Mutation of a Zinc-Binding Residue in the Glycine Receptor $\alpha 1$ Subunit Changes Ethanol Sensitivity In Vitro and Alcohol Consumption In Vivo^S

Lindsay M. McCracken,¹ Yuri A. Blednov, James R. Trudell, Jillian M. Benavidez, Heinrich Betz,² and R. Adron Harris

The Waggoner Center for Alcohol and Addiction Research, The University of Texas at Austin, Austin, Texas (L.M.M., Y.A.B., J.M.B., R.A.H.); Stanford University School of Medicine, Stanford, California (J.R.T.); Max-Planck Institute for Brain Research, Frankfurt, Germany (H.B.)

Received June 22, 2012; accepted September 5, 2012

ABSTRACT

Ethanol is a widely used drug, yet an understanding of its sites and mechanisms of action remains incomplete. Among the protein targets of ethanol are glycine receptors (GlyRs), which are potentiated by millimolar concentrations of ethanol. In addition, zinc ions also modulate GlyR function, and recent evidence suggests that physiologic concentrations of zinc enhance ethanol potentiation of GlyRs. Here, we first built a homology model of a zinc-bound GlyR using the D80 position as a coordination site for a zinc ion. Next, we investigated in vitro the effects of zinc on ethanol action at recombinant wild-type (WT) and mutant $\alpha 1$ GlyRs containing the D80A substitution, which eliminates zinc potentiation. At D80A GlyRs, the effects of 50 and 200 mM ethanol were reduced as compared with WT receptors. Also, in contrast to what was seen with WT

GlyRs, neither adding nor chelating zinc changed the magnitude of ethanol enhancement of mutant D80A receptors. Next, we evaluated the in vivo effects of the D80A substitution by using heterozygous *Gla1*(D80A) knock-in (KI) mice. The KI mice showed decreased ethanol consumption and preference, and they displayed increased startle responses compared with their WT littermates. Other behavioral tests, including ethanol-induced motor incoordination and strychnine-induced convulsions, revealed no differences between the KI and WT mice. Together, our findings indicate that zinc is critical in determining the effects of ethanol at GlyRs and suggest that zinc binding at the D80 position may be important for mediating some of the behavioral effects of ethanol action at GlyRs.

Introduction

Strychnine-sensitive glycine receptors (GlyRs) are members of the Cys-loop superfamily of ligand-gated ion channels. In the spinal cord (Legendre, 2001) and in many brain regions, including the cortex, hippocampus, amygdala, nucleus accumbens, striatum, ventral tegmental area (Baer et al., 2009; Jonsson et al., 2009), brain stem (Legendre, 2001), and cerebellum (Takahashi et al., 1992), GlyRs mediate inhibitory neurotransmission (Legendre, 2001).

There are four known GlyR α subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 4$) that are transcribed and translated from four distinct genes (*gla1*, *gla2*, *gla3*, and *gla4*), and there is one known β subunit (β from *glrb*) (Lynch, 2004; Betz and Laube, 2006)

that can assemble to form pentameric homomers or heteromers containing an integral chloride channel. Structurally, each subunit contains a large extracellular N terminus, an extracellular C terminus, a large intracellular loop, and four transmembrane segments that collectively constitute a transmembrane domain.

Several agents act as allosteric modulators to enhance GlyR function, including ethanol (and other alcohols), volatile anesthetics, and inhaled drugs of abuse (Mihic et al., 1997; Beckstead et al., 2002). In addition to these exogenous modulators, endogenous agents like zinc ions, which are present in cerebrospinal fluid at tonic baseline concentrations in the low nanomolar range (Frederickson et al., 2006), also modulate GlyR function. Notably, zinc acts in a biphasic manner such that nanomolar and low micromolar concentrations of zinc enhance GlyR function, whereas higher micromolar zinc concentrations produce GlyR inhibition (Bloomenthal et al., 1994; Laube et al., 1995; Miller et al., 2005b).

In addition to modulating glycine-activated currents, recent evidence from in vitro investigations of recombinant GlyRs suggests that zinc also modulates ethanol action at GlyRs.

This research was supported by National Institutes of Health [Grant 5R01AA006399] to (RAH), [Grant 5R01AA013378] to (JRT), and [Grant 1F31AA019852] to (LMM).

¹Current affiliation: Columbia University, New York, New York.

²Current affiliation: Max-Planck Institute for Medical Research, Heidelberg, Germany.

dx.doi.org/10.1124/jpet.112.197707.

^S Supplemental material is available online at jpet.aspetjournals.org.

ABBREVIATIONS: ANOVA, analysis of variance; CHARMM, Chemistry at Harvard Macromolecular mechanics; CNS, central nervous system; DS 3.1, Discovery Studio 3.1; GluCl, glutamate receptor chloride channel; GlyR, glycine receptor; KI, knock-in; LORR, loss of righting reflex; MBS, modified Barth's solution; WT, wild type.

More specifically, zinc chelation by tricine decreases the magnitude of ethanol enhancement of $\alpha 1$ GlyR currents, whereas the addition of physiologically relevant nanomolar concentrations of zinc enhances ethanol potentiation of GlyRs (McCracken et al., 2010b).

Zinc modulation and ethanol potentiation of GlyR function have been shown to be mediated by independent binding sites (Laube et al., 2000). This is consistent with mutational studies locating the high-affinity potentiating and low-affinity inhibitory binding sites for zinc in the extracellular domain of GlyR $\alpha 1$ outside of the agonist binding site (Harvey et al., 1999; Laube et al., 2000; Miller et al., 2005a,b; Grudzinska et al., 2008), whereas ethanol potentiation is thought to involve an alcohol-binding pocket that likely extends from residues of loop 2 in the extracellular domain (Crawford et al., 2008; Perkins et al., 2009; Perkins et al., 2012) to the thoroughly investigated residues of the helical segments in the transmembrane domain (Mihic et al., 1997; Mascia et al., 2000; Lobo et al., 2004, 2006, 2008; McCracken et al., 2010a). Additionally, there is evidence that residues of the intracellular loop of the $\alpha 1$ GlyR subunit may be important for ethanol action at GlyRs (Yevenes et al., 2011; Castro et al., 2012).

