressing $\gamma 2$ GABA_AR USERS 1, 2, and 3 with $\alpha 1\beta 2\gamma 2$ composition exhibited a bimodal response to ethanol (denoted by black curves). The first curve followed an inverse U pattern that extended from 0.1 to 3 mM ethanol, and the second curve extended across higher concentrations beginning at 3 mM ethanol with concentration-dependent increases in potentiation until 50 mM. Values for ethanol potentiation are presented as the percentage of GABA EC₂ control. The GABA EC₂ concentrations used were 5 μ M for USERS and 10 μ M for WT. There was a significant decrease in threshold for ethanol sensitivity and increase in the magnitude of ethanol response compared with WT. $\gamma 2$ GABA_AR USER 4 was insensitive to ethanol across the tested concentration range. Each data point represents the mean $\pm$ S.E.M. from at least 4–9 oocytes. (B) Manipulation of loop 2 partially restores the agonist concentration response for $\gamma 2$ GABA_AR USER 3. GABA-induced Cl⁻ currents were normalized to the maximal current activated by a saturating concentration of GABA (1000–10,000 μ M). The curves represent nonlinear regression analysis of the GABA concentration responses in $\gamma 2$ GABA_AR USERS and WT. There was a significant left shift in the agonist concentration-response curve for $\gamma 2$ GABA_AR USERS 1, 2, and 4 relative to WT. The agonist concentration-response curve for $\gamma 2$ GABA_AR USER 3 was partially restored to WT. However, there was a statistically significant difference between the EC₅₀ values of $\gamma 2$ GABA_AR USER 3 and WT (Table 1). Each data point represents the mean $\pm$ S.E.M. from at least 4–23 oocytes.

in $\gamma 2$ GABA_AR USER 2 by reverting serine at the homologous position in $\gamma 2$ GABA_AR USER 1 back to WT asparagine (S66N) to test whether this would increase ethanol sensitivity.

Similar to results for $\gamma 2$ GABA_AR USER 1, the resultant $\gamma 2$ GABA_AR USER 2 showed an increase in ethanol sensitivity compared with WT, with decreased ethanol threshold and increased magnitude of ethanol potentiation (Fig. 3A; Table 3). However, the ethanol threshold and magnitude of potentiation between the two mutant $\gamma 2$ GABA_AR USERS remained similar (Fig. 3A). The ethanol concentration response followed a bimodal pattern for $\gamma 2$ GABA_AR USER 2 similar to that of $\gamma 2$ GABA_AR USER 1.

Agonist Sensitivity of $\alpha 1$ GlyR and $\gamma 2$ GABA_AR USERS 1 and 2 Were Modestly Altered Compared with Respective WT

Glycine produced inward Cl⁻ currents in a concentration-dependent manner (Fig. 2B). $\alpha 1$ GlyR WT values for glycine EC₅₀, Hill slope, and $I_{\max}$ are shown in Table 1 and were consistent with results previously reported (Crawford et al., 2007; Perkins et al.,

2009). In both $\alpha 1$ GlyR USERS (USERS 1 and 2), loop 2 mutations produced a left shift in the agonist concentration-response curve relative to $\alpha 1$ GlyR WT with a significant decrease in EC₅₀ (Fig. 2B; Table 1). There were no significant differences in Hill slope and $I_{\max}$ between $\alpha 1$ GlyR USERS 1 and 2 and $\alpha 1$ GlyR WT (Table 1).

GABA produced inward Cl⁻ currents in a concentration-dependent manner (Fig. 3B). Values for EC₅₀, Hill slope, and $I_{\max}$ of $\alpha 1\beta 2\gamma 2$ GABA_AR WT are shown in Table 1 and are consistent with results previously reported (Perkins et al., 2009). In both $\gamma 2$ GABA_AR USERS 1 and 2, loop 2 mutations produced a left shift in the agonist concentration-response curve relative to GABA_AR WT with a significant decrease in EC₅₀ (Fig. 3B; Table 1). There were no significant differences in Hill slope and $I_{\max}$ between $\gamma 2$ GABA_AR USERS 1 and 2 and GABA_AR WT (Table 1).

