Cysteine Substitution of Transmembrane Domain Amino Acids Alters the Ethanol Inhibition of GluN1/GluN2A N-Methyl-d-Aspartate Receptors

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ABSTRACT
N-Methyl-d-aspartate receptors (NMDARs) are inhibited by behaviorally relevant concentrations of ethanol, and residues within transmembrane (TM) domains of NMDARs, including TM3 GluN1 phenylalanine 639 (F639), regulate this sensitivity. In the present study, we used cysteine (C) mutagenesis to determine whether there are additional residues within nearby TM domains that regulate ethanol inhibition on NMDARs. GluN1(F639C)/GluN2A receptors were less inhibited by ethanol than wild-type receptors, and inhibition was restored to wild-type levels following treatment with ethanol-like methanethiosulfonate reagents. Molecular modeling identified six residues in the GluN1 TM1 domain (valine V566; serine S569) and the GluN2A TM4 domain (methionine, M817; V820, F821, and leucine, L824) that were in close vicinity to the TM3 F639 residue, and these were individually mutated to cysteine and tested for ethanol inhibition and receptor function. The F639C-induced decrease in ethanol inhibition was blunted by co-expression of GluN1 TM1 mutants V666C and S669C, and statistically significant interactions were observed for ethanol inhibition among V666C, F639C, and GluN2A TM4 mutants V820C and F821C and S669C, F639C, and GluN2A TM4 mutants F821C and L824C. Ethanol inhibition was also reduced when either GluN1 TM1 mutant V666C or S669C was combined with GluN2A V820C, suggesting a novel TM1:TM4 intrasubunit site of action for ethanol. Cysteines substituted at TM3 and TM4 sites previously suggested to interact with ethanol had less dramatic effects on ethanol inhibition. Overall, the results from these studies suggest that interactions among TM1, TM3, and TM4 amino acids in NMDARs are important determinants of ethanol action at these receptors.

Introduction
Ethanol, one of the world’s oldest and most widely used psychoactive substances, induces a variety of behavioral effects, including reduced anxiety, feelings of well-being, and increased impulsivity following acute ingestion (reviewed by Woodward, 2013). As concentrations continue to rise, ethanol’s depressant effects begin to emerge, characterized by motor incoordination, loss of cognitive skills, and sedation. The mechanisms that underlie these behaviors are not completely known, but are thought to result primarily from ethanol’s interaction with ion channels that regulate neuronal excitability. Of these, the N-methyl-d-aspartate (NMDA) subtype of glutamate receptor has received considerable attention due to its essential role in fast glutamatergic transmission and involvement in many aspects of neuronal plasticity (Szmulinski and Woodward, 2014). NMDA receptors in almost all biologic systems are reliably inhibited by behaviorally relevant concentrations of ethanol, although the extent of inhibition varies depending on a wide range of factors (Dopico and Lovinger, 2009). These include the anatomic location of neurons containing NMDA receptors, their developmental profile, and the state of various signaling pathways such as phosphorylation cascades that alter NMDA function (Ron and Messing, 2013).

Over the last 20 years, there has been significant progress toward defining the sites and mechanisms for ethanol’s effect on a variety of ion channels, including NMDA receptors, and studies with recombinant NMDA subunits have been particularly useful for testing specific hypotheses regarding the specificity and selectivity of ethanol inhibition of NMDA function. NMDA receptors are obligate heterotetramers composed of two glycine-binding GluN1 subunits interleaved with two glutamate-binding GluN2 subunits (Traynelis et al., 2010), and results from studies of both native and recombinant NMDA receptors suggest that ethanol acts as an allosteric modulator of receptor function (Chu et al., 1995; Mirshahi and Woodward, 1995; Peoples et al., 1997; Xu et al., 2012). Understanding where and how this allosteric modulation takes place is still not well understood, although recent studies by this laboratory and others have revealed potential sites of ethanol action within the transmembrane (TM) domains of the NMDA receptor (Ronald et al., 2001; Honse et al., 2004). Thus, discrete sites within the TM3 and TM4 domains of GluN1 and GluN2 subunits have been identified that, when mutated, alter the sensitivity of the receptor to ethanol. The first of these sites identified is a phenylalanine (F) 639 that resides in the TM3 domain of...
the GluN1 subunit. When mutated to alanine, the ethanol sensitivity of recombinant receptors is significantly reduced (Ronald et al., 2001; Smothers and Woodward, 2006). Knock-in mice carrying the GluN1 F639A mutation also show reduced ethanol inhibition of synaptic NMDA currents as well as selective alterations in various ethanol-related behaviors and consumption (den Hartog et al., 2013).