Several lines of evidence highlight the physiologic relevance of free zinc in the central nervous system (CNS) and its ability to modulate GlyR function in the brain and spinal cord. Most notable are results obtained with *Gla1D80A* knock-in (KI) mice, which contain a point mutation (an aspartate to alanine substitution) within a putative high-affinity zinc-binding site (D80) located in the N-terminal domain of the $\alpha 1$ GlyR subunit (Hirzel et al., 2006) (Fig. 1). Mice homozygous for *Gla1D80A* display a neuromotor phenotype resembling human startle disease, and in vitro recordings from spinal neurons and brainstem slices obtained from these animals have disclosed significant impairments in the enhancement of spontaneous glycinergic currents by zinc (Hirzel et al., 2006). The availability of these D80A mice affords the opportunity to study the potential role of zinc binding to GlyRs in modulating alcohol drinking and other alcohol-related behaviors.

In the present study, we sought to investigate whether the high-affinity zinc-binding position, D80, on the $\alpha 1$ GlyR subunit is important for the enhancing effects of zinc on ethanol modulation of GlyR function. We first conducted in vitro experiments using two-electrode voltage clamp electrophysiology to test the effects of ethanol, zinc, and other sedative agents on mutant $\alpha 1D80A$ GlyRs expressed in *Xenopus* oocytes. Next, by using the *Gla1D80A* KI mouse as an animal model, we evaluated the effects of this GlyR mutation on alcohol consumption and other related behaviors in mice. The combination of these in vitro and in vivo techniques were used to test the hypothesis that high-affinity zinc binding to GlyRs is an important determinant of both ethanol potentiation of the receptor current and ethanol consumption.

Materials and Methods

GlyR Homology Modeling

A homology model of the GlyR $\alpha 1$ subunit was built to explore the location of the zinc-binding site at the D80 position on the full pentameric receptor. As a template for the homology model, we used the most recent x-ray structure of the eukaryotic glutamate receptor

chloride ion channel (GluCl, PDB ID 3RHW) and the Modeler module of Discovery Studio 3.1 (DS 3.1) (Accelrys Inc., San Diego, CA). GlyR $\alpha 1$ and GluCl share high sequence identity (34%), so few adjustments in the alignment were required, and our model was constructed based on a recent sequence alignment of GlyR $\alpha 1$ and GluCl (Hibbs and Gouaux, 2011). More specifically, we built 15 homology models and chose the best model based on the total force field energy determined with the Chemistry at Harvard Macromolecular Mechanics (CHARMM) force field. This model was improved with the Side Chain Refinement module of DS 3.1 to optimize the possible side chain rotomers. We then imposed a harmonic restraint on all backbone atoms of 10 kcal/(mol $\times$ $\text{\AA}^2$) and ran 1,000,000 1-femtosecond steps of molecular dynamics at 300 K with this backbone restraint. Finally, we optimized the last structure to a gradient of 0.0001 kcal/(mol $\times$ $\text{\AA}$) with the same CHARMM force field.

In the resulting model, the D80 residue was identified, and a zinc atom was positioned (by assigning a formal and partial charge of +2 and potential function ID MXN with the CHARMM force field) in close proximity to the carboxy oxygens of D80 in one subunit. First, the default optimization module of DS 3.1 was used to dock the zinc atom near the carboxylate moiety of D80. Because the resulting orientation was not the expected straight-on arrangement (it was at 90 degrees to the carboxylate moiety), the resulting model was subjected to 10,000 steps of molecular dynamics at 300 K, with all atoms of GlyR fixed to explore the preferred orientation of the zinc atom with respect to D80.

Site-Directed Mutagenesis

Site-directed mutagenesis was used to introduce a single point mutation in the GlyR $\alpha 1$ cDNA to create the $\alpha 1D80A$ mutant GlyR. This was accomplished using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA) and commercially engineered mutagenesis primers (Integrated DNA Technology, San Diego, CA). Successful mutagenesis was verified using automated fluorescent DNA sequencing (The University of Texas at Austin DNA Core Facility, Austin, TX).

Xenopus Oocyte Isolation and cDNA Injection

Partial ovariectomies were performed on sexually mature female *Xenopus laevis* obtained from Xenopus Express (Brooksville, FL). Manual isolation of individual oocytes from ovary fragments, cDNA injection of isolated oocytes, and incubation of injected oocytes were performed as previously described (McCracken et al., 2010b).

Two-Electrode Voltage Clamp Electrophysiology

For electrophysiology recordings, oocytes were impaled in the animal poles with two high-resistance (>1 M Ω) glass electrodes containing 3 M KCl and voltage-clamped at -70 mV using a Warner OC-725C oocyte clamp (Warner Instruments, Hamden, CT). A Masterflex USA peristalsis pump (Cole-Parmer Instrument Corporation, Vernon Hills, IL) was used to deliver buffer solutions to oocytes via bath perfusion at a rate of 2 ml/min. Clamping currents were recorded on LabChart Pro software (Colorado Springs, CO), which was interfaced to the oocyte voltage-clamp apparatus via a PowerLab 4/30 data acquisition system (AD Instruments, Colorado Springs, CO). Recordings were performed on oocytes expressing wild-type (WT) $\alpha 1$ or mutant $\alpha 1D80A$ mutant GlyRs approximately 1–7 days post cDNA injection. All electrophysiology protocols were run using modified Barth's solution (MBS) [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.91 mM CaCl₂] and oocytes harvested from at least two different frogs.