Since receptor characteristics of GlyR and GABA_AR USERS 1 and 2 were modestly altered compared with WT, we next restored additional loop 2 residues in GlyR and GABA_AR USER 1 back to WT to determine if these changes would normalize EC₅₀ and restore WT-like agonist sensitivity.

Manipulation of the Loop 2 Structure Results in GlyR and GABA_AR USERS with Normalized Receptor Characteristics

$\alpha 1$ GlyR USER 3. The loop 2 sequence of $\alpha 1$ GlyR USER 1 spans across both exons 3 (positions 50–55) and 4 (positions 56–59) of the human GLRA1 (glycine receptor, $\alpha 1$) gene (Table 1). To avoid possible complications for gene incorporation and functional expression of USERS in vivo, we developed a mutant receptor that limited the mutations in $\alpha 1$ GlyR USER 1 to exon 3 (Table 1).

There was no significant change in agonist sensitivity in $\alpha 1$ GlyR USER 3 compared with $\alpha 1$ GlyR WT, as indicated by similar EC₅₀ values (Fig. 2B; Table 1). Thus, the agonist concentration-response curve for $\alpha 1$ GlyR USER 3 was right shifted relative to $\alpha 1$ GlyR USERS 1 and 2 (Fig. 2B; Table 1). The other receptor characteristics of $\alpha 1$ GlyR USER 3, including Hill slope and $I_{\max}$, did not differ significantly from $\alpha 1$ GlyR WT (Table 1).

TABLE 3

Threshold for ethanol sensitivity of $\gamma 2$ and $\alpha 1$ GABA_AR USERS is bimodal and lower than $\alpha 1\beta 2\gamma 2$ GABA_AR WT

$\gamma 2$ GABA_AR USERS 1, 2, and 3, and $\alpha 1$ GABA_AR USER 1 exhibited a bimodal pattern in response to ethanol. Two threshold concentrations are denoted for USERS to represent the bimodal effect at ultra-low and higher (>3 mM) ethanol concentrations. The threshold for ethanol sensitivity was reduced from 50 mM ethanol in $\alpha 1\beta 2\gamma 2$ GABA_AR WT to 0.25 and 10 mM in $\gamma 2$ GABA_AR USERS 1, 2, and 3, and $\alpha 1$ GABA_AR USER 1. The $\gamma 2$ GABA_AR USER 4 produced ethanol-insensitive receptors. All GABA_AR USERS represent the $\alpha 1\beta 2\gamma 2$ isoform. Each data point represents the mean $\pm$ S.E.M. from at least 4–13 oocytes. All threshold values were statistically significant with $P < 0.05$.

	Lower Ethanol Threshold	Upper Ethanol Threshold
	mM	
$\alpha 1\beta 2\gamma 2$ GABA _A R WT	n/a	50
$\gamma 2$ GABA _A R USER 1	0.25	10
$\gamma 2$ GABA _A R USER 2	0.25	10
$\gamma 2$ GABA _A R USER 3	0.25	10
$\gamma 2$ GABA _A R USER 4	n/a	n/a
$\alpha 1$ GABA _A R USER 1	0.25	10

n/a, not applicable.

$\alpha 1$ GlyR USER 3 demonstrated an increase in ethanol sensitivity compared with $\alpha 1$ GlyR WT and $\alpha 1$ GlyR USERS 1 and 2, by decreasing the ethanol threshold to 0.05 mM (Fig. 2A; Table 2). At ultra-low ethanol concentrations, $\alpha 1$ GlyR USER 3 demonstrated an increase in the magnitude of ethanol response compared with $\alpha 1$ GlyR WT and USERS. Remarkably, at 0.1 mM ethanol, the magnitude of response in this USER was equivalent to that of 30 mM ethanol in $\alpha 1$ GlyR WT (Fig. 2A). Across high ethanol concentrations, the magnitude of ethanol response was lower than that of $\alpha 1$ GlyR USERS 1 and 2, but comparable to $\alpha 1$ GlyR WT (Fig. 2A). As with the other $\alpha 1$ GlyR USERS, the ethanol concentration response for $\alpha 1$ GlyR USER 3 was bimodal.

