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TARGETS FOR ETHANOL ACTIONS AND ANTAGONISM IN LOOP 2 OF THE EXTRACELLULAR DOMAIN OF GLYCINE RECEPTORS

Daya I. Perkins¹, James R. Trudell², Daniel K. Crawford^{1,3}, Ronald L. Alkana^{1,#}, and Daryl L. Davies¹

¹Alcohol and Brain Research Laboratory, Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy and Neuroscience Graduate Program, University of Southern California, Los Angeles, CA, 90089

²Department of Anesthesia and Beckman Program for Molecular and Genetic Medicine, Stanford School of Medicine, Palo Alto, CA, 94305

Abstract

The present studies used increased atmospheric pressure in place of a traditional pharmacological antagonist to probe the molecular sites and mechanisms of ethanol action in GlyRs. Based on previous studies, we tested the hypothesis that physical-chemical properties at position 52 in extracellular domain Loop 2 of $\alpha 1$ GlyRs, or the homologous $\alpha 2$ GlyR position 59, determine sensitivity to ethanol and pressure antagonism of ethanol. Pressure antagonized ethanol in $\alpha 1$ GlyRs that contain a non-polar residue at position 52, but did not antagonize ethanol in receptors with a polar residue at this position. Ethanol sensitivity in receptors with polar substitutions at position 52 was significantly lower than GlyRs with non-polar residues at this position. The $\alpha 2$ T59A mutation switched sensitivity to ethanol and pressure antagonism in the WT $\alpha 2$ GlyR, thereby making it $\alpha 1$ -like. Collectively, these findings indicate that: 1) polarity at position 52 plays a key role in determining sensitivity to ethanol and pressure antagonism of ethanol; 2) the extracellular domain in $\alpha 1$ - and $\alpha 2$ GlyRs is a target for ethanol action and antagonism and 3) there is structural-functional homology across subunits in Loop 2 of GlyRs with respect to their roles in determining sensitivity to ethanol and pressure antagonism of ethanol. These findings should help in the development of pharmacological agents that antagonize ethanol.

#Address correspondence to: Ronald L. Alkana, University of Southern California, School of Pharmacy, 1985 Zonal Avenue PSC 506, Los Angeles, CA 90089, USA. Phone: (323)442-1429; Fax: (323)442-1704; alkana@usc.edu.

³Current address: Department of Neurology, Neuroscience Research Building 1, Room 479, 635 Charles E. Young Drive South, Los Angeles, CA 90095-733422 (D.K.C.)

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Keywords

Increased atmospheric pressure; two-electrode voltage clamp; ethanol sites of action; *Xenopus* oocytes; ion channels; glycine receptor

INTRODUCTION

Alcohol (ethanol) abuse represents a major problem in the United States with an estimated 14 million people being affected (Grant et al. 2004). To address this issue, considerable attention has begun to focus on the development of medications to prevent and treat alcoholism (Heilig M. and Egli M. 2006; Steensland et al. 2007; Johnson et al. 2007). The development of such medications would be aided by a clear understanding of the sites and mechanisms of ethanol action.

Traditionally, the mechanisms and sites of drug action are studied using the appropriate receptor agonists and antagonists. To be used in this way, the mechanism of the antagonism must be direct (mechanistic not physiological) and selective. When these criteria are met, the site of antagonism is synonymous with and defines the site causing drug action. However, the physical-chemical mechanism of action as well as the low affinities of ethanol for its targets limit the utility of traditional pharmacological receptor agonist and antagonist ligands as tools for investigating ethanol's sites of action (Eckenhoff and Johansson 1997; Davies et al. 2003).

Prior studies indicate that increased atmospheric pressure (pressure) is an ethanol antagonist that can help fill this gap. This work found that low level hyperbaric exposure (pressure up to twelve times normal atmospheric pressure—12 ATA) directly antagonizes the behavioral and biochemical actions of ethanol (Alkana and Malcolm 1981; Alkana et al. 1992; Bejanian et al. 1993; Davies and Alkana 1998; Davies and Alkana 2001). The antagonism occurred without causing changes in baseline behavior or central nervous system excitation (Syapin et al. 1988; Davies et al. 1994; Davies et al. 1999) that called into question the direct mechanism of earlier studies investigating high pressure reversal of anesthesia (Kendig 1984; Bowser-Riley et al. 1988; Wann and MacDonald 1988; Franks and Lieb 1994; Little 1996). The low level hyperbaric studies also demonstrated that pressure was selective for allosteric modulators (Alkana et al. 1995; Davies et al. 1996; Davies et al. 2003). More recent hyperbaric two-electrode voltage clamp studies demonstrated that pressure antagonized ethanol potentiation of $\alpha 1$ Glycine receptor (GlyR) function in a direct, reversible, concentration and pressure dependent manner that was selective for allosteric modulation by alcohols (Davies et al. 2003; Davies et al. 2004). Taken together, these findings indicate that pressure is a direct, selective ethanol antagonist that can be used, in place of a traditional pharmacological antagonist, as a tool to help identify the sites of ethanol action.

This notion is supported by recent studies using pressure to identify novel targets for ethanol in GlyRs. Glycine is a major inhibitory neurotransmitter in the mammalian central nervous system. GlyRs are a member of the superfamily of ligand-gated ion channels (LGICs), known as Cys-loop receptors (Ortells and Lunt 1995; Karlin 2002). Other members of this receptor family include γ -aminobutyric acid type-A receptor (GABA_AR), nicotinic

acetylcholine receptor (nAChR) and 5-hydroxytryptamine₃ receptor (5HT₃R), all of which assemble to form ion channels with a pentameric structure (Schofield et al. 1987). Glycine causes inhibition in the adult central nervous system by activating the strychnine-sensitive GlyR. Five GlyR subunits have been cloned ($\alpha 1 - \alpha 4$ and β). The pentamer formed can be homo- or heteromeric (Betz 1991; Rajendra et al. 1997). Native adult GlyRs contain both $\alpha 1$ and β subunits, while native neonatal GlyRs contain both $\alpha 2$ and β subunits ((Malosio et al. 1991; Mascia et al. 1996a; Rajendra et al. 1997; Eggers et al. 2000)

Studies over the last decade have pointed to a role for GlyRs in mediating the effects of ethanol. This work includes studies that have shown that behaviorally relevant concentrations of ethanol positively modulate GlyR function measured in a variety of brain and spinal cord preparations (Engblom and Åkerman 1991; Aguayo and Pancetti 1994; Tapia et al. 1998; Eggers et al. 2000; Tao and Ye 2002; Ye et al. 2002; McCool et al. 2003; Ziskind-Conhaim et al. 2003). More recent studies also suggest that GlyRs in the nucleus accumbens are targets for ethanol that are involved in ethanol-induced mesolimbic dopamine release (Molander and Söderpalm 2005; Molander et al. 2007), thus linking GlyRs to the rewarding effects of ethanol.

The molecular targets and mechanisms that initiate ethanol action in GlyRs represent an active area of investigation (Mascia et al. 1996a; Mihic et al. 1997; Valenzuela et al. 1998; Ye et al. 2002; Davies et al. 2004; Yevenes et al. 2006; Crawford et al. 2007; Lobo et al. 2007). Considerable research has focused on potential targets for ethanol action in the transmembrane (TM) domains of GlyRs. Several of these studies employed the substituted cysteine accessibility method (SCAM) (Karlin and Akabas 1998) in combination with the anesthetic-like propyl methanethiosulfonate (PMTS) (Mascia et al. 2000). The authors proposed that PMTS would covalently bind to the substituted cysteine residue and change the normal effect of PMTS (reversible potentiation) to irreversible potentiation if the actions of PMTS and alcohols result from binding at this site. The results and subsequent studies fit this prediction and provide solid evidence that position 267 and, possibly other sites in the TM domain of GlyRs, are sites of ethanol action and contribute to an ethanol “binding/action” pocket (Mihic et al. 1997; Ye et al. 1998; Ye et al. 2002; Lobo et al. 2007).