F639 is a non–pore-facing amino acid and lies C-terminal to the highly conserved SYTANLAAP domain in the TM3 domain of GluN subunits that form a helical bundle crossing above the entrance to the channel pore (Traynelis et al., 2010). Ligand-induced rearrangements of TM3 domains remove the barrier to ion flow, and ethanol’s ability to reduce mean open time and frequency of channel opening (Wright et al., 1996) may reflect its interaction with one or more of these steps. Recently published crystal structures of NMDA receptors in complex with site-selective antagonists (Karakas and Furukawa, 2014; Lee et al., 2014) show that the ethanol-sensitive F639 residue lies in close proximity to amino acids in the GluN1 TM1 and GluN2 TM4 domains that most likely influence receptor activity. In the present study, we use cysteine substitutions and thiol-reactive agents to explore whether residues within these domains can modulate F639’s effect on ethanol inhibition of NMDA receptors and to identify new sites of action for ethanol.

Materials and Methods

Molecular Biology. The NMDA receptor cDNAs used in these experiments were provided by S. Nakanishi (Kyoto University, Kyoto, Japan) and P. Seeburg (Max-Planck Institute for Medical Research, Heidelberg, Germany) and were subcloned in mammalian expression vectors as needed. Site-directed mutagenesis was performed using the QuikChange XL mutagenesis kit (Agilent Technologies, Santa Clara, CA), and mutants were confirmed by DNA sequencing (GeneWiz, South Plainfield, NJ). NMDA structures were rendered using MacPyMOL (PyMOL Molecular Graphics System, Version 1.5.0.4; Schrödinger, New York, NY) using a homology model of the GluN1/GluN2A receptor described in a previous study (Xu et al., 2012).

Human Embryonic Kidney 293 Cell Maintenance and Receptor Expression. Human embryonic kidney (HEK293 cells were obtained from American Type Culture Collection (Manassas, VA) and grown according to the provided protocol. In brief, cells were cultured in Dulbecco’s minimum Eagle’s medium (Life Technologies, Grand Island, NY), supplemented with 10% fetal calf serum (HyClone, Logan, UT), and grown at 37°C in a 5% CO2 environment. Twenty-four hours following plating of low-density cultures (approximately 5 × 10^4 cells per dish) onto 35-mm dishes coated with poly-L-lysine, cells were transfected with equal amounts of subunit cDNA (1 µg) using the Lipofectamine 2000 reagent (Life Technologies). To identify transfected cells, 1 µg pMAX-GFP (Lonza, Basel, Switzerland) was added to the transfection mixture. After transfection, the NMDA antagonist 2-amino-5-phosphonovaleric acid (AP5; 200 µM) was added to Dulbecco’s minimum Eagle’s medium to prevent glutamate-mediated excitotoxicity. Cells were used for electrophysiological recordings 48 hours following transfection, and media containing AP5 was removed by extensive washing just prior to recording.

Electrophysiological Recording Conditions. All recordings were performed as previously described (Smothers and Woodward, 2006). Briefly, cells were perfused with an external solution containing (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl2, 5 HEPES, 0.01 EDTA, and 10 glucose (pH adjusted to 7.4 with NaOH, and osmolality adjusted to 315–325 mOsm/kg with sucrose). Patch electrodes (tip resistance 3–6 MΩ) were fabricated from thick-walled borosilicate glass (B150; WPI, Sarasota, FL) and filled with internal solution composed of (in mM): 140 CsCl, 2 NaATP, 2 MgCl2, 10 HEPES, and 5 EGTA (pH was adjusted to 7.2 with CaOH, and osmolality was adjusted to 290–300 mOsm/kg with sucrose). All internal solutions used for each experiment were from frozen stocks. All drug solutions were prepared fresh for each experiment from frozen stocks in external solution. Stock solutions of glycine and glutamate were prepared in water and diluted into external solution. Ethanol was purchased from Pharmco-Aaper (Shelbyville, KY).

Whole-cell voltage-clamp recordings (Hamill et al., 1981) were performed at room temperature using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Cells were voltage-clamped at –60 mV, and current records were filtered at 1 kHz (eight-pole Bessel filter) and digitized at 2 kHz using an ITC-16 interface (Instrutech, Port Washington, NY). Software control of data acquisition was provided by AxographX software (AxographX, New South Wales, Australia). A three-barrel perfusion apparatus (barrel ID 0.6 mm; SF-77B; Warner Instruments, Hamden, CT) was used to switch between control and drug-containing solutions. Solution exchange times were determined to be between 6 and 8 milliseconds, as calculated by measuring changes in the liquid junction current across an open electrode when switching between solutions with different ionic strength.