$\gamma 2$ GABA_AR USER 3. Crawford et al. (2008) demonstrated that removal of the negative charge associated with the glutamate residue at position 53 in $\alpha 1$ GlyRs caused a right shift in agonist sensitivity with respect to $\alpha 1$ GlyR WT, indicating that the physical-chemical properties at this position may influence agonist sensitivity. Therefore, we hypothesized that substituting the charged glutamate at position 67 (homologous to position 53 in $\alpha 1$ GlyRs) with the neutral WT alanine in GABA_AR USER 1 would right shift and thus normalize agonist sensitivity compared with GABA_AR WT.

As predicted, the GABA concentration-response curve for this USER was right shifted compared with $\gamma 2$ GABA_AR USERS 1 and 2 (Fig. 3B). However, $\gamma 2$ GABA_AR USER 3 was significantly more sensitive to agonist compared with GABA_AR WT (Fig. 3B; Table 1). Thus, these loop 2 manipulations in $\gamma 2$ GABA_AR USER 3 partially restored agonist sensitivity. Hill slope and $I_{\max}$ did not differ significantly from GABA_AR WT in $\gamma 2$ GABA_AR USER 3 (Table 1).

The ethanol threshold in $\gamma 2$ GABA_AR USER 3 was significantly reduced to 0.25 mM compared with 50 mM in GABA_AR WT (Table 3). The magnitude of ethanol response at ultra-low concentrations was higher than GABA_AR USER 1. The ethanol concentration response for $\gamma 2$ GABA_AR USER 3 followed a bimodal pattern similar to that of other $\gamma 2$ GABA_AR USERS (Fig. 3A).

$\gamma 2$ GABA_AR USER 4 (Null Mutant). Since $\alpha 1$ GlyR USER 3 was ultra sensitive to ethanol with WT-like agonist response, we hypothesized that substitution of the terminal loop 2 residues of $\alpha 1$ GlyR USER 3 in $\gamma 2$ GABA_AR USERS would also increase ethanol sensitivity while normalizing agonist response (Table 1). However, these mutations left shifted the agonist concentration response and thus did not normalize agonist sensitivity (Fig. 3B; Table 1). Hill slope and $I_{\max}$ of $\gamma 2$ GABA_AR USER 4 did not differ significantly from GABA_AR WT (Table 1). In contrast to the expected increase in ethanol sensitivity, this USER was insensitive to ethanol across all concentrations tested (Fig. 3A; Table 3).

Loop 2 Manipulations in Multiple Subunits of GlyRs and GABA_ARs Result in USERS

To demonstrate the applicability of USERS across multiple subunits and receptors of the Cys-loop superfamily, we developed USERS in two new subunits: $\alpha 2$ GlyR and $\alpha 1$ GABA_AR. $\alpha 2$ GlyRs are the predominant receptor subtype found in the adult brain, and are believed to play an important role in ethanol-induced reward (Delaney et al., 2010; Jonsson et al., 2012). In addition, emerging evidence suggests that, unlike $\alpha 1$ GlyRs, which are predominantly expressed as heteromers ($\alpha 1\beta$) in the spinal cord, $\alpha 2$ GlyRs are expressed as homomers in the

brain (Eichler et al., 2009; Ademark et al., 2011; Chen et al., 2011; Weltzien et al., 2012). $\alpha 1\beta 2\gamma 2$ GABA_ARs are the predominantly expressed form of GABA_ARs in the brain, and are believed to play a role in producing ethanol-induced behaviors (Borghese et al., 2006; Werner et al., 2006; Kumar et al., 2009).