Studies using pressure indicate that the extracellular domain also contains important targets for ethanol. These studies found that a naturally occurring mutation in Loop 2 of the extracellular domain of $\alpha 1$ GlyRs (A52S) (Ryan et al. 1994; Saul et al. 1994) rendered $\alpha 1$ GlyRs insensitive to pressure antagonism of 100mM ethanol (Davies et al. 2004). Prior work has also shown that A52S mutant $\alpha 1$ GlyRs are less sensitive to ethanol than WT GlyRs (Mascia et al. 1996b; Davies et al. 2004; Lobo et al. 2007). Follow-up studies at normal atmospheric pressure, which utilized the SCAM technique in combination with PMTS, added two key elements to the evidence supporting the extracellular domain as an ethanol target (Crawford et al. 2007). This work found that PMTS exposure caused irreversible alcohol-like potentiation in $\alpha 1$ A52C GlyRs and, thus, suggested a cause-effect relationship between ethanol acting on this site in the extracellular domain and alcohol-like GlyR modulation (Crawford et al. 2007). Further studies found that PMTS binding to cysteines in A52C and S267C $\alpha 1$ GlyRs decreased the alcohol cutoff in a manner consistent with the notion that position 52 in the extracellular domain and position 267 in the TM

domain form part of the same alcohol-action pocket (Crawford et al. 2007). These findings with position 52 of the α_1 GlyR parallel earlier findings that established the TM domain as a target for ethanol action. Collectively, these findings indicate that ethanol acts on targets in both the TM and extracellular domains, and that positions 52 and 267 are part of the same alcohol pocket (Crawford et al. 2007).

Interestingly, hyperbaric and other findings in WT and mutant α_1 GlyRs suggest that there may be a relationship between the physical chemical characteristics of the amino acid at position 52 and sensitivity to ethanol and to pressure antagonism of ethanol. As discussed above, substituting a polar serine for the non-polar alanine in WT α_1 GlyRs reduced ethanol sensitivity and eliminated sensitivity to pressure antagonism of ethanol (Davies et al. 2004). Moreover, sequence analysis of α_1 GlyRs and α_2 GlyRs indicates that α_2 GlyRs have a polar threonine at position 59, which is homologous to position 52 in WT α_1 GlyRs. Like A52S α_1 GlyRs, α_2 GlyRs have reduced ethanol sensitivity and are insensitive to pressure antagonism of ethanol compared to α_1 GlyRs (Davies et al. 2004). Taken together, these findings suggest that the polarity of the amino acid at position 52 in α_1 GlyRs, and the homologous position in α_2 GlyRs, plays a key role in determining the sensitivity of the receptor to ethanol and pressure antagonism of ethanol. Prior studies found a significant correlation between the molecular volume of the amino acid at TM position 267 and ethanol sensitivity (Ye et al. 1998), which suggests that other physical-chemical parameters at position 52 might also influence sensitivity of the receptor to ethanol and pressure antagonism of ethanol.

The present studies test the hypothesis that the physical-chemical properties of the specific residue at position 52 in WT α_1 GlyRs (A52) or its homologous position in WT α_2 GlyRs (T59), are determinants of the receptor's sensitivity to ethanol and pressure antagonism of ethanol. The findings support this hypothesis, increase our understanding of the molecular targets for ethanol action and antagonism, and should help in the development of novel prevention and treatment strategies for alcohol-related problems.

MATERIALS AND METHODS

Materials

Adult female *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI). Penicillin, streptomycin, gentamicin, 3-aminobenzoic acid ethyl ester, glycine, ethanol, and collagenase were purchased from Sigma (St. Louis, MO). All other chemicals used were of reagent grade. Certified premixed gases were supplied by Specialty Air Technologies (Long Beach, CA).

Expression in Oocytes

Xenopus oocytes were isolated and injected with either human α_1 , α_2 or mutant α_1 A52C, α_1 A52F, α_1 A52I, α_1 A52S, α_1 A52T, α_1 A52V, α_1 A52W, α_1 A52Y, or α_2 T59A cDNAs (1 ng per 32 nl) cloned into the mammalian expression vector pCIS2 or pBKCMV as previously described (Davies et al. 2003) and verified by partial sequencing (DNA Core Facility, University of Southern California, USA). Mutagenesis of the alanine at position 52

in $\alpha 1$ GlyRs or the threonine at position 59 in $\alpha 2$ GlyRs was performed as previously described (Ryan et al. 1994). After injection, oocytes were stored individually in incubation medium (MBS supplemented with 2mM sodium pyruvate, 0.5mM theophylline, 10,000 U/l penicillin, 10 mg/l streptomycin and 50 mg/l gentamicin) in petri dishes (VWR, San Dimas, CA). All solutions were sterilized by passage through 0.22 μ M filters. Oocytes, stored at 18°C, usually expressed GlyRs the day after injection. Oocytes were used in electrophysiological recordings for 3–7 days after cDNA injection.

Atmospheric conditions

Atmospheric conditions were established as described previously (Davies et al. 1999; Davies et al. 2003). Briefly, we tested the 1 ATA air condition (control) in a hyperbaric chamber with the lid removed or by using compressed air with the chamber sealed. Pilot studies determined that there was no measurable difference between the results obtained by these two methods. To achieve the 12 ATA heliox condition, the chamber was purged with heliox and then pressurized to the experimental ATA at the rate of 1 ATA/min using premixed certified compressed gases. The heliox mixture consisted of 1.7% oxygen and 98.3% helium resulting in a 0.2 ATA partial pressure for oxygen at 12 ATA; this mixture provides normal oxygenation. We found previously that this mixture avoids complications from higher oxygen partial pressures (Alkana and Malcolm 1981). We replaced the nitrogen in our gas mixture with helium to avoid the depressant effect of nitrogen at increased atmospheric pressure (Alkana and Malcolm 1982).

Hyperbaric Two-Electrode Whole Cell Voltage Clamp Recording

Two-electrode voltage clamp recording was performed using techniques similar to those previously reported (Davies et al. 2003). Briefly, electrodes pulled (P-30, Sutter Instruments, Novato, CA) from borosilicate glass [1.2mm thick-walled filamented glass capillaries (WPI, Sarasota, FL)] were back-filled with 3M KCl to yield resistances of 0.5–3 M Ω . All electrophysiological recordings were conducted within a specially designed hyperbaric chamber that contains a vibration resistant platform that supports an oocyte bath, two micro positioners (WPI, Sarasota, FL or Narishige International USA, Inc East Meadow, NY) and bath clamp (Davies et al. 2003). Oocytes were perfused in a 100 μ l oocyte bath with MBS $\pm$ drugs via a custom high pressure drug delivery system (Alcott Chromatography, Norcross, GA) at 2 ml/min using 1/16 OD high pressure PEEK tubing (Upchurch Scientific, Oak Harbor, WA). Oocytes were voltage clamped at a membrane potential of -70 mV using a Warner Instruments Model OC-725C (Hamden, CT) oocyte clamp. Individual oocytes were tested at both control and experimental atmospheric conditions and checked for normal function during pressurization and depressurization. The order of testing control and atmospheric conditions was counterbalanced to minimize the effects of testing order and to determine if pressure effects were reversible.

Hyperbaric ethanol experiments

Previous work found that ethanol potentiation of GlyR function is more robust and reliable when tested in the presence of low concentrations of glycine (typically EC₂₋₁₀) (Davies et al. 2003). Based on these studies, we used a concentration of glycine producing $2 \pm 0.3\%$ of the maximal effect (EC₂). Pilot experiments found that WT and mutant GlyR responses using a

1 min glycine application reached maximal response, which did not differ appreciably from results using 30s applications. Therefore, we used the shorter application time to increase efficiency and to minimize desensitization at the higher glycine concentrations. When testing ethanol potentiation, the oocytes were preincubated with ethanol for 60 sec prior to co-application of ethanol and glycine (Davies et al. 2004). Washout periods (5–15 min) were allowed between drug applications to ensure complete resensitization of receptors. The hyperbaric experiments for WT $\alpha 1$, $\alpha 1A52S$, and $\alpha 2T59A$ were measured across a full ethanol concentration range (25–200mM). For the $\alpha 1A52C$, $\alpha 1A52F$, $\alpha 1A52I$, $\alpha 1A52T$, $\alpha 1A52V$, $\alpha 1A52W$ and $\alpha 1A52Y$ mutants, we tested 100mM ethanol because this concentration produced a degree of potentiation that was functionally equivalent to the potentiation produced by ethanol in WT $\alpha 1$ GlyRs that were antagonized by pressure (Davies et al. 2003; Davies et al. 2004). All experiments testing mutant GlyRs included WT control receptors expressed in the same batch of oocytes as the respective mutant GlyRs. A chart recorder (Barnstead/Thermolyne, Dubuque, IA) continuously plotted the clamped currents. The peak currents were measured and used in data analysis. All experiments were performed at room temperature (20–23° C).