Thiol-Modifying Treatments. Several methods were used to treat cells with thiol-modifying reagents. In some studies, dishes containing transfected cells were pretreated for 2 minutes with recording solution containing fresh-made methanethiosulfonate reagents (MTS; 80 µM final concentration; TRC, Toronto, ON, Canada) in the absence or presence of 100 µM glutamate/100 µM glycine (Sigma-Aldrich, St. Louis, MO). Dishes were then rinsed with fresh recording solution and then allowed to recover for 1 hour in the CO2 incubator in HEK cell-feeding media containing 100 µM AP5 before the electrophysiological recording. For experiments involving dithiothreitol (DTT; Sigma-Aldrich) treatment, several control currents (10 µM glutamate/10 µM glycine) were obtained to establish a stable baseline response, and then the patched cell was exposed to 10 mM DTT in the presence of 100 µM AP5 using a local perfusion barrel. After a 1-minute treatment, the perfusion barrel was immediately stepped from the DTT solution to one containing 10 µM glutamate/10 µM glycine to elicit currents.

Western Blotting. SDS-PAGE was used to determine whether cysteine substitutions induced intersubunit cross-linking (Lee and Gouaux, 2011). Twenty-four hours following transfection with cDNAs encoding NMDA subunits, HEK293 cells were solubilized in homogenization buffer (50 mM NaCl, 50 mM Tris-HCl, 10 mM EGTA, 5 mM EDTA, 2 mM Na-pyrophosphate, 1 mM NaF, 1 mM Na-orthovanadate, 40 mM n-dodecyl-β-β-maltoside, and a protease inhibitor (Halt; Thermo Scientific, Waltham, MA). Whole-cell extracts were centrifuged for 30 minutes at 25,000 rpm at 4°C, and the supernatant was collected. Samples (10 µg per lane) were loaded onto Tris-acetate polyacrylamide gels (3–8% gels; Life Technologies) and run under either reducing (addition of DTT to 100 mM final concentration) or nonreducing conditions (no DTT) to separate proteins. Proteins were transferred onto polyvinylidene difluoride membrane and probed with an anti-GluN1 antibody (catalogue 75-272; Antibodies, Davis, CA) at a 1:5000 dilution. Secondary antibody (goat anti-mouse; Southern Biotech, Birmingham, AL) was used at a dilution of 1:1000, and bands were detected by enhanced chemiluminescence (Clarity Western ECL; Bio-Rad, Hercules, CA) using a ChemiDoc MP Imager (Bio-Rad).

Data and Statistical Analysis. AxographX software was used to measure NMDA receptor current parameters. Responses were evoked by a 5-second agonist application and were measured at two time points, peak and steady state. Peak current amplitude was determined as the difference between the current immediately prior to agonist application and at the point where inward current was maximal. Steady-state current amplitude was determined as the difference between current immediately prior to agonist application and the last 500 milliseconds of agonist application where currents were stable. Macroscopic desensitization was calculated as the ratio of the current obtained at the steady-state and the peak current. Ethanol-induced
inhibition of receptor currents ($I_{\text{Control}}$) was calculated using the following formula:

$$[1 - (I_{\text{Agonist} + \text{Etoh}} / I_{\text{Control}})] \times 100,$$

where $I_{\text{Agonist} + \text{Etoh}}$ represents the response to coapplication of agonist + ethanol, and $I_{\text{Control}}$ represents the mean of two responses to agonist, one before and one after the coapplication of ethanol. Leak currents were continually monitored as an indicator of seal and cell integrity. Cells that showed unstable leak currents were not included in the data analysis.

Statistical analyses were performed using the software program Prism 6.0 (GraphPad Software, San Diego, CA) or SPSS (v22, IBM, Armonk, NY). In SPSS, a General Linear Model was used to conduct a three-way analysis of the effects of mutations in TM1, TM3, and TM4 domains. Posthoc pairwise comparisons were then performed using a Bonferroni correction. All inhibition data values are expressed as percent inhibition and represent the mean ± S.E.M. In DTT potentiation experiments, a one-sample t test was used to analyze for enhancement of currents following DTT treatment.