$\alpha 2$ GlyR WT. Ethanol produced concentration-dependent potentiation of glycine-induced Cl[−] currents in $\alpha 2$ GlyR WT with significant effects starting at 50 mM ethanol (Fig. 4A; Table 2). There were no significant effects of ethanol on these WT receptors at concentrations below 50 mM.

Glycine produced inward Cl[−] currents in a concentration-dependent manner (Fig. 4B). Values for glycine EC₅₀, Hill slope, and $I_{\max}$ of $\alpha 2$ GlyR WT are shown in Table 1.

$\alpha 2$ GlyR USER 1. Since $\alpha 1$ GlyR USER 3 demonstrated increased ethanol sensitivity without altering agonist response, we applied the same rationale to limit loop 2 mutations to exon 3 in $\alpha 2$ GlyR USER 1 to develop USERS with similar characteristics.

As predicted, $\alpha 2$ GlyR USER 1 demonstrated an increase in ethanol sensitivity by decreasing the ethanol threshold from 50 mM in $\alpha 2$ GlyR WT to 0.25 mM (Fig. 4A; Table 2). At higher concentrations, ethanol produced a concentration-dependent effect with significant potentiation in $\alpha 2$ GlyR USER 1 beginning at 10 mM (Fig. 4A; Table 2). As with other GlyR USERS, the ethanol concentration response followed a bimodal pattern (Fig. 4A). No changes in agonist sensitivity relative to $\alpha 2$ GlyR WT were observed, as indicated by similar EC₅₀ values (Fig. 4B; Table 1). The other receptor characteristics of $\alpha 2$ GlyR USER 1, including Hill slope and $I_{\max}$, did not differ significantly from $\alpha 2$ GlyR WT (Table 1).

$\alpha 1$ GABA_AR USER 1. To explore the development of $\alpha 1$ GABA_AR USERS, we first produced the homolog of $\gamma 2$ GABA_AR USER 1. As expected, $\alpha 1$ GABA_AR USER 1 had markedly increased ethanol sensitivity compared with GABA_AR WT (Fig. 5A), with an ethanol sensitivity threshold at 0.25 mM compared with 50 mM in GABA_AR WT (Fig. 5A; Table 3). At ultra-low ethanol concentrations, $\alpha 1$ GABA_AR USER 1 demonstrated an increase in the magnitude of ethanol response compared with WT (Fig. 5A). The ethanol concentration response exhibited a bimodal pattern, consistent with other GlyR and GABA_AR USERS (Fig. 5A; Table 3).

Importantly, $\alpha 1$ GABA_AR USER 1 produced no change in agonist sensitivity relative to GABA_AR WT, as indicated by similar EC₅₀ values (Fig. 5B; Table 1). The other receptor characteristics of $\alpha 1$ GABA_AR USER 1, including Hill slope and $I_{\max}$, did not differ significantly from GABA_AR WT (Table 1).

$\gamma 2$ and $\alpha 1$ GABA_AR USERS do not have altered sensitivity to the neurosteroid Allopregnanolone (3 α ,5 α -THP)

Allopregnanolone is an active metabolite of progesterone, and selectively enhances GABA_A receptor function (Turner and Simmonds, 1989; Smith et al., 1998; Evers et al., 2010). To determine whether loop 2 mutations in the $\gamma 2$ subunit and/or the $\alpha 1$ subunit of the GABA_AR interfere with the effects of 3 α ,5 α -THP, we tested the effects of 20 and 100 nM 3 α ,5 α -THP against two GABA_AR USERS that demonstrated the greatest increase in ethanol sensitivity relative to WT— $\gamma 2$ GABA_AR USER 3 and $\alpha 1$ GABA_AR USER 1. As illustrated, 3 α ,5 α -THP significantly potentiated GABA-induced Cl[−] currents in WT GABA_ARs and $\gamma 2$ GABA_AR USER 3 and $\alpha 1$ GABA_AR USER 1 at 20 and 100 nM (Fig. 6). Notably, there were no significant