Data Analysis

Data for each experiment were obtained from oocytes from at least two different frogs. The n refers to the number of oocytes tested. Results are expressed as mean $\pm$ SEM. Where no error bars are shown, they are smaller than the symbols. We used Prism (GraphPAD Software, San Diego, CA) to perform curve fitting and statistical analyses. Concentration response data were analyzed using non-linear regression analysis: $[I = I_{\max} [A]^{n_H} / ([A]^{n_H} + EC_{50}^{n_H})]$ where I is the peak current recorded following application of a range of agonist concentrations, $[A]$; $I_{\max}$ is the estimated maximum current; EC_{50} is the glycine concentration required for a half-maximal response and n_H is the Hill slope. Data were subjected to t-tests, one- or two-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison or Bonferroni post tests when warranted. For the correlation analyses, the change in ethanol potentiation upon exposure to 12ATA (P) was determined by subtracting mean ethanol % potentiation at 12ATA from that observed at 1ATA. Polarity values were assigned to amino acid residues using the Zimmerman polarity scale (Zimmerman et al. 1968). Hydrophobicity values were assigned using the Eisenberg hydrophobicity scale (Eisenberg et al. 1984). Molecular volumes were calculated with the Spartan Molecular Modeling Program (Wave Function, San Diego, CA). Statistical significance was defined as $p < 0.05$.

RESULTS

Glycine EC50s in WT and Mutant $\alpha 1$ GlyRs

The effect of point mutations at position 52 on glycine sensitivity of $\alpha 1$ GlyRs is shown in Figure 1A. Inward chloride currents were evoked in a concentration-dependent manner by glycine in WT and all mutant GlyRs. We found significant right shifts in glycine EC50 from WT $\alpha 1$ GlyR for the polar $\alpha 1A52S$, $\alpha 1A52T$ and $\alpha 1A52W$ GlyRs and significant left shifts for the non-polar $\alpha 1A52I$ and $\alpha 1A52V$ GlyRs, but not for other substitutions. EC50 values of WT and mutant $\alpha 1$ GlyRs did not correlate significantly with polarity, hydrophobicity,

molecular volume, or weight (Data not shown). No significant differences were observed between WT and mutant GlyRs in Hill slope (n_H) and/or maximal current amplitude (I_{max}) (Table 1).

Ethanol Sensitivity in WT and Mutant $\alpha 1$ GlyRs

The effect of point mutations at position 52 in $\alpha 1$ GlyRs on sensitivity to 100mM ethanol is shown in Figure 2. Ethanol responses for highly polar substitutions at position 52 - $\alpha 1$ A52S, $\alpha 1$ A52T, $\alpha 1$ A52W and $\alpha 1$ A52Y were significantly reduced compared to WT $\alpha 1$ GlyRs. Ethanol responses for the intermediate-polar $\alpha 1$ A52C and non-polar $\alpha 1$ A52I, $\alpha 1$ A52V and $\alpha 1$ A52F mutants were not significantly reduced compared to WT $\alpha 1$ GlyRs. There was a significant inverse correlation between polarity and receptor sensitivity to ethanol (Figure 3A). In addition, we found a significant direct correlation between hydrophobicity of the residue at position 52 and receptor sensitivity to ethanol (Figure 3B). The r^2 values from the correlation analyses indicate that polarity explained approximately 88% of the variability in the sensitivity of $\alpha 1$ GlyRs to ethanol while hydrophobicity accounted for approximately 69% of the variability. In contrast to polarity and hydrophobicity, the molecular volume and weight of the residues at position 52 did not appear to affect the sensitivity of $\alpha 1$ GlyRs to ethanol (Figure 3C–D).

Pressure Antagonism Sensitivity in WT and Mutant $\alpha 1$ GlyRs

We began testing the hypothesis that the physical-chemical properties at position 52 in $\alpha 1$ GlyRs play a role in determining sensitivity to pressure antagonism of four different concentrations of ethanol by testing A52S GlyRs, in which the non-polar alanine in WT receptors was replaced by a polar serine. Oocytes expressing $\alpha 1$ WT or A52S GlyRs were voltage-clamped and tested with glycine $\pm$ (25–200mM) ethanol under both control and pressure conditions. Consistent with previous findings (Davies et al. 2004), the alanine to serine mutation at position 52 eliminated sensitivity to pressure antagonism of 100mM ethanol seen in WT $\alpha 1$ GlyRs. This lack of the antagonist's effect was evident across a broad range of ethanol concentrations (Figure 4).

We expanded the investigation to test the effects of mutations with different physical chemical parameters at position 52 on the sensitivity to pressure antagonism of ethanol in $\alpha 1$ GlyRs. Based on the results with ethanol sensitivity (Figures 2 and 3), we focused on polarity and hydrophobicity, but also investigated substitutions that altered molecular weight and volume.

Oocytes expressing the polar $\alpha 1$ A52T, $\alpha 1$ A52W and $\alpha 1$ A52Y GlyRs, the intermediate-polar $\alpha 1$ A52C GlyRs and the non-polar $\alpha 1$ A52F, $\alpha 1$ A52I and $\alpha 1$ A52V GlyRs were voltage-clamped and tested with glycine $\pm$ (100mM) ethanol under both control and pressure conditions. We found that exposure to 12 ATA heliox did not significantly affect ethanol potentiation in the polar $\alpha 1$ A52C, $\alpha 1$ A52T, $\alpha 1$ A52W or $\alpha 1$ A52Y GlyRs (Figure 5A–D). In contrast, pressure did significantly antagonize ethanol potentiation of 100mM in the non-polar $\alpha 1$ A52F, $\alpha 1$ A52I and $\alpha 1$ A52V GlyRs (Figure 5E–G). Moreover, as with sensitivity to ethanol, there was a significant inverse correlation between polarity of the residue at position 52 and receptor sensitivity to pressure antagonism of ethanol (Figure 6A) and a

significant inverse correlation between hydrophobicity of the residue at position 52 and sensitivity to pressure antagonism of ethanol (Figure 6B). The r^2 indicated that polarity explained approximately 84% of the variability in the sensitivity of $\alpha 1$ GlyRs to pressure antagonism of ethanol while hydrophobicity accounted for approximately 53% of the variability. In contrast to polarity and hydrophobicity, the molecular volume and weight of the residues at position 52 did not correlate with sensitivity to ethanol or pressure antagonism of ethanol (Figure 6C–D). Collectively, these findings support the hypothesis that the polarity/hydrophobicity, but not the molecular weight or volume, of the residue at position 52 in $\alpha 1$ GlyRs are key factors in determining the sensitivity of the receptor to pressure antagonism of ethanol.