Glycine Concentration-Response Curves. Glycine concentration-response curves were generated with oocytes expressing WT $\alpha 1$ or mutant $\alpha 1D80A$ GlyRs by measuring the chloride currents elicited by a series of glycine concentrations (10 μ M–100 mM). Each concentration of glycine was applied for ~ 30 seconds followed by a 7-minute washout. The concentration of glycine that elicited the largest response was determined to be maximal, and the effects of

Fig. 1. Homology model of the GlyR $\alpha 1$ subunit highlighting the aspartate residue at position 80 (D80; carbon, oxygen, hydrogen, and zinc atoms are rendered as gray, red, white, and green space filling surfaces, respectively). (A) The entire pentameric receptor is shown with a transparent molecular surface colored according to the electrostatic potential; more red is negative, and more blue is positive. Each subunit is rendered as a cartoon ribbon and colored individually to help distinguish them. For orientation, the position of D80 and the TM4 α helix of the same subunit (pink) are indicated with an arrow. (B) A single GlyR $\alpha 1$ subunit rendered as a cartoon ribbon. The ion pore of the receptor is to the right of the TM2 α helix; very distant from D80. (C) An expanded view of the interaction of a zinc atom with D80. The center-to-center zinc-to-oxygen distances are approximately 2.1 Å.

the remaining glycine concentrations were calculated and recorded as a percentage of the maximal current ($I_{\max}$). To confirm decreased sensitivity of $\alpha 1$ D80A GlyRs to enhancing concentrations of zinc, additional glycine concentration-response curves were generated using a similar protocol except that all MBS and glycine solutions contained 1 μ M added zinc, which produces an approximately maximal enhancing effect of zinc on glycine-activated currents (Bloomenthal et al., 1994).

Taurine Concentration-Response Curves. Concentration-response curves for the GlyR partial agonist taurine were generated for WT and mutant $\alpha 1$ GlyRs. A series of taurine concentrations (100 μ M–100 mM) was tested; each concentration was applied for ~30 seconds, and a 7-minute washout period separated each application of taurine. The amount of current elicited by each taurine concentration was calculated and recorded as a percentage of the maximal glycine effect.

Zinc-Dependent Modulation of Wild-Type GlyRs by Ethanol. The effects of three different enhancing concentrations of zinc (100 nM, 500 nM, and 1 μ M) on the modulation of WT receptors by ethanol were tested. Oocytes were first perfused with two ~15-second applications of 10 mM glycine each, followed by a 7-minute washout period. The peak current elicited by the second application of glycine was considered to be the maximal response and was used to determine a concentration of glycine that produced ~5%–10% (EC_{5-10}) of $I_{\max}$. This experimentally derived EC_{5-10} concentration of glycine was then applied, followed by a 7-minute washout period. Next, 200 mM ethanol was first applied alone for 1 minute, then applied concurrently with the experimentally derived EC_{5-10} concentration of glycine for ~45 seconds. After another 7-minute washout, the EC_{5-10}

concentration of glycine was again applied alone for 45 seconds. This procedure was carried out first in regular MBS, and then repeated in MBS containing 10 mM tricine to chelate any contaminating free zinc, in MBS plus 100 nM added zinc, in MBS plus 500 nM added zinc, and in MBS plus 1 μ M added zinc. In all conditions, the effects of ethanol were determined as percent potentiation of the glycine EC_{5-10} response.

Ethanol Sensitivity in Mutant versus WT GlyRs. Oocytes expressing either WT $\alpha 1$ or mutant $\alpha 1$ D80A GlyRs were first perfused with two ~15-second applications of 10 mM glycine each, followed by a 7-minute washout period. The peak current elicited by the second application of glycine was considered to be the maximal response and was used to determine a concentration of glycine that produced ~5%–10% (EC_{5-10}) of the maximal glycinergic effect. This experimentally derived EC_{5-10} concentration of glycine was then applied. Following a 7-minute washout period, ethanol was first applied alone for 1 minute, and then applied concurrently with the experimentally derived EC_{5-10} concentration of glycine for ~45 seconds. After another 7-minute washout, the EC_{5-10} concentration of glycine was again applied alone for 45 seconds. This protocol was used to test 50 mM and 200 mM ethanol.

To determine how chelating any contaminating zinc would affect ethanol modulation of WT $\alpha 1$ and mutant $\alpha 1$ D80A GlyRs that lacked enhancement by physiologic concentrations of zinc, we repeated the same protocol in the presence of 10 mM tricine, which was added to the buffer solution as previously described (McCracken et al., 2010b). Because the level of free zinc is very low in this solution, we refer to it as “zinc free.” In addition, to test the hypothesis that added zinc would produce the opposite effect of a zinc chelator, this procedure was

repeated for testing ethanol sensitivity in MBS containing 100 nM added zinc. In all three conditions, the effects of ethanol were determined as percent potentiation of the glycine EC₅₋₁₀ response.

GlyR Modulation by Nonalcohol Sedatives. The effects of flurazepam, ketamine, pentobarbital, and isoflurane in MBS were tested on WT $\alpha 1$ and mutant $\alpha 1D80A$ GlyRs. The same procedure that was used to test ethanol sensitivity was used to test WT and mutant GlyR sensitivity to other nonalcohol sedatives.

Mouse Breeding and Genotyping

Heterozygous *Gla1D80A* KI mice [backcrossed to C57BL/6 for more than five generations (Hirzel et al., 2006)] were bred at The University of Texas Animal Resource Center from heterozygous breeding pairs provided by the Max-Planck Institute for Brain Research, Frankfurt, Germany. Following weaning, mice were housed under a 12-hour light/dark cycle (lights on at 7:00 AM) with ad libitum access to standard rodent chow and water. All mice, male and female, used in behavioral tests were between 8 and 12 weeks of age, were ethanol naïve at the start of each experiment, and were used only once (i.e., animals were not used in more than one behavioral test). Due to poor viability and testing limitations resulting from the functional impairments of homozygous *Gla1D80A* KI mice, only heterozygous KI animals and their WT littermate controls were included in the behavioral experiments. The protocols used for behavioral testing were approved by The University of Texas Institutional Animal Care and Use Committee and were conducted in accordance with National Institutes of Health guidelines with regard to the use of animals in research.

Alcohol Consumption and Preference (24-Hour Access)

A standard two-bottle choice drinking protocol similar to those previously described (Blednov et al., 2003, 2012) was used. Mice were individually housed and given a 1-week acclimation period. Two drinking tubes (one containing water and one containing an ethanol solution) were continuously available to mice. The tubes were weighed, and their left/right positions were alternated daily (to avoid side preference). In addition, the mice were weighed every fourth day. Mice were offered 3% (v/v) ethanol versus water for 2 days. This was repeated successively for 6%, 9%, 12%, and 15% (v/v) ethanol. The quantity of ethanol and water consumed by each mouse (g/kg body weight/24 hours) was calculated, and the values for each respective concentration of ethanol were averaged. Throughout the experiment, estimates of spillage/evaporation were calculated daily from drinking tubes (one containing water and one containing the appropriate ethanol solution) placed in an empty cage on each row of the cage rack.