Fig. 4. (A) $\alpha 2$ GlyR USERS have increased ethanol sensitivity and bimodal response. Ethanol-induced potentiation of glycine EC₂-activated Cl⁻ currents in *Xenopus* oocytes expressing $\alpha 2$ GlyR USER 1 exhibited a bimodal response to ethanol (indicated by black curves). The first curve followed an inverse U pattern that extended from 0.1 to 3 mM ethanol, and the second curve extended across higher concentrations beginning at 3 mM ethanol with concentration-dependent increases in potentiation until 50 mM. Values for ethanol potentiation are presented as a percentage of glycine EC₂ control. The glycine EC₂ concentrations used ranged from 5 to 10 μ M for USERS and WT. There was a significant decrease in the threshold for ethanol sensitivity and increase in the magnitude of ethanol response in $\alpha 2$ GlyR USERS compared with WT. Each data point represents the mean $\pm$ S.E.M. from at least 4–16 oocytes. (B) Manipulation of loop 2 produces normal agonist concentration response in $\alpha 2$ GlyR USER 1. Glycine-induced Cl⁻ currents were normalized to the maximal current activated by a saturating concentration of glycine (1000–10,000 μ M). The curves represent nonlinear regression analysis of the glycine concentration responses in $\alpha 2$ GlyR USERS and WT. There was no significant difference in the agonist concentration-response curves for $\alpha 2$ GlyR USER 1 and WT. Each data point represents the mean $\pm$ S.E.M. from at least 4–5 oocytes.

differences between $3\alpha,5\alpha$ -THP sensitivity in USERS relative to WT GABA_ARs as determined by measuring changes in the magnitude of receptor potentiation (Fig. 6) and threshold of activation ($P > 0.05$).

Discussion

The present study demonstrated that manipulation of the physical-chemical characteristics of extracellular loop 2 produced a library of homomeric $\alpha 1$ and $\alpha 2$ GlyR USERS and heteromeric $\alpha 1$ and $\gamma 2$ GABA_AR USERS of the $\alpha 1\beta 2\gamma 2$ isoform. These USERS were over 100-fold more sensitive to ethanol compared with WT receptors, with increased magnitude of ethanol response and decreased threshold for ethanol sensitivity (Figs. 1–5; Tables 2 and 3). We also developed an ethanol-insensitive receptor in the $\gamma 2$ subunit of GABA_ARs (Fig. 3; Table 3). Notably, we demonstrated that loop 2 manipulations that increase ethanol sensitivity in USERS do not necessarily alter general receptor characteristics such as EC₅₀, I_{max} , and Hill slope relative to WT (Table 1). In addition, we found that loop 2 mutations did not significantly alter sensitivity to the

neurosteroid allopregnanolone ($3\alpha,5\alpha$ -THP) (Fig. 6). Finally, our findings indicate that USERS can be produced across multiple receptor subunits of GlyRs and GABA_ARs (Figs. 4 and 5).

The significance of these findings is attributable to the fact that the threshold concentrations for ethanol sensitivity of USERS were ≤ 0.25 mM (BEC $\leq 0.001\%$). Behavioral effects of ethanol in humans can be detected at BECs as low as 0.03% (w/v) (7 mM) (Ogden and Moskowitz, 2004; Kumar et al., 2009). Thus, USERS are sensitive to ethanol concentrations that are far below those that produce known responses in vivo or in vitro (Aguayo and Pancetti, 1994; Mascia et al., 1996b; Davies et al., 2003; Ogden and Moskowitz, 2004; Hancher et al., 2005, 2006; Olsen et al., 2007; Perkins et al., 2009). Testing at low concentrations would minimize the probability of activating multiple targets of ethanol and associated behaviors, thus allowing activation of specific receptor subunits.