Ethanol and Pressure Antagonism Sensitivity in $\alpha 2$ GlyRs

As described above, $\alpha 1$ and $\alpha 2$ GlyRs differ in their sensitivity to ethanol ($\alpha 1 > \alpha 2$) and sensitivity to pressure antagonism of ethanol ($\alpha 1$ —pressure antagonism sensitive; $\alpha 2$ —pressure antagonism insensitive) (Davies et al. 2004). Sequence alignment between the Loop 2 regions of the receptor subtypes revealed that the WT $\alpha 2$ GlyR has a polar threonine at position 59, the position homologous to position 52 in $\alpha 1$ GlyRs (Table 2). Given that substituting the non-polar alanine for a polar threonine reduces ethanol sensitivity and eliminates sensitivity to pressure antagonism of ethanol in $\alpha 1$ GlyRs (Figures 2 and 5A), we predicted that mutating this polar threonine in $\alpha 2$ GlyRs to the non-polar alanine would convert the response of $\alpha 2$ GlyRs to ethanol and pressure to be similar to those of $\alpha 1$ GlyRs. The $\alpha 2T59A$ mutation yielded functional receptors with glycine concentration responses that were left shifted compared to WT $\alpha 2$ GlyRs and were similar to WT $\alpha 1$ GlyRs (Figure 7A). As predicted, the alanine substitution at position 59 changed WT $\alpha 2$ GlyRs from being insensitive to pressure antagonism of ethanol to being sensitive to the antagonism (Figure 7B). Overall, the findings in $\alpha 1$ and $\alpha 2$ GlyRs demonstrate that switching between non-polar and polar residues at homologous positions 52 and 59 can switch the response of $\alpha 1$ GlyRs to ethanol and pressure to be $\alpha 2$ -like and vice-versa.

DISCUSSION

Considerable attention has begun to focus on the development of medications to prevent or treat alcoholism. This focus is, in part, due to the marked increase in knowledge regarding the neurochemical targets for ethanol's effects on brain function. This includes identification of neurotransmitter systems that appear to be initial targets for ethanol such as LGICs (e.g., GABA_A, glycine, glutamate, 5HT₃) and other targets that may play a role in mediating physical dependence and craving (Deitrich et al. 1989; Zhou and Lovinger 1996; Mihic et al. 1997; Ye et al. 1998; Cardoso et al. 1999; Harris 1999; Davies and Alkana 2001). However, this information has not resulted in comparable success in the development of pharmacological treatments for alcoholism.

Current approved therapeutics for alcoholism focus on deterring drinking by making alcohol ingestion aversive (e.g., disulfiram) or reducing the craving for alcohol (e.g., naltrexone and acamprostate) (Heilig M. and Egli M. 2006). However, the modest success rate of these drugs reflects, at least in part, their proposed mechanisms, which do not focus on blocking

the actions of ethanol at initial targets. Rather, these drugs target neurochemical and neuropeptide systems in the downstream cascades leading to craving and dependence. Recently, varenicline, an approved pharmacotherapeutic for smoking cessation, demonstrated significant reduction in both acute and chronic alcohol consumption in an animal model (Steensland et al. 2007). Other drugs currently under investigation for alcoholism include topiramate (Johnson et al. 2007) and rimonabant (Colombo et al. 2007). But, these drugs also, theoretically, target downstream actions of ethanol.

An alternative approach that we have taken focuses on strategies to develop agents that are designed to block the initial actions of ethanol at its different neurotransmitter targets, thus potentially yielding novel prevention and treatment approaches (Alkana and Malcolm 1980; Davies and Alkana 1998). Recent studies along these lines have investigated the benzodiazepine receptor inverse agonist RO 15–4513 as a novel method of antagonizing ethanol action in GABA_ARs (Wallner et al. 2006).

Presently, we do not know enough about where and how ethanol acts to develop medications that antagonize ethanol directly. Part of the difficulty lies in the physical-chemical nature of ethanol's mechanism of action, which limits the ability to use standard pharmacological approaches to study ethanol's actions and effects, and to identify direct mechanistic ethanol antagonists (Deitrich et al. 1989). Findings over the last fifteen years suggesting that ethanol acts by "binding" to "pockets" (Franks and Lieb 1994; Mihic et al. 1997; Franks and Lieb 1997; Ye et al. 1998; Wick et al. 1998; Mascia et al. 2000; Crawford et al. 2007) seem to have blurred the mechanistic distinction between intoxicant-anesthetics, such as ethanol and other psychoactive drugs. However, the primary determinant of intoxicant-anesthetic potency remains hydrophobicity, not molecular structure. Furthermore, the high, millimolar ethanol concentrations required for its biological actions are inconsistent with high affinity sites of action and suggests that ethanol acts simultaneously by the same mechanism on different types of initial sites (Deitrich et al. 1989). Hence, ethanol's physical-chemical mechanism of action is fundamentally different from the selective, high affinity binding mechanism that is known to initiate the behavioral effects of most psychoactive drugs (Dunn et al. 1999). This atypical mechanism, and resultant lack of high affinity and pharmacological specificity, precludes the classical approach of using "ethanol receptor" antagonists to identify the sites and mechanisms of ethanol action.

The present studies used increased atmospheric pressure in place of a traditional pharmacological antagonist to probe the molecular sites and mechanisms of ethanol action in GlyRs. We focused on position 52 in Loop 2 in the extracellular domain of GlyRs based on prior work which indicated that this position is part of an ethanol action pocket and is also a site of ethanol antagonism (Davies et al. 2004; Crawford et al. 2007).

We tested the hypothesis that the physical-chemical properties at position 52 in $\alpha 1$ GlyRs or the homologous position 59 in $\alpha 2$ GlyRs determine sensitivity to ethanol and pressure antagonism of ethanol. As predicted, we found that pressure antagonized the effects of ethanol in $\alpha 1$ GlyRs that contain a non-polar residue at position 52, but did not antagonize the receptors that had a polar residue at this position. Moreover, ethanol sensitivity in the

receptors with polar substitutions at position 52 was significantly lower compared to the GlyRs with non-polar residues at this position.

These findings indicate that the polarity and the hydrophobicity of the residue at position 52 plays a key role in determining the responsiveness of $\alpha 1$ GlyRs to both ethanol and pressure antagonism of ethanol. Correlation analyses presented in the present paper support this conclusion in $\alpha 1$ GlyRs. In addition, the $\alpha 2$ T59A mutation restored sensitivity to ethanol and pressure antagonism of ethanol in the $\alpha 2$ GlyR, thereby making it $\alpha 1$ -like in these respects. Collectively, these findings in $\alpha 1$ and $\alpha 2$ GlyRs point to Loop 2 in the extracellular domain of GlyRs as a potential target for the development of alcohol antagonists. They also indicate that there is structural functional homology across subunits in Loop 2 of the extracellular domain with respect to the role they play in determining sensitivity to ethanol and pressure antagonism of ethanol. These findings in the extracellular region differ from previous studies in the TM region that showed a role for molecular volume at position 267 in controlling ethanol sensitivity in $\alpha 1$ GlyRs (Ye et al. 1998), and suggest that different physical-chemical properties may influence ethanol action, respectively, in the extracellular and TM domains.

Although polarity and hydrophobicity are related, and are often thought to be interchangeable, examining separately their correlations with potency revealed some insights into the nature of the ethanol binding sites. The Zimmerman polarity scale used in the present study (Zimmerman et al. 1968) is based on the difference in electronegativity between atoms in the amino acids tested, and is not directly determined by oil-water partitioning/partition coefficients. However, hydrophobicity is determined by the oil-water partitioning of the respective amino acids. Hence, these differences in the physical chemical parameters likely underlie the respective differences in the strength of the correlations with sensitivity to ethanol and to pressure antagonism of ethanol.

For example, tryptophan is considered to be a non-polar amino acid due to the hydrophobic character of its indole ring, which is reflected in its relatively high positioning in the hydrophobicity scale. In contrast, the electron pair on the indole nitrogen allows it to form a hydrogen bond and causes it to behave like a polar amino acid. In addition, the indole ring in tryptophan (and the aromatic rings in A52F and A52Y) could act as an acceptor in a cation- π interaction (Dougherty 1996) and this ability is not reflected in the hydrophobicity scale. In the present study, tryptophan appears to be a key determinant of the strength of the correlations with sensitivity to both ethanol and pressure antagonism of ethanol. Specifically, when its polarity is used, it fits nicely in the linear correlation for both sensitivity to ethanol, and pressure antagonism of ethanol. In contrast, when its hydrophobicity is used, tryptophan is an outlier in both cases. This suggests that polarity, and not hydrophobicity, is the key determinant. Taken together, these findings suggest that the physical-chemical factors at position 52 that influence sensitivity to ethanol and sensitivity to pressure antagonism of ethanol are influenced strongly by polarity and that polarity may be the underlying factor that gives hydrophobicity its correlation.