Nonalcohol Tastant Consumption and Preference (24-Hour Access)

In addition to ethanol, consumption of and preference for two other tastant solutions, saccharin and quinine, were tested in WT and mutant KI mice to test for sweet or bitter taste preferences. In a two-bottle choice paradigm, mice were serially offered saccharin (0.0165% and 0.033%) and quinine hemisulfate (0.03 mM and 0.066 mM) and their intakes were calculated daily. Each respective concentration was offered for 2 consecutive days, and the bottle positions were alternated daily. For each tastant, the lower concentration was always presented first, followed by the higher concentration, and 1 week separated tastant testing during which mice had access to two bottles, both containing water.

Drug Preparation and Injection

All drug solutions were prepared as previously described (Blednov et al., 2012). Ethanol (Aaper Alcohol and Chemical, Shelbyville, KY, or Pharmco, Brookfield, CT) solutions were prepared in 0.9% saline (20% v/v) and injected i.p. at a dosing volume of 0.2 ml/10 g body

weight. Flurazepam (Sigma-Aldrich, St. Louis, MO) and pentobarbital (Sigma/RBI, Natick, MA) were dissolved in 0.9% saline and were injected i.p. at 0.01 ml/g body weight. Strychnine (Sigma-Aldrich) was prepared in 0.9% saline and injected i.p. at a volume of 10 ml/kg body weight.

Loss of Righting Reflex

WT and KI mice were tested for sensitivity to the sedative effects of ethanol (3.4 and 3.8 g/kg) and other CNS depressants including flurazepam (225 mg/kg), pentobarbital (50 mg/kg), and ketamine (175 mg/kg) using a standard duration of LORR (loss of righting reflex) (sleep time) assay as previously described for other GlyR KI mice (Blednov et al., 2012). Once ataxic, mice were placed in the supine position in V-shaped plastic troughs until they were capable of righting themselves three times within 30 seconds; sleep time was defined as the time from when they were placed in the supine position until they regained their righting reflex.

Rotarod

Mice were trained on a fixed speed (5 rpm) Economex rotarod (Columbus Instruments, Columbus, OH). Training was considered complete when mice could remain on the rotarod for 60 seconds. To test the effects of ethanol on motor coordination, WT and KI mice were administered ethanol (2.0 g/kg i.p.), and 15 minutes post injection they were placed back on the rotarod. Their latency to fall was recorded until they were capable of remaining on the rotarod for 60 seconds.

Acoustic Startle Response

SR-LABORATORY test stations and software (San Diego Instruments, San Diego, CA) were used to test acoustic startle responses in WT and mutant KI mice. Startle responses elicited by a series of auditory stimuli were recorded as previously described (Findlay et al., 2003; Blednov et al., 2012). First, mice were placed in Plexiglas holding chamber for a 5-minute acclimation period. Then, in the following 8 minutes, mice were presented with seven trial types across five discrete blocks of trials for a total of 30 trials. There was a 10- to 20-second inter-trial interval. To control for baseline movement, one trial type measured the response to no stimulus, whereas the remaining six trial types measured the response to startle stimuli. This consisted of 40 ms sound bursts of 90, 95, 100, 105, 110, or 115 db. Starting at the onset of startle stimuli, startle amplitude was measured every 1 millisecond during a 65 millisecond period. The six trial types were presented to mice in a pseudorandom order such that each trial type was presented once within a block of six trials. The maximum startle amplitude (V_{max}) over this sampling period was taken as the dependent variable. A background noise level of 70 db was maintained throughout the duration of the test session.

Strychnine-induced Convulsions

WT and KI mice were injected with a series of doses of the GlyR antagonist strychnine to test for differences in chemically-induced seizure activity. For each dose of strychnine, a group of five mice was used. During testing, mice were individually placed into observation chambers (20 cm × 10 cm × 10 cm Plexiglas cage), and were continuously observed following injections of strychnine (up to 30 minutes post injection) for latency to clonic and tonic convulsions. The data were recorded and tabulated as previously described (Blednov et al., 2012) as the number of mice exhibiting the behavioral signs as well as the latency to first seizure occurrence. The dose of strychnine that elicited convulsive activity in 50% of the group (ED₅₀) was calculated using regression analyses.

Statistical Analysis

For experiments on recombinant GlyRs, nonlinear regression analyses were performed to calculate glycine EC₅₀ values and Hill coefficients for glycine concentration-response curves, and *t* tests and one-way ANOVAs (analysis of variance) followed by post-hoc analyses were used to determine differences in ethanol sensitivity and the effects of other sedative agents. In addition, two-way ANOVAs (with Dunnett's or Bonferroni post hoc tests), Student's *t* tests, and regression analyses were conducted to detect differences between groups in the behavioral experiments. Overall, statistical differences were determined at $P < 0.05$, and all analyses were conducted using GraphPad PRISM software (San Diego, CA).

Results

GlyR Homology Model of Position D80

The molecular model in Fig. 1 shows that D80 is located at the upper and outer edge of the GlyR protein, which is readily accessible to the extracellular milieu including metal ions, such as zinc. To explore the environment of D80, we built a smooth molecular surface over the GlyR model, made it transparent, and colored the surface with the electrostatic potential derived from the atomic partial charges on the molecular surface. The position of the zinc atom (Fig. 1, B and C) was essentially identical after docking with a simple minimization algorithm and after a short molecular dynamics simulation to test for the stability of the pose shown in Fig. 1A. In Fig. 1C, the zinc atom is positioned between the two carboxylate oxygens of D80 and both center to center distances were approximately 2.1 Å. This configuration is appropriate for a tight electrostatic bond between D80 and zinc.