Notably, exposure to ultra-low ethanol concentrations can produce responses in both GlyR and GABA_AR USERS that are similar in magnitude to those produced in WT receptors at higher ethanol concentrations (Figs. 2–5A). These results suggest the exciting possibility that KI animals expressing

Fig. 5. (A) $\alpha 1$ GABA_AR USERS have increased ethanol sensitivity and bimodal response to ethanol. Ethanol-induced potentiation of GABA EC₂-activated Cl⁻ currents in *Xenopus* oocytes expressing $\alpha 1$ GABA_AR USER 1 with $\alpha 1\beta 2\gamma 2$ composition exhibited a bimodal response to ethanol (indicated by black curves). The first curve followed an inverse U pattern that extended from 0.1 to 3 mM ethanol, and the second curve extended across higher concentrations beginning at 3 mM ethanol with concentration-dependent increases in potentiation until 50 mM. Values for ethanol potentiation are presented as a percentage of GABA EC₂ control. The GABA EC₂ concentrations used were 8–10 μ M for USERS and WT. There was a significant decrease in the threshold for ethanol sensitivity and increase in the magnitude of ethanol response in $\alpha 1$ GABA_AR USER 1 compared with WT. Each data point represents the mean $\pm$ S.E.M. from at least 4–13 oocytes. (B) Manipulation of loop 2 produces normal agonist concentration response for $\alpha 1$ GABA_AR USER 1. GABA-induced Cl⁻ currents were normalized to the maximal current activated by a saturating concentration of GABA (1000–10,000 μ M). The curves represent nonlinear regression analysis of the GABA concentration responses in $\alpha 1$ GABA_AR USER 1 and WT. There was no significant difference in the agonist concentration-response curves for $\alpha 1$ GABA_AR USER 1 and WT. Each data point represents the mean $\pm$ S.E.M. from at least 4–13 oocytes.

Fig. 6. Loop 2 mutations do not alter sensitivity to 3α,5α-THP for γ2 GABA_AR USER 3 and α1 GABA_AR USER 1. 3α,5α-THP potentiated EC₂ GABA-induced Cl⁻ currents in α1β2γ2 GABA_AR WT and γ2 GABA_AR USER 3 and α1 GABA_AR USER 1. There was no significant difference in the magnitude of 3α,5α-THP potentiation between WT and mutant receptors. Each data point represents the mean ± S.E.M. from 6–13 oocytes.

USERS should respond similarly to ultra-low-dose ethanol as if they received higher ethanol doses in the 30–50 mM range. Furthermore, the development of KI animals expressing the ethanol-insensitive γ2 GABA_AR USER 4 would help validate findings from current KI strategies that express ethanol-insensitive receptors in vivo by direct inactivation of a single receptor subunit population. Results from our studies would complement and add to the findings identified in KI and KO studies of GlyRs and GABA_ARs, which use higher, intoxicating ethanol concentrations that affect multiple targets and downstream cascades (Werner et al., 2006). Thus, USERS in conjunction with current transgenic approaches can be used to increase our understanding of the pharmacological and physiologic effects of ethanol action by establishing precise links between specific receptor subunits and behavioral outcomes.

In addition to GlyRs and GABA_ARs, other members of the Cys-loop superfamily of LGICs, including the neuronal nicotinic cholinergic and serotonergic receptors, have been linked to a number of behavioral effects of ethanol administration (Knapp and Pohorecky, 1992; Hodge et al., 2004; Kamens et al., 2010a,b). Since the sequence homology among subunits of Cys-loop receptors is on the order of 30% (Olsen and Sieghart, 2008), this suggests that the applicability of USER technology can be extended to other receptors within the Cys-loop superfamily.