There are several possible explanations for the antagonistic effects of 12 ATA pressure observed in the present study. Fortunately, previous studies help to define the most likely

mechanisms. Direct effects of high pressure on protein structure have been studied extensively. However, these studies typically use 1000 to 10,000 ATA to observe effects (Weber G and Drickamer HG 1983). Indeed, in the present studies no effect was observed at 12 ATA in the response of GlyRs in the absence of ethanol. The effect of pressure on membrane fluidity (Trudell et al. 1973;Mastrangelo et al. 1979) and phase transitions (Trudell et al. 1974) in phospholipid bilayers has also been studied, but approximately 100 ATA was required to observe significant effects.

More pertinent to interpretation of the present results are studies in which approximately 10 ATA of Xenon or nitrous oxide was sufficient to displace water molecules from solvated cavities in proteins. For example, Xenon displaced water molecules from the pore of the cartilage oligomeric matrix protein (COMP) with little change in the structure of the water-soluble five stranded coil (Malashkevich et al. 1996). However, in the case of cavities engineered into T4-lysozyme (Quillin et al. 2000), occupancy of the cavities by Xenon resulted in swelling of the cavity volume. Although the latter low pressure studies demonstrated significant effects at 8–12 ATA, there is an important distinction between the soluble gases used in them and the relatively insoluble heliox used in the present study: Raising the equilibrium pressure of Xenon from 1 to 10 ATA raises the local concentration of the gas that could enter a cavity by the same ratio. This increase in activity of Xenon is sufficient to explain its ability to displace water from a binding site.

In contrast, raising the pressure of heliox makes only a small increase in the concentration of the gas in the cavity, because helium is relatively insoluble in water and lipid. Moreover, the short period that the solutions used in the present study were exposed to hyperbaric heliox further reduces the possibility that helium was dissolved in the solutions. Thus, the antagonism by 12 ATA heliox cannot be explained by an increase in the local concentration of heliox that displaces water and/or ethanol from the cavity, but is a direct effect of hydrostatic pressure.

Ethanol could act on GlyRs by creating excess partial molar volume. That is, the volume of the total system with alcohol present would be greater than the sum of the volumes of ethanol, water, and GlyR. This hypothesis that alcohols and anesthetics create excess partial molar volume has been presented in various forms by several authors (Miller et al. 1973;Mori et al. 1984;Imai T et al. 2006) and is consistent with the swelling of cavities by Xenon described above for T4-lysozyme. Given that exposure to 12 ATA heliox did not change WT or mutant receptor function in the absence of ethanol, it is reasonable to conclude that pressure does not change the way that water hydrates the exterior of the GlyR or fills its internal cavities. This notion is supported by previous work that shows that glycine response under 12 ATA heliox remains constant with multiple applications of glycine over prolonged periods, which indicates that pressure antagonism cannot be explained by receptor desensitization or rundown (Davies et al. 2003;Davies et al. 2004). Therefore, the most likely explanation of pressure antagonism of ethanol in GlyRs is that pressure offsets the increase in partial molar volume produced by ethanol. Pressure could do this by displacing ethanol and/or an ethanol-water complex from an active site.

The above notion is consistent with our present and previous data that suggest a continuous thermodynamic opposition between the effects of ethanol and pressure (Davies et al. 2004). That is, ethanol causes potentiation of $\alpha 1$ GlyR, pressure opposes this potentiation, but the pressure effect can be overcome by increasing the ethanol concentration (Figure 4A). Moreover, the differences in sensitivity to ethanol, based on the physical chemical properties of mutations at homologous positions 52 and 59 in $\alpha 1$ and $\alpha 2$ GlyRs, add key information to this model. The mutation of A52 to more polar residues reduced the potentiation by ethanol. This change can be explained by the differences in the forces attracting water and ethanol in the WT and mutant receptors. That is, water is attracted to alanine (non-polar) in WT A52 $\alpha 1$ GlyRs by a combination of London dispersion and van der Waals forces; whereas, water is attracted to serine (polar) in mutant A52S $\alpha 1$ GlyRs by these forces plus hydrogen bonding. Therefore, it would be harder for ethanol to displace water in order to access positions 52 when the respective position contains a polar versus non-polar residue. These differences in the accessibility of ethanol to a site of action could explain the differences in ethanol sensitivity of WT and mutant GlyRs.

In conclusion, the findings presented in this report represent the initial steps towards defining a site and mechanism of ethanol action and antagonism in GlyRs. Additional studies will increase our knowledge regarding the physical-chemical characteristics necessary for pressure antagonism, and in turn will aid in the development of molecules that mimic the ethanol antagonizing properties of pressure. Given the homology between GlyRs and other LGICs (GABA_AR, 5HT₃R and nAChRs), the current findings should also provide insight into the molecular mechanisms and targets of ethanol action and antagonism in other LGICs in the Cys loop superfamily of receptors.

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Abbreviations

5HT₃R	5-hydroxytryptamine ₃ receptor
COMP	cartilage oligomeric matrix protein
GlyR	glycine receptor
GABA_AR	γ -aminobutyric acid type-A receptor
LGIC	ligand-gated ion channel
nAChR	nicotinic acetylcholine receptor
PMTS	propyl methanethiosulfonate
SCAM	substituted cysteine accessibility method

TM	transmembrane
WT	wild type

References

1. Aguayo LG, Pancetti FC. Ethanol modulation of the γ -aminobutyric acid_A and glycine-activated Cl⁻ current in cultured mouse neurons. *J Pharmacol Exp Ther.* 1994; 270:61–69. [PubMed: 8035343]
2. Alkana RL, Davies DL, Morland J, Parker ES, Bejanian M. Low level hyperbaric exposure antagonizes locomotor effects of ethanol and n-propanol but not morphine in C57BL mice. *Alcohol Clin Exp Res.* 1995; 19:693–700. [PubMed: 7573795]
3. Alkana RL, Jones BL, Palomares ML, Crabbe JC, Bejanian M. Mechanism of low level hyperbaric ethanol antagonism: Specificity versus other drugs. *Alcohol Alcohol Suppl.* 1992; 1:63.
4. Alkana RL, Malcolm RD. The effects of low level hyperbaric treatment on acute ethanol intoxication. *Adv Exp Med Biol.* 1980; 126:499–507. [PubMed: 7190768]
5. Alkana RL, Malcolm RD. Low-level hyperbaric ethanol antagonism in mice. Dose and pressure response. *Pharmacology.* 1981; 22:199–208. [PubMed: 7208603]
6. Alkana RL, Malcolm RD. Hyperbaric ethanol antagonism in mice: studies on oxygen, nitrogen, strain and sex. *Psychopharmacology.* 1982; 77:11–16. [PubMed: 6812114]
7. Bejanian M, Jones BL, Alkana RL. Low-level hyperbaric antagonism of ethanol-induced locomotor depression in C57BL/6J mice: dose response. *Alcohol Clin Exp Res.* 1993; 17:935–939. [PubMed: 8279677]
8. Betz H. Glycine receptors: heterogeneous and widespread in the mammalian brain. *TINS.* 1991; 14:458–461. [PubMed: 1722365]
9. Bowser-Riley F, Daniels S, Smith EB. Investigations into the origin of the high pressure neurological syndrome: the interaction between pressure, strychnine and 1,2- propandiol in the mouse. *Br J Pharmacol.* 1988; 94:1069–1076. [PubMed: 3207974]
10. Cardoso RA, Brozowski SJ, Chavez-Noriega LE, Harpold M, Valenzuela CF, Harris RA. Effects of ethanol on recombinant human neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther.* 1999; 289:774–780. [PubMed: 10215652]
11. Colombo G, Orrù A, Lai P, Cabras C, Maccioni P, Rubio M, Gessa GL, Carai MA. The cannabinoid CB1 receptor antagonist, rimonabant, as a promising pharmacotherapy for alcohol dependence: preclinical evidence. *Mol Neurobiol.* 2007; 36:102–112. [PubMed: 17952655]
12. Crawford DK, Trudell JR, Bertaccini E, Li KX, Davies DL, Alkana RL. Evidence that ethanol acts on a target in Loop 2 of the extracellular domain of $\alpha 1$ glycine receptors. *J Neurochem.* 2007; 102:2097–2109. [PubMed: 17561937]
13. Davies DL, Alkana RL. Direct antagonism of ethanol's effects on GABA_A receptors by increased atmospheric pressure. *Alcohol Clin Exp Res.* 1998; 22:1689–1697. [PubMed: 9835282]
14. Davies DL, Alkana RL. Ethanol enhances GABA_A receptor function in short sleep and long sleep mouse brain membranes. *Alcohol Clin Exp Res.* 2001; 25:478–483. [PubMed: 11290862]
15. Davies DL, Bejanian M, Parker ES, Morland J, Bolger MB, Brinton RD, Alkana RL. Low-level hyperbaric antagonism of diazepam's locomotor depressant and anticonvulsant properties in mice. *J Pharmacol Exp Ther.* 1996; 276:667–675. [PubMed: 8632335]
16. Davies DL, Bolger MB, Brinton RD, Finn DA, Alkana RL. In vivo and in vitro hyperbaric studies in mice suggest novel sites of action for ethanol. *Psychopharmacology.* 1999; 141:339–350. [PubMed: 10090641]
17. Davies DL, Crawford DK, Trudell JR, Mihic SJ, Alkana RL. Multiple sites of ethanol action in $\alpha 1$ and $\alpha 2$ glycine receptors suggested by sensitivity to pressure antagonism. *J Neurochem.* 2004; 89:1175–1185. [PubMed: 15147510]
18. Davies DL, Morland J, Jones BL, Alkana RL. Low-level hyperbaric antagonism of ethanol's anticonvulsant property in C57BL/6J mice. *Alcohol Clin Exp Res.* 1994; 18:1190–1195. [PubMed: 7847605]