Recombinant GlyRs Expressed in Oocytes

Glycine Sensitivity. We first generated agonist (glycine and taurine) concentration-response curves for $\alpha 1$ and $\alpha 1D80A$ GlyRs expressed in oocytes to determine differences in agonist sensitivity. For the full agonist glycine, introducing the D80A mutation in the $\alpha 1$ subunit resulted in a rightward shift in the glycine concentration-response curve for the mutant compared with WT GlyRs (Fig. 2A). To confirm the results of previous studies showing that neutral substitutions of the $\alpha 1D80$ position strongly reduce the enhancement of glycine-activated currents by low micromolar and nanomolar concentrations of zinc (Laube et al., 2000; Hirzel et al., 2006), we also generated glycine concentration-response curves in the presence of 1 μ M added zinc. This concentration of zinc, which is approximately maximally enhancing on WT GlyRs (Bloomenthal et al., 1994), did not produce any shifts in the glycine concentration-response curve of mutant $\alpha 1D80A$ GlyRs, indicating no enhancement of the glycine-activated currents. In addition, there was no significant difference in the maximal glycine-activated currents in mutant versus WT receptors in either normal MBS or in the presence of 1 μ M added zinc.

Taurine Sensitivity. Concentration-response curves for the partial agonist taurine suggested that it might be less efficacious on mutant $\alpha 1D80A$ GlyRs compared with WT GlyRs, however, this trend was not statistically significant (Fig. 2B).

Zinc Modulation of Ethanol Sensitivity in WT GlyRs. Because the D80 position is among the known high-affinity

Fig. 2. Agonist concentration-response curves for mutant $\alpha 1D80A$ and WT $\alpha 1$ GlyRs. Normalized current responses to the indicated glycine (A) and taurine (B) concentrations are shown. Note that there were no significant differences in the maximal glycine-activated currents between mutant versus WT receptors in either normal MBS or in the presence of 1 μ M added zinc.

sites important for enhancement of GlyR function by low physiologic concentrations of zinc (Laube et al., 2000), we tested the hypothesis that mutation of this site would produce changes in the effects of ethanol by performing recordings on mutant $\alpha 1D80A$ GlyRs expressed in oocytes. First, we tested the effects of 200 mM ethanol on WT $\alpha 1$ GlyRs in the presence of different concentrations of zinc, ranging from a zinc-free MBS solution that contained the zinc chelating agent tricine to 1 μ M zinc. This resulted in significant differences in the magnitude of ethanol's enhancing effect on WT $\alpha 1$ GlyR function (Fig. 3A). In zinc-free MBS, which contained 10 mM tricine, 200 mM ethanol produced the smallest potentiation of glycine-activated currents, whereas it was greatest in the presence of 100 nM added zinc. Intermediate degrees of enhancement by 200 mM ethanol were observed in the presence of 500 nM and 1 μ M added zinc.

Ethanol Sensitivity of Mutant D80A GlyRs. Next, we investigated whether mutation of the aspartate at position 80 to alanine in the $\alpha 1$ GlyR would result in GlyRs with decreased sensitivity to ethanol. The enhancement of WT $\alpha 1$ and mutant $\alpha 1D80A$ GlyRs by 50 and 200 mM ethanol in standard MBS solutions was measured. Fig. 3, B and C show that the mutant compared with WT GlyRs demonstrated decreased sensitivity to 50 mM [$t(7) = 2.4$; $P = 0.046$] and 200 mM [$t(14) = 2.36$; $P = 0.033$] ethanol, respectively.

Zinc/Ethanol Interactions in Wild Type and D80A GlyRs. To determine if the decreased ethanol sensitivity of the D80A mutant might be due to the loss of the enhancing

Fig. 3. Modulation of mutant D80A and WT $\alpha 1$ GlyRs by ethanol (EtOH) in the presence and absence of zinc. (A) The effects of 200 mM ethanol on $\alpha 1$ GlyRs were tested in standard MBS, in tricine, and in three different zinc concentrations (100 nM, 500 nM, and 1 μ M). (B) Modulation of D80A and WT GlyRs by 50 mM ethanol was tested in standard MBS, in tricine, and in 100 added nM zinc. (C) Ethanol modulation of WT and D80A GlyRs was tested as in (B), but 200 mM ethanol was used. Manipulating the concentration of zinc ions present in MBS resulted in changes in the magnitude of ethanol enhancement of WT but mutant D80A GlyR function.

effects of zinc on ethanol action, we subsequently tested the effects of the same ethanol concentrations (50 and 200 mM) in mutant and WT receptors first in the presence of the chelating agent tricine and then in the presence of 100 nM added zinc, which in the previous experiment we determined to produce the greatest increase in the magnitude of ethanol's effect on WT $\alpha 1$ GlyRs. Manipulating the concentrations of zinc present

in our buffers did not change in the magnitude of ethanol enhancement of mutant $\alpha 1$ D80A GlyRs by either 50 or 200 mM ethanol [for 50 mM ethanol: $F(2,9) = 1.15$; $P = 0.36$; for 200 mM ethanol: $F(2,12) = 0.60$; $P = 0.56$] (Fig. 3, B and C). In contrast, for WT GlyRs, the presence of tricine significantly decreased the degree of enhancement by 50 and 200 mM ethanol, and the opposite effect was observed such that 100 nM added zinc significantly increased the effects of both 50 mM and 200 mM ethanol on WT GlyR function [for 50 mM ethanol: $F(2,7) = 191$; $P < 0.0001$; for 200 mM ethanol: $F(2,17) = 8.9$; $P = 0.002$].

Sensitivity of Mutant D80A GlyRs to Other Sedative Agents. In addition to ethanol, we tested the effects of other sedative agents on WT $\alpha 1$ and $\alpha 1$ D80A GlyRs. There were no significant differences in the enhancement of WT or mutant GlyRs by flurazepam, pentobarbital, ketamine, or isoflurane (unpublished data).

Behavioral tests in GlyR KI Mice

Mouse Breeding. Heterozygous breeding pairs of *Gla1D80A* KI mice were used to produce the animals tested in this study, because mice homozygous for the D80A mutation displayed low viability in our breeding population. All behavioral tests described below were conducted using heterozygous *Gla1D80A* mice and their respective WT littermates. More specifically, of 138 mice that we produced 46 mice or 33% were WT (+/+), 71 mice or 52% were heterozygous (+/-) for the D80A mutation, and 21 mice or 15% were *Gla1D80A* homozygotes (-/-). χ^2 analysis of the number of mice we produced with each respective genotype reveals that the actual genotype ratios that we generated were significantly different than those predicted by Mendelian genetics [$\chi^2(2, N = 138) = 9.2$; $P = 0.01$]. Most homozygotic D80A mice did not survive more than three to four weeks past birth, and those that did displayed neuromotor impairments (see Hirzel et al., 2006) that would have precluded reliable measurements in all the behavioral tests used.