USER technology will also add to recent advances in brain circuitry research strategies including optogenetics and designer receptors exclusively activated by designer drugs (DREADD). Optogenetics involves microbial opsins that allow photoactivation or inhibition of defined neuron populations, axonal pathways, or brain regions, whereas DREADD involves pharmacological activation of DREADD-expressing neurons using clozapine *N*-oxide (Aston-Jones and Deisseroth, 2013). Similarly, USERS rely on ethanol as a pharmacological probe, analogous to a specific receptor agonist or antagonist, to directly activate receptor populations sensitized to ultra-low ethanol concentrations or inactivate receptor populations without affecting other targets. Thus, USERS could serve as an advantageous brain-mapping tool with direct, noninvasive and bidirectional capabilities to provide further insight into specific neural cascades at the resolution of the receptor subunit level. Taken together, USERS along with optogenetics and DREADD provide researchers with a novel tool to

determine the interplay between neural pathways, thereby enhancing our understanding of the neurobiological basis of behaviors.

Mutational studies in extracellular loop 2 of GlyRs and GABA_ARs already have and will continue to inform the construction of molecular receptor models that increase our understanding of the targets of ethanol action and structure-function relationships underlying receptor activation and ethanol-induced modulation (Crawford et al., 2007; Perkins et al., 2009, 2012). The bimodal pattern of ethanol response observed in GlyR and GABA_AR USERS (Figs. 2–5A), combined with findings from prior studies (Davies et al., 2004; Crawford et al., 2007), supports the existence of multiple potentiating and inhibitory sites of ethanol action. A potential explanation for this bimodal effect could be that these loop 2 mutations lead to the creation of an ultra-sensitive ethanol site that produces robust potentiation of the receptor at submillimolar concentrations. At concentrations between 1 and 3 mM, ethanol could act at inhibitory sites that potentially reduce and/or mask the contribution of the ultra-sensitive site. Then, at higher concentrations (>3 mM), ethanol could act at WT potentiating sites. The interplay between inhibitory and potentiating sites observed in USERS is similar to that reported for other Cys-loop allosteric modulators, such as zinc, propofol, pentobarbital, and neurosteroids (Morrow et al., 1990; Bloomenthal et al., 1994; Laube et al., 1995; Maksay and Biro, 2002; Evers et al., 2010). Thus, future studies could explore the actions of other allosteric agents on these USERS.

The selectivity of the GABA_AR USERS is further strengthened by the lack of significant changes in 3α,5α-THP potentiation and change in threshold activity on GABA_ARs. Specifically, in this investigation, we demonstrated that neither the highly ethanol-sensitive γ2 GABA_AR USER 3 nor the α1 GABA_AR USER 1 showed any significant difference in sensitivity to 3α,5α-THP compared with α1β2γ2 GABA_AR WT (Fig. 6). The lack of significant change in sensitivity to 3α,5α-THP suggests that the extracellular loop 2 region may not be a critical site of neurosteroid action. Further studies are necessary to determine if these findings extend to all neurosteroids.

Based on the findings, we propose new molecular models that highlight the structural differences between loop 2 of the ethanol-ultra-sensitive γ2 GABA_AR USER 1 and ethanol-insensitive γ2 GABA_AR USER 4 (Supplemental Material). Mutation of positions 71 and 73 in loop 2 of γ2 GABA_AR USER 4 eliminated ethanol sensitivity. This may be explained by the distortion of the β hairpin structure of loop 2 (indicated in yellow) in γ2 GABA_AR USER 4 compared with that of γ2 GABA_AR USER 1 (Fig. 7). This distortion could be due to the formation of new electrostatic interactions between the flanking residues of loop 2 (H64 and R73). Specifically, hydrogen bond interaction(s) may form between the arginine at position 73 and the histidine at position 64. Furthermore, additional interactions may form between positions 71 and 73 with the transmembrane region, adding to the change in the overall loop 2 structure. These new electrostatic interactions, which are not present in γ2 GABA_AR WT, could increase the rigidity of the loop 2 structure and change its shape (Perkins et al., 2009). As a result, these changes may affect the actions of ethanol molecules on loop 2 and its surrounding regions. Thus, the differences in ethanol sensitivity between γ2