19. Davies DL, Trudell JR, Mihic SJ, Crawford DK, Alkana RL. Ethanol potentiation of glycine receptors expressed in *Xenopus* oocytes antagonized by increased atmospheric pressure. *Alcohol Clin Exp Res*. 2003; 27:1–13. [PubMed: 12543998]
20. Deitrich R, Dunwiddie T, Harris RA, Erwin VG. Mechanism of action of ethanol: initial central nervous system actions. *Pharmacol Rev*. 1989; 41:489–537. [PubMed: 2700603]
21. Dougherty DA. Cation- π interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. *Science*. 1996; 271:163–168. [PubMed: 8539615]
22. Dunn SMJ, Davies M, Muntoni AL, Lambert JJ. Mutagenesis of the rat $\alpha 1$ subunit of the γ -aminobutyric acid_A receptor reveals the importance of residue 101 in determining the allosteric effects of benzodiazepine site ligands. *Mol Pharmacol*. 1999; 56:768–774. [PubMed: 10496960]
23. Eckenhooff RG, Johansson JS. Molecular interactions between inhaled anesthetics and proteins. *Pharmacol Rev*. 1997; 49:343–367. [PubMed: 9443162]
24. Eggers ED, O'Brien JA, Berger AJ. Developmental changes in the modulation of synaptic glycine receptors by ethanol. *J Neurophysiol*. 2000; 84:2409–2416. [PubMed: 11067983]
25. Eisenberg D, Weiss RM, Terwilliger TC. The hydrophobic moment detects periodicity in protein hydrophobicity. *Proc Natl Acad Sci U S A*. 1984; 81:140–144. [PubMed: 6582470]
26. Engblom AC, Åkerman KE. Effect of ethanol on γ -aminobutyric acid and glycine receptor-coupled Cl^- fluxes in rat brain synaptoneurosomes. *J Neurochem*. 1991; 57:384–390. [PubMed: 1712827]
27. Franks NP, Lieb WR. Molecular and cellular mechanisms of general anaesthesia. *Nature*. 1994; 367:607–614. [PubMed: 7509043]
28. Franks NP, Lieb WR. Inhibitory synapses: Anaesthetics set their sites on ion channels. *Nature*. 1997; 389:334–335. [PubMed: 9311772]
29. Grant BF, Dawson DA, Stinson FS, Chou P, Dufour MC, Pickering RP. The 12-month prevalence and trends in DSM-IV alcohol abuse and dependence: United States, 1991–1992 and 2001–2002. *Drug Alcohol Depend*. 2004; 74:223–234. [PubMed: 15194200]
30. Harris RA. Ethanol actions on multiple ion channels: which are important? *Alcohol Clin Exp Res*. 1999; 23:1563–1570. [PubMed: 10549986]
31. Heilig M, Egli M. Pharmacological treatment of alcohol dependence: target symptoms and target mechanisms. *Pharmacol Ther*. 2006; 111:855–876. [PubMed: 16545872]
32. Imai T, Isogai H, Seto T, Kovalenko A, Hirata F. Theoretical study of volume changes accompanying xenon-lysozyme binding: implications for the molecular mechanism of pressure reversal of anesthesia. *Journal of Physical Chemistry B*. 2006; 110:12149–12154.
33. Johnson BA, Rosenthal N, Capece JA, Wiegand F, Mao L, Beyers K, McKay A, Ait-Daoud N, Anton RF, Ciraulo DA, Kranzler HR, Mann K, O'Malley SS, Swift R. Topiramate for Treating Alcohol Dependence: A Randomized Controlled Trial. *JAMA*. 2007; 298:1641–1651. [PubMed: 17925516]
34. Karlin A. Emerging structure of the nicotinic acetylcholine receptors. *Nat Reviews, Neurosci*. 2002; 3:102–1.
35. Karlin A, Akabas MH. Substituted-Cysteine Accessibility Method. *Methods Enzymol*. 1998; 293:123–145. [PubMed: 9711606]
36. Kendig JJ. Pressure dependence of excitable cell function. *TINS*. 1984; 7:483–486.
37. Little HJ. How has molecular pharmacology contributed to our understanding of the mechanism(s) of general anesthesia? *Pharmacol Ther*. 1996; 69:37–58. [PubMed: 8857302]
38. Lobo IA, Harris RA, Trudell JR. Cross-linking of sites involved with alcohol action between transmembrane segments 1 and 3 of the glycine receptor following activation. *J Neurochem*. 2007; 104:1649–1662. [PubMed: 18036150]
39. Malashkevich VN, Kammerer RA, Efimov VP, Schulthess T, Engel J. The crystal structure of a five-stranded coiled coil in COMP: A prototype ion channel? *Science*. 1996; 274:761–765. [PubMed: 8864111]
40. Malosio ML, Marquèze-Pouey B, Kuhse J, Betz H. Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *EMBO J*. 1991; 10:2401–2409. [PubMed: 1651228]