**Results**

**MTS Modulation of Ethanol Inhibition.** A total of 43 different receptor combinations was tested, and all showed reproducible agonist-induced responses that varied in amplitude depending on the subunit combination tested. In the first set of studies, the effect of ethanol on GluN1(F639C)/GluN2A receptors was tested before or after treatment with alcohol-like MTS reagents. In the absence of MTS treatment, GluN1 (F639C)/GluN2A receptors were significantly less sensitive to inhibition by 100 mM ethanol as compared with wild-type receptors (Fig. 1, A and B). A brief pretreatment (90 seconds) of cells with propyl-MTS (C3) applied in the absence of agonists had no effect on the subsequent magnitude of ethanol inhibition in either wild-type or GluN1(F639C)-containing receptors. As some studies have shown that thiol modification of TM amino acids is state dependent (Beck et al., 1999), we repeated these studies and applied the MTS reagent in the presence of a saturating concentration of glutamate and glycine (each at 100 μM) prior to determining ethanol inhibition. Following treatment with propyl (C3), pentylyl (C5), octyl (C8), or decyl (C10) MTS reagents, the ethanol inhibition of GluN1 (F639C)/GluN2A receptors increased to levels similar to that of controls. Ethanol’s inhibition of wild-type receptors was largely unaffected by MTS treatment, although a trend toward a decrease was observed following treatment with the C10-MTS. MTS treatment also produced a general decline in amplitude in both wild-type and the GluN1(F639C) receptors with a particular effect of pentylyl-MTS on GluN1(F639C) receptors. However, due to the significant variability in current amplitudes, the effect of MTS treatment on current amplitude was not statistically significant (two-way analysis of variance; effect of treatment, $F_{4,97} = 1.16, P = 0.33$; effect of mutation, $F_{1,97} = 0.33, P = 0.57$; interaction, $F_{4,97} = 0.083, P = 0.99$).

**Cysteine Cross-Linking Mutants.** To determine whether amino acids in nearby TM domains interact with the TM3 F639 residue to influence ethanol inhibition, we used our previously reported GluN1/GluN2A homology model (Xu et al., 2012) to map residues that are in close proximity to F639. This model is based on the crystal structure of the GluA2 hommeric AMPA receptor (Sobolevsky et al., 2009) and has high homology particularly in the TM domains with the recently solved crystal structures of the GluN1/GluN2B receptor (Karakas and Furukawa, 2014; Lee et al., 2014). Analysis of the GluN1/GluN2A model (Fig. 2A) revealed two residues within TM1 of the GluN1 subunit (V566, S569) and four residues within TM4 of the GluN2A subunit (M817, V820, F821, L824) that could potentially interact with the TM3 F639 residue. Cysteines were substituted at each of these sites (Fig. 2B), and, in the case of the GluN1 subunit, double mutants containing the indicated
residue and the TM3 F639C mutation in the same GluN1 subunit were also generated. In addition to these sites, we also examined three additional mutants containing cysteines substituted at pairs of residues in TM3 and TM4 domains previously reported to influence ethanol sensitivity (Ren et al., 2012). All mutants were expressed in HEK293 cells, and glutamate-activated currents were measured in the absence and presence of 100 mM ethanol using whole-cell patch-clamp electrophysiology.

Representative currents from selected mutants are shown in Fig. 2C and suggest that residues within TM1, TM3, and TM4 of NMDA receptor (NMDAR) subunits may combine to influence receptor function and the degree of ethanol inhibition of glutamate-induced currents. To explore this in a systematic fashion, we used the General Linear Model analysis module in SPSS to analyze how the reduction in ethanol inhibition by the TM3 F639C mutation was affected by cysteine substitutions in the GluN1 TM1 and GluN2 TM4 domains. A single ethanol concentration (100 mM) that is near the IC50 value for wild-type GluN1/GluN2A receptors (Ren et al., 2003; Xu et al., 2012) was used to screen the various mutants for ethanol inhibition. The results of these experiments are summarized in Fig. 3, which shows ethanol inhibition and current amplitude for each of the results of these experiments are summarized in Fig. 3, which shows ethanol inhibition and current amplitude for each of the glutamate-activated currents. To explore this in a systematic fashion, we used the General Linear Model analysis module in SPSS to analyze how the reduction in ethanol inhibition by the TM3 F639C mutation was affected by cysteine substitutions in the GluN1 TM1 and GluN2 TM4 domains. A single ethanol concentration (100 mM) that is near the IC50 value for wild-type GluN1/GluN2A receptors (Ren et al., 2003; Xu et al., 2012) was used to screen the various mutants for ethanol inhibition. The results of these experiments are summarized in Fig. 3, which shows ethanol inhibition