Fig. 7. A molecular model of the $\alpha 1\beta 2\gamma 2$ GABA_AR with mutations in loop 2 of the $\gamma 2$ subunit. (A) A model of the full receptor $\alpha 1\beta 2\gamma 2$ GABA_AR, but with the foreground two subunits removed to reveal loop 2 in the $\gamma 2$ subunit with the USER 1 mutation (see Table 1 for sequences). The view is at the plane of the membrane looking outward from the center of the ion pore. The α helices are rendered as cylinders and the β strands as ribbons. The ribbon for loop 2 in $\gamma 2$ is highlighted in yellow. (B) A zoomed view of loop 2 from (A). The residues that differ between the ethanol-sensitive USER 1 and ethanol-insensitive USER 4 are rendered with a space-filling surface, whereas the other residues are rendered in stick form; carbon, hydrogen, oxygen, nitrogen, and sulfur are colored gray, white, red, blue, and orange, respectively. (C) The two mutations that remove sensitivity to ethanol, E71D and T73R, are highlighted. Since USERS 1 and 4 have relatively similar sensitivity to GABA (Table 2), it is likely that mutations in this region of loop 2 influence ethanol potentiation of GABA, rather than directly influencing agonist sensitivity and/or receptor gating.

GABA_AR USERS 1 and 4 may be explained by the introduction of new electrostatic interactions.

Interestingly, $\gamma 2$ GABA_AR USERS 1 and 4 demonstrate similar agonist sensitivities (Fig. 3B), thus it is likely that mutations at the terminal region of loop 2 influence ethanol potentiation of GABA, rather than agonist sensitivity. These findings represent new evidence suggesting that the physical, chemical, and structural properties of loop 2 that regulate ethanol and agonist sensitivity are different. In addition, the findings distinguish regions that mediate ethanol sensitivity from those that mediate agonist sensitivity. Thus, we propose that residues in loop 2 play a role in the activation pathway from ligand binding to opening of the ion pore. We suggest that these mutations do not alter agonist binding, but rather provide a site for ethanol modulation by the amino acid side chains involved in the transition between the resting, open, or desensitized states of the receptor. However, the factors that influence these properties may not be completely independent of each other. Rather, the factors that mediate ethanol and agonist sensitivity likely depend on the overall structure of loop 2 and the interplay between different residues that are within and surrounding this region.

Taken together, these loop 2 models, in conjunction with those proposed earlier (Crawford et al., 2007; Perkins et al., 2009, 2012), will aid in defining the key drivers that will determine the architecture of ethanol action sites of GlyRs and GABA_ARs. Whereas these studies focus primarily on the structural mechanisms of GlyR and GABA_AR USERS in response to ethanol, future studies may characterize channel kinetics to determine the functional elements of GlyR and GABA_AR USER-mediated synaptic currents in response to ethanol. In addition, since these studies address USER technology for the $\alpha 1\beta 2\gamma 2$ GABA_AR isoform, future studies should investigate additional isoforms of Cys-loop receptors that are known to mediate ethanol-induced behaviors.

In conclusion, USER technology will reveal the roles played by specific receptor subunits in AUDs, thus providing new

targets for the development of novel drugs to prevent and/or treat alcohol-related problems. The development of our new molecular model will provide additional insights regarding the specific sites and mechanisms of ethanol action, and identify potential pharmacophores for drug development by mimicking, blocking, or otherwise modulating the actions of ethanol on key receptor subunits identified by USER technology.

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Authorship Contributions

Participated in research design: Alkana, Naito, Muchhala, Asatryan, Davies, Homanics, Perkins.

Conducted experiments: Naito, Muchhala.

Contributed new reagents or analytic tools: Trudell.

Performed data analysis: Alkana, Naito, Muchhala.

Wrote or contributed to the writing of the manuscript: Alkana, Naito, Muchhala, Asatryan, Trudell, Homanics, Davies, Perkins.

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