41. Mascia MP, Machu TL, Harris RA. Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. *Br J Pharmacol.* 1996a; 119:1331–1336. [PubMed: 8968539]
42. Mascia MP, Mihic SJ, Valenzuela CF, Schofield PR, Harris RA. A single amino acid determines differences in ethanol actions on strychnine-sensitive glycine receptors. *Mol Pharmacol.* 1996b; 50:402–406. [PubMed: 8700149]
43. Mascia MP, Trudell JR, Harris RA. Specific binding sites for alcohols and anesthetics on ligand-gated ion channels. *Proc Natl Acad Sci USA.* 2000; 97:9305–9310. [PubMed: 10908659]
44. Mastrangelo CJ, Kendig JJ, Trudell JR, Cohen EN. Nerve membrane lipid fluidity: Opposing effects of high pressure and ethanol. *Undersea Biomed Res.* 1979; 6:47–53. [PubMed: 223266]
45. McCool BA, Frye GD, Pulido MD, Botting SK. Effects of chronic ethanol consumption on rat GABA_A and strychnine-sensitive glycine receptors expressed by lateral/basolateral amygdala neurons. *Brain Res.* 2003; 963:165–177. [PubMed: 12560122]
46. Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL. Sites of alcohol and volatile anaesthetic action on GABA_A and glycine receptors. *Nature.* 1997; 389:385–389. [PubMed: 9311780]
47. Miller KW, Paton WDM, Smith RA, Smith EB. The pressure reversal of anaesthesia and the critical volume hypothesis. *Mol Pharmacol.* 1973; 9:131–143. [PubMed: 4711696]
48. Molander A, Lidö HH, Löf E, Ericson M, Söderpalm B. The glycine reuptake inhibitor Org 25935 decreases ethanol intake and preference in male wistar rats. *Alcohol Alcohol.* 2007; 42:11–18. [PubMed: 17098748]
49. Molander A, Söderpalm B. Accumbal strychnine-sensitive glycine receptors: an access point for ethanol to the brain reward system. *Alcohol Clin Exp Res.* 2005; 29:27–37. [PubMed: 15654288]
50. Mori T, Matubayasi N, Ueda I. Membrane expansion and inhalation anesthetics. Mean excess volume hypothesis. *Mol Pharmacol.* 1984; 25:123–130. [PubMed: 6546781]
51. Ortells MO, Lunt GG. Evolutionary history of the ligand-gated ion- channel superfamily of receptors. *TINS.* 1995; 18:121–127. [PubMed: 7754520]
52. Quillin ML, Breyer WA, Griswold IJ, Matthews BW. Size versus polarizability in protein-ligand interactions: binding of noble gases within engineered cavities in phage T4 lysozyme. *J Mol Biol.* 2000; 302:955–977. [PubMed: 10993735]
53. Rajendra S, Lynch JW, Schofield PR. The glycine receptor. *Pharmacol Ther.* 1997; 73:121–146. [PubMed: 9131721]
54. Ryan SG, Buckwalter MS, Lynch JW, Handford CA, Segura L, Shiang R, Wasmuth JJ, Camper SA, Schofield PR, O'Connell P. A missense mutation in the gene encoding the $\alpha 1$ subunit of the inhibitory glycine receptor in the spasmodic mouse. *Nature Genet.* 1994; 7:131–135. [PubMed: 7920629]
55. Saul B, Schmieden V, Kling C, Mülgardt C, Gass P, Kuhse J, Becker CM. Point mutation of glycine receptor $\alpha 1$ subunit in the spasmodic mouse affects agonist responses. *FEBS Lett.* 1994; 350:71–76. [PubMed: 8062927]
56. Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, Seeburg PH, Barnard EA. Sequence and functional expression of the GABA_A receptor shows a ligand-gated receptor super-family. *Nature.* 1987; 328:221–227. [PubMed: 3037384]
57. Steensland P, Simms JA, Holgate J, Richards JK, Bartlett SE. Varenicline, an $\{\alpha\}4\beta 2$ nicotinic acetylcholine receptor partial agonist, selectively decreases ethanol consumption and seeking. *Proc Natl Acad Sci U S A.* 2007; 104:12518–12523. [PubMed: 17626178]
58. Syapin PJ, Chen J, Finn DA, Alkana RL. Antagonism of ethanol-induced depression of mouse locomotor activity by hyperbaric exposure. *Life Sci.* 1988; 43:2221–2229. [PubMed: 3210903]
59. Tao L, Ye JH. Protein Kinase C Modulation of Ethanol Inhibition of Glycine-Activated Currnt in Dissociated Neurons of Rat Ventral Tegmental Area. *J Pharmacol Exp Ther.* 2002; 300:967–975. [PubMed: 11861805]
60. Tapia JC, Aguilar LF, Sotomayor CP, Aguayo LG. Ethanol affects the function of a neurotransmitter receptor protein without altering the membrane lipid phase. *Eur J Pharmacol.* 1998; 354:239–244. [PubMed: 9754925]

61. Trudell JR, Payan DG, Chin JH, Cohen EN. Pressure-induced elevation of phase transition temperature in dipalmitoylphosphatidylcholine bilayers: an electron spin resonance measurement of the enthalpy of phase transition. *Biochim Biophys Acta*. 1974; 373:436–443. [PubMed: 4373076]
62. Trudell JR, Hubbell WL, Cohen EN. Pressure reversal of inhalation anesthetic-induced disorder in spin-labeled phospholipid vesicles. *Biochim Biophys Acta*. 1973; 291:328–334. [PubMed: 4347902]
63. Valenzuela CF, Cardoso RA, Wick MJ, Weiner JL, Dunwiddie TV, Harris RA. Effects of ethanol on recombinant glycine receptors expressed in mammalian cell lines. *Alcohol Clin Exp Res*. 1998; 22:1132–1136. [PubMed: 9726286]
64. Wallner M, Hancher HJ, Olsen RW. Low-dose alcohol actions on $\alpha 4\beta 3\delta$ GABAA receptors are reversed by the behavioral alcohol antagonist Ro15–4513. *Proc Natl Acad Sci U S A*. 2006; 103:8540–8545. [PubMed: 16698930]
65. Wann KT, MacDonald AG. Actions and interactions of high pressure and general anesthetics. *Prog Neurobiol*. 1988; 30:271–307. [PubMed: 2449704]
66. Weber G, Drickamer HG. The effect of high pressure upon proteins and other biomolecules. *Q Rev Biophys*. 1983; 16:89–112. [PubMed: 6348832]
67. Wick MJ, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, Ye Q, Harrison NL, Harris RA. Mutations of γ -aminobutyric acid and glycine receptors change alcohol cutoff: Evidence for an alcohol receptor? *Proc Natl Acad Sci U S A*. 1998; 95:6504–6509. [PubMed: 9600996]
68. Ye JH, Tao L, Krnjevic K, McArdle JJ. Decay of Ethanol-Induced Suppression of Glycine-Activated Current of Ventral Tegmental Area Neurons. *Neuropharmacology*. 2002; 43:788–798. [PubMed: 12367623]
69. Ye Q, Koltchine VV, Mihic SJ, Mascia MP, Wick MJ, Finn SE, Harrison NL, Harris RA. Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position $\alpha 267$. *J Biol Chem*. 1998; 273:3314–3319. [PubMed: 9452448]
70. Yevenes GE, Moraga-Cid G, Guzmán L, Haeger S, Oliveira L, Olate J, Schmalzing G, Aguayo LG. Molecular determinants for G protein betagamma modulation of ionotropic glycine receptors. *J Biol Chem*. 2006; 281:39300–39307. [PubMed: 17040914]
71. Zhou Q, Lovinger DM. Pharmacologic characteristics of potentiation of 5-HT₃ receptors by alcohols and diethyl ether in NCB-20 neuroblastoma cells. *J Pharmacol Exp Ther*. 1996; 278:732–740. [PubMed: 8768725]
72. Zimmerman JM, Eliezer N, Simha R. The characterization of amino acid sequences in proteins by statistical methods. *J Theor Biol*. 1968; 21:170–201. [PubMed: 5700434]
73. Ziskind-Conhaim L, Gao BX, Hinckley C. Ethanol dual modulatory actions on spontaneous postsynaptic currents in spinal motoneurons. *J Neurophysiol*. 2003; 89:806–813. [PubMed: 12574458]

Figure 1. Concentration-response curves for glycine (10–3,000 μM) activated chloride currents in *Xenopus* oocytes expressing WT and mutant $\alpha 1\text{GlyR}$ subunits

Glycine induced chloride currents were normalized to the maximal current activated by a saturating concentration of glycine (3 mM) when tested under 1 ATA air conditions. The curves represent non-linear regression analysis of the glycine concentration responses in the $\alpha 1$ mutant GlyRs compared to WT $\alpha 1\text{GlyRs}$. Details of EC_{50} , Hill Slope and Maximal current amplitude (I_{max}) are provided in Table 1. Glycine was applied for 30 sec. Washout time was 5–15 min after application of glycine. Each data point represents the mean $\pm$ SEM from 4 different oocytes.