Alcohol Consumption and Preference. A continuous access, two-bottle choice drinking paradigm was used to test for differences in alcohol consumption and preference between D80A GlyR KI mice and WT littermate controls. Female *Gla1D80A* mice consumed significantly less ethanol than WT females. A significant main effect and an interaction between ethanol concentration and genotype were detected using a two-way ANOVA. A Bonferroni post-hoc analysis revealed a significant difference in ethanol consumption at the 9% concentration [main effect: $F(4, 64) = 2.9$; $P = 0.029$; $t(17) = 3.06$; $P < 0.05$ at 9% ethanol]. The female mutant mice also exhibited decreased preference for alcohol [$F(4,64) = 9.5$; $P < 0.0001$]. However, this effect was sex-specific as there were no differences in ethanol consumption [$F(4,85) = 1.1$; $P = 0.38$] or preference [$F(4,85) = 0.70$; $P = 0.59$] between male WT and mutant mice. There were also no significant differences in total fluid intake detected between the two genotypes for males or females [for males: $F(4,85) = 0.24$; $P = 0.91$; for females: $F(4,64) = 0.30$; $P = 0.87$ (Fig. 4)].

Non-alcohol Tastant Consumption and Preference. To test for differences between mutant and WT mice with respect to sweet or bitter tastants, we used a continuous access two-bottle choice paradigm to measure saccharin and quinine consumption and preference. There were no significant differences detected

Fig. 4. Ethanol consumption and preference in mutant and WT mice. Ethanol consumption (g/kg/24 hours) and preference were measured in heterozygous D80A KI mice and their WT littermates. (A and B) Both ethanol consumption and preference were decreased in female D80A mutant mice, whereas there were no differences between male WT and D80A mice (D and E). (C and F) Total fluid intake was not different between genotypes. * $P < 0.05$.

between D80A KI and WT male or female mice for either saccharin consumption [for females: $F(1,16) = 0.68$; $P = 0.42$; for males: $F(1,16) = 0.04$; $P = 0.85$], preference [for females: $F(1,16) = 0.84$; $P = 0.37$; for males: $F(1,16) = 0.0$; $P = 0.97$], or total fluid intake [for females: $F(1,16) = 0.47$; $P = 0.50$; for males: $F(1,16) = 0.13$; $P = 0.72$] (Supplemental Fig. 1). In addition, female D80A KI and WT mice did not differ in their consumption [$F(1,16) = 0.0$; $P = 0.96$] or preference [$F(1,16) = 0.0$; $P = 0.99$] for quinine (Supplemental Fig. 2). However, in this assay the mutant D80A mice did show less total fluid intake compared with WT females [$F(1,16) = 7.48$; $P = 0.01$]. Male mutant and WT mice did not demonstrate any differences in either quinine

consumption [$F(1,16) = 0.24$; $P = 0.63$], preference [$F(1,16) = 0.59$; $P = 0.45$], or total fluid intake [$F(1,16) = 0.13$; $P = 0.72$] (Supplemental Fig. 2).

Loss of Righting Reflex. Differences between mutant and WT mice in the duration of loss of righting reflex were measured following the injection of four sedative agents, ethanol, flurazepam, pentobarbital, or ketamine. For ethanol (Fig. 5, A and B), there was no effect of genotype on sleep time for either males [$t(16) = 0.15$; $P = 0.88$] or females [$t(16) = 2.0$; $P = 0.06$] (Fig. 5, A and B). However, male D80A KI demonstrated increased LORR compared with their WT male littermates in response to pentobarbital [$t(8) = 4.0$; $P = 0.004$],

Fig. 5. Loss of righting reflex induced by sedative agents. The duration of LORR induced by ethanol, pentobarbital, flurazepam, and ketamine was measured in D80A KI and WT mice. (A and B) There were no differences in ethanol sleep time between mutant and WT mice. However, both male and female D80A mice had increased durations of LORR induced by pentobarbital (C and D), flurazepam (E and F), and ketamine (G and H). ** $P < 0.01$; *** $P < 0.001$.

flurazepam [$t(9) = 10.5$, $P < 0.0001$], and ketamine [$t(10) = 7.2$; $P < 0.0001$] (Fig. 5, C, E, and G). Likewise, Fig. 5, D, F, and H shows that the same effect was observed in female mice in response to pentobarbital [$t(12) = 7.5$; $P < 0.001$], flurazepam [$t(15) = 4.0$; $P = 0.001$], and ketamine [$t(12) = 11.4$; $P < 0.0001$], respectively.

Acoustic Startle Response. Behavioral characterizations of GlyR KI mice (Findlay et al., 2003; Blednov et al., 2012), including D80A homozygotes (Hirzel et al., 2006), have shown that impairments in GlyR function are accompanied by changes in startle responses. Here, we compared this behavior in the heterozygotic D80A KI and WT mice. In both sexes, the mutant mice exhibited increased startle responses compared with WT controls. There were no main effects for males [$F(4, 110) = 0.67$; $P = 0.61$] or females [$F(4, 60) = 2.14$; $P = 0.09$]. However, there were differences detected at individual decibel levels for males [$t(24) = 2.8$; $P < 0.05$; at 115 db] and females [$t(13) = 3.5$; $P < 0.01$ at 110 db; $t(13) = 4.4$; $P < 0.01$ at

115 db] (Fig. 6, A and B). In an additional series of experiments, we further tested whether ethanol modulation of acoustic startle responses was changed in mutant D80A KI mice and discovered that ethanol (0.5 or 1.0 g/kg, i.p.) had no effect on startle responses of female [$F(10, 222) = 0.10$; $P = 1.0$] and male [$F(10, 156) = 0.67$; $P = 0.75$] mutant KI mice (Fig. 6, C and E). However, both ethanol doses increased startle responses in male WT mice [$t(25) = 2.7$; $P < 0.05$ for 0.5 g/kg ethanol; $t(25) = 2.8$; $P < 0.05$ for 1.0 g/kg ethanol] (Fig. 6D), whereas only 0.5 g/kg ethanol increased startle responses in female WT mice [$t(21) = 3.6$; $P < 0.01$] (Fig. 6F).