Figure 2. Polarity of the residue at position 52 in α 1GlyRs determines sensitivity to ethanol
Figure shows ethanol % potentiation of glycine induced chloride current at 100mM ethanol. White bars represent non-polar substitutions at position 52 in α 1 GlyRs and grey bars represent polar substitutions. Ethanol responses for the polar mutants α 1A52S, α 1A52T, α 1A52Y and α 1A52W GlyRs were significantly reduced compared to WT α 1GlyRs. There were no significant differences among the polar mutants with respect to ethanol sensitivity at 100mM. Significance is * $p < 0.05$ vs. WT α 1GlyRs.

Figure 3. The polarity and hydrophobicity of substitutions at position 52 are significantly correlated with sensitivity of $\alpha 1$ GlyRs to ethanol

The physical-chemical properties of the residues at position 52 (A) Polarity, (B) Hydrophobicity, (C) Molecular Volume and (D) Molecular Weight respectively, were correlated against sensitivity to 100mM ethanol.

Figure 4.

(A). Pressure significantly antagonizes ethanol potentiation in $\alpha 1$ GlyRs: The figure depicts WT $\alpha 1$ GlyRs tested at both 1 ATA air control (white bars) and 12 ATA heliox (black bars). In agreement with previous findings, pressure antagonized ethanol potentiation from 50–200mM but not at 25mM; **(B) Pressure Does not Antagonize Ethanol potentiation in mutant $\alpha 1A52S$ GlyRs:** The figure depicts results from 25–100 mM ethanol in the mutant (A52S) $\alpha 1$ GlyRs from the same batches of oocytes shown in (A), tested at both 1 ATA air control (white bars) and 12 ATA heliox (black bars). Data is presented as mean $\pm$ SEM of 4–6 oocytes. Data was subjected to two-way repeated measures ANOVA followed by Bonferroni post-hoc analyses. Statistical significance is * $p < 0.05$ or *** $p < 0.001$.

Figure 5. Pressure antagonizes ethanol in $\alpha 1$ GlyRs that have a non-polar residue at position 52
 The figure depicts mutant $\alpha 1$ GlyRs tested at both 1 ATA air control (white bars) and 12 ATA heliox (black bars). Exposure to 12 ATA heliox did not significantly affect ethanol potentiation at 100mM in (A) $\alpha 1$ A52C (intermediate, intermediate-polar) (B) $\alpha 1$ A52T (intermediate, polar), (C) $\alpha 1$ A52W (bulky, polar) or (D) $\alpha 1$ A52Y (bulky, polar) GlyRs. Pressure did significantly antagonize ethanol potentiation of (E) $\alpha 1$ A52F (bulky, non-polar), (F) $\alpha 1$ A52I (intermediate, non-polar) and (G) $\alpha 1$ A52V (small, non-polar) GlyRs. Data were analyzed using unpaired t-tests to determine the effect of atmospheric condition on ethanol potentiation of glycine-activated chloride currents. Statistical significance is ** $p < 0.01$. Data are presented as mean $\pm$ SEM of 4–6 oocytes.

Figure 6. The polarity of the residue at position 52 in $\alpha 1$ GlyRs determines the receptors sensitivity to pressure antagonism of ethanol

The physical-chemical properties of the residues at position 52 (A) Polarity, (B) Hydrophobicity, (C) Molecular Volume and (D) Molecular Weight respectively, were correlated against change in ethanol potentiation upon exposure to 12ATA (ΔP).

Figure 7.

(A) Concentration-response curves for glycine (10–3,000 μ M) activated Cl⁻ currents in *Xenopus* oocytes expressing WT and mutant α 2 glycine receptor subunits: Glycine induced chloride currents were normalized to the maximal current activated by a saturating concentration of glycine (3mM) when tested under 1 ATA air conditions. The curves represent non-linear regression analysis of the glycine concentration responses in the α 2 WT and mutant GlyRs compared to WT α 1GlyRs. Details of EC₅₀, Hill Slope and Maximal current amplitude (I_{max}) are provided in Table 1. Glycine was applied for 30 sec. Washout

time was 5–15 min after application of glycine. Each data point represents the mean $\pm$ SEM from 4 different oocytes; **(B) Replacing the polar threonine at position 59 in WT α 1GlyRs with the non-polar alanine changes the α 2GlyRs to be α 1GlyR-like in response to ethanol and pressure antagonism of ethanol:** The figure depicts mutant α 2T59A GlyRs tested at both 1ATA air control (white bars) and 12 ATA heliox (black bars). As predicted, pressure antagonized ethanol potentiation from 50–200mM but not at 25mM. The ethanol responses in the mutant were similar to WT α 1GlyRs. Data were subjected to two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni post-hoc analyses. Statistical significance is * $p < 0.05$ or *** $p < 0.001$. Data are presented as mean $\pm$ SEM of 4–5 oocytes.

Table 1
Summary of non-linear regression analysis results for glycine concentration responses and differences in ethanol sensitivity WT and mutant $\alpha 1$ and $\alpha 2$ GlyRs shown in terms of increasing polarity of residue at position 52 or 59

Glycine EC50, Hill Slope (n_H) and maximal current amplitude (I_{max}) are presented as mean $\pm$ SEM from 4 different oocytes (as shown in Figure 1). Statistical significance from WT $\alpha 1$ GlyRs was assessed using one-way ANOVA with Dunnett's post test. GlyRs containing polar substitutions at position 52 were significantly right shifted from $\alpha 1$ WT and not significantly different than WT $\alpha 2$ GlyRs in terms of EC50 with the exception of A52W. No significant differences were observed from WT $\alpha 1$ GlyRs for Hill Slope (n_H) or maximal current amplitude (I_{max}).

	Receptor (relative polarity)	I _{max} (nA)	Hill Slope	EC50 (μ M)
Non-polar	$\alpha 1$ A52V	17800 $\pm$ 1842	2.2 $\pm$ 0.3	31 $\pm$ 4
	$\alpha 1$ A52I	18425 $\pm$ 2072	2.5 $\pm$ 0.4	34 $\pm$ 1
	$\alpha 1$ A52F	18625 $\pm$ 5525	2.3 $\pm$ 0.3	199 $\pm$ 62
	$\alpha 1$ WT (A52)	19875 $\pm$ 2932	2.1 $\pm$ 0.3	130 $\pm$ 48
	$\alpha 2$ T59A	17750 $\pm$ 4033	1.6 $\pm$ 0.4	123 $\pm$ 45
Intermediate polar	$\alpha 1$ A52C	18250 $\pm$ 2322	2.4 $\pm$ 0.3	148 $\pm$ 23
Polar	$\alpha 2$ WT (T59)	20687 $\pm$ 7026	2.3 $\pm$ 0.3	374 $\pm$ 61
	$\alpha 1$ A52T	18187 $\pm$ 4360	2.9 $\pm$ 0.5	410 $\pm$ 32
	$\alpha 1$ A52S	18375 $\pm$ 5572	3.4 $\pm$ 0.4	387 $\pm$ 10
	$\alpha 1$ A52Y	16687 $\pm$ 2809	2.8 $\pm$ 0.9	239 $\pm$ 58
	$\alpha 1$ A52W	16750 $\pm$ 2809	1.4 $\pm$ 0.1	839 $\pm$ 112

Table 2
Alignment of a portion of the amino terminal regions from human α 1GlyR, α 1A52S GlyR and α 2GlyR subunit sequences

Asterisks indicate residues that differ between WT α 1 and α 2GlyRs. Black boxed in region represents Loop 2. Residues in bold represent position 52 in α 1GlyRs and homologous position 59 in α 2GlyR respectively. Relative sensitivity to ethanol potentiation (EtOH) and whether pressure can antagonize the effects of ethanol (Pressure) are shown.

Subunit	Loop 2 in Extracellular Amino Terminal Region										EtOH	Pressure
			*	*								
α 1GlyR	50	S	I	A	E	T	T	M	D	Y R 60	++++	Yes
α 1A52S	50	S	I	S	E	T	T	M	D	Y R 60	+++	No
α 2GlyR	57	S	V	T	E	T	T	M	D	Y R 68	+++	No