Ethanol Rotarod. To measure the motor-incoordinating effects of ethanol (2 g/kg, i.p.), we used the rotarod test. Although this dose of ethanol impaired motor coordination, there were no measureable differences between female [$F(7, 80) = 0.63$; $P = 0.7271$] or male [$F(9, 100) = 0.23$, $P = 0.9889$] mutant and WT mice in their respective recoveries from ethanol-induced incoordination (not shown).

Fig. 6. Acoustic startle responses in mutant and WT mice. The data represent the maximum startle amplitude (V_{max}) as a function of sound intensity in decibels (dB). (A and B) The startle responses of both male and female D80A mice were increased compared with WT littermates. (C and D) Ethanol increased the startle responses in male WT but not mutant D80A mice. Likewise, female WT mice had increased startle responses following injections of ethanol, but this was not observed in female D80A mice (E and F). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Strychnine Convulsions. We measured tonic convulsions induced by injections of the GlyR antagonist strychnine in both WT and mutant D80A KI mice. There were no differences observed between WT and KI mice in sensitivity (ED_{50}) to tonic convulsions in either females (0.49 mg/kg for WT versus 0.54 mg/kg for KI mice) or males (0.53 mg/kg for WT versus 0.59 for KI mice) (data not shown).

Discussion

Several amino acid residues responsible for the enhancing and inhibiting effects of zinc on GlyR function have been identified in the N-terminal domain of the $\alpha 1$ subunit. The potentiating effects of zinc, generally seen at concentrations in the nanomolar to low micromolar range ($<10 \mu M$), require

high-affinity binding to amino acids at positions aspartate-80 (D80), threonine-151 (T151), glutamate-192 (E192), aspartate-194 (D194), and histidine-215 (H215) (Laube et al., 2000; Miller et al., 2005b).

In the present study, we first characterized in vitro the effects of introducing a mutation (D80A) at one of the residues required for high-affinity zinc binding. The increase in the EC_{50} value of glycine that we observed in the $\alpha 1$ D80A mutant versus WT GlyRs is consistent with the reduced glycine affinity seen with WT receptors in the presence of the zinc-chelating agent tricine. McCracken et al. (2010b) used tricine to eliminate low nanomolar concentrations of contaminating zinc and showed that this resulted in WT GlyRs with right-shifted glycine concentration-response curves similar to those obtained with the $\alpha 1$ D80A GlyRs in our current study. In addition, the lack of difference observed between the taurine concentration-response curves of WT and mutant GlyRs is consistent with previous reports that the $\alpha 1$ D80A mutation reduces enhancement of glycine-activated, but not taurine-activated currents by nanomolar and low micromolar concentrations of zinc (Miller et al., 2005b). Furthermore, neither manipulating zinc concentrations nor introducing the D80A mutation produced changes in maximal glycine-activated currents, which serves as further evidence that zinc modulates GlyR function via allosteric binding sites (Laube et al., 2000).

The position of D80 in Fig. 1 is at the cusp of the long loop between beta strands 2 and 3 identified in the x-ray structure of our template for homology modeling, the glutamate-gated chloride channel (Hibbs and Gouaux, 2011). Fig. 1B shows that the D80A site is located at the tip of loop 3, which is positioned between beta strands 2 and 3 in the ligand-binding domain of the GlyR $\alpha 1$ subunit (Brejc et al., 2001). As such, it could link directly to loop 2 by inducing tension on beta strand 3. Loop 2 is thought to be involved in electrostatic interactions with the transmembrane domain (Kash et al., 2003), as well as part of a binding pocket for ethanol (Mascia et al., 1996; Perkins et al., 2010, 2012). Because D80 is over 20 Å from the surface of the extracellular membrane, and an even greater distance from the putative ethanol binding pocket, we hypothesize that this position indirectly affects ethanol action and channel gating via electrostatic interactions. Additionally, Fig. 1C shows the zinc atom is positioned between the two carboxylate oxygens of D80; both center-to-center distances were approximately 2.1 Å. This configuration is appropriate for a tight electrostatic bond between D80 and zinc, suggesting that this position contributes to an allosteric binding site for zinc ions.

Although zinc concentrations in the brain exceed those present in other organs, most brain zinc is protein-bound (Mathie et al., 2006). In its free or rapidly exchangeable form, zinc exists in the cerebrospinal fluid at tonic baseline concentrations ranging from approximately 5–25 nM (Frederickson et al., 2006). However, in the CNS, zinc is also secreted from neurons (Takeda et al., 2001), and this can result in transient zinc concentrations in excess of 1 μ M following presynaptic release from GABAergic, glutamatergic, or glycinergic terminals (Frederickson and Bush, 2001). Thus, at least some of the potentiating zinc-binding sites in GlyRs are likely to be occupied in vivo.

Because physiologic concentrations of zinc enhance the magnitude of ethanol's effects on $\alpha 1$ GlyRs (McCracken et al., 2010b), we tested whether mutant $\alpha 1$ D80A GlyRs would

confer decreased sensitivity to ethanol. The enhancing effects of both 50 and 200 mM ethanol were reduced in mutant D80A as compared with WT GlyRs. These reduced effects of ethanol enhancement on D80A mutant GlyRs mimicked the reduced potentiation by ethanol observed at WT $\alpha 1$ GlyRs in the presence of tricine.

We determined that the reduced sensitivity of $\alpha 1$ D80A GlyRs to ethanol was due to the elimination of the enhancing effects of zinc on ethanol action, confirming that the D80 position on the $\alpha 1$ GlyR subunit is critical for zinc modulation of ethanol action (Laube et al., 2000). In addition, the recent pharmacologic characterization of another GlyR $\alpha 1$ subunit mutant (M287L), also with decreased ethanol sensitivity, revealed that zinc did not enhance the effects of ethanol on mutant receptors at nanomolar concentrations that were sufficient for increasing ethanol potentiation at WT GlyRs (Borghese et al., 2012). This further indicates that zinc is crucial in determining the sensitivity of GlyRs to ethanol and highlights the importance of including zinc in studies of ethanol actions.

Several studies in rodent models of ethanol consumption provide compelling evidence for a role of GlyRs in alcohol drinking and reinforcement. For example, bilateral infusions of glycine into the nucleus accumbens increase dopamine release and reduce ethanol drinking and preference, and injections of glycine reuptake inhibitors reduce ethanol intake and preference (Molander et al., 2005, 2007; Lido et al., 2011). Reduction in alcohol intake by injection of glycine appears contradictory to our results, showing that mutations that reduce alcohol potentiation of GlyR function also reduce drinking (Blednov et al., 2012). However, the GlyR alpha subunits may have distinct effects on alcohol drinking. Another possibility is that reduced GlyR function produced by the KI mutations results in changes in GABA or other neuronal signaling networks, which alter alcohol consumption as well as sensitivity to flurazepam and other drugs.

The apparent importance of both GlyRs and zinc in alcohol consumption, paired with our in vitro data, indicates a critical role for zinc in modulating ethanol action at GlyRs and provides a rationale to study the potential role of zinc signaling at GlyRs in alcohol consumption and other alcohol-related behaviors. In addition, *Gla1*D80A KI mice, which contain the same zinc-binding site substitution (D80A) that we characterized in vitro, provided us with an animal model for investigating zinc/ethanol interactions at the behavioral level. In these experiments, significant differences were observed between mutant KI and WT animals. First, D80A KI mice showed decreased consumption of and preference for ethanol compared with their littermate controls. However, this effect was only observed in females, which is in agreement with findings from previous studies showing sex differences in reduced alcohol consumption and preference for other GlyR KI mice (Findlay et al., 2003; Blednov et al., 2012).

Additional behavioral differences between D80A KI and WT mice include LORR induced by pentobarbital, flurazepam, and ketamine (Table 1). Both female and male mutant mice had increased sleep times following injections of these drugs. These effects also are consistent with the results of behavioral tests in other GlyR mutant mice (Findlay et al., 2003; Blednov et al., 2012). However, previous characterization of mice homozygous for the D80A mutation did not show differences in strychnine binding between these mutant and WT littermates, suggesting

TABLE 1

Summary of the alcohol-related behaviors tested in WT and heterozygous GlyR D80A KI mice. Increases in behavior are indicated by ↑, decreases are represented with ↓, and = denotes no significant difference between the mutant mice and their WT littermate controls.

Test	Behavior	Male	Female
Startle reflex		↑	↑
Ethanol activation		↓	↓
LORR			
Ethanol	3.8 g/kg	=	=
Pentobarbital	50 mg/kg	↑	↑
Ketamine	175 mg/kg	↑	↑
Flurazepam	225 mg/kg	↑	↑
2-Bottle choice			
Ethanol 3%–15%	Ethanol intake	=	↓
	Preference	=	↓
	Fluid intake	=	=
Saccharin	Preference	=	=
	Fluid intake	=	=
Quinine	Preference	=	↓
	Fluid intake	=	↓
Rotarod (recovery)	EtOH (2 g/kg)	=	=
Convulsions	Strychnine	=	=

EtOH, ethanol; LORR, loss of righting reflex.

that mice with the D80A substitution do not contain altered numbers of GlyRs (Hirzel et al., 2006). Together, these findings suggest that impairments in normal GlyR function potentially lead to compensatory changes in the receptors or other neurotransmitter systems, such as GABA or glutamate.

Multiple GlyR mutations in mammals, both naturally occurring and engineered, result in enhanced startle phenotypes (Findlay et al., 2003; 2005; Harvey et al., 2008; Blednov et al., 2012). Like these other GlyR mutants, D80A KI mice also showed increased acoustic startle responses compared with WT mice, which is consistent with similar results obtained with the homozygous D80A mutants (Hirzel et al., 2006). In addition, we tested the effects of ethanol on startle responses and found that it had no effect on the startle responses of mutant KI mice but increased the startle responses of WT mice. This is consistent with the proposal that zinc binding at the D80 position of the $\alpha 1$ GlyR is important for the effect of ethanol on startle responses.

Homozygous KI mice carrying each of the previously characterized GlyR KI mutations (S267Q, Q266I, and M287L) were not viable, and therefore all ethanol-related behavioral tests of these mutations have been limited to heterozygous animals (Findlay et al., 2002, 2003, 2005; Blednov et al., 2012). Likewise, here we tested WT and heterozygous *Gla1D80A* mice because our colony yielded only a low number of homozygotes, and the motor impairments of those born would have interfered with our behavioral assays. The overall level of impairment observed in homozygous D80A KI mice (Hirzel et al., 2006) highlights the importance of GlyR modulation by endogenous zinc in normal nervous system function.

In this study, we focused on one of the amino acid residues of the $\alpha 1$ GlyR subunit that is known to be important for the enhancing actions of zinc ions on GlyR function. Other sites that are important for zinc potentiation of GlyR function include glutamate-E192, aspartate-194 (D194), threonine-151 (T151), and histidine-215 (H215) (Miller et al., 2005a,b). However, additional residues of the GlyR $\alpha 1$ subunit, in particular histidine-107 (H107), histidine-109 (H109), threonine-112 (T112), and threonine-133 (T133), are thought

to contribute to lower-affinity binding sites and are necessary for inhibition of GlyR function by higher micromolar concentrations of zinc ($>10 \mu\text{M}$) (Harvey et al., 1999; Laube et al., 2000; Miller et al., 2005a).

Overall, our findings demonstrate the significance of low physiologic concentrations of zinc in modulating the effects of ethanol on GlyR function and, more specifically, the crucial role of the D80 position in the $\alpha 1$ GlyR subunit in regulating ethanol actions, both in vitro and in vivo.

Acknowledgments

The authors thank Chelsea Geil, Hannah Stern, Mendy Black, Nadia Khan, Dr. Rebecca Howard, Joseph Coskey, and Kathryn Ondrichuk.

Authorship Contributions

Participated in research design: McCracken, Harris, Blednov, Trudell.

Conducted experiments: McCracken, Blednov, Benavidez, Trudell.

Contributed new reagents: Betz.

Performed data analysis: McCracken, Blednov, Trudell.

Wrote or contributed to the writing of the manuscript: McCracken, Harris, Betz, Trudell.

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Address Correspondence to: Dr. R. Adron Harris, 1 University Station A4800, Austin, TX 78712. E-mail: harris@austin.utexas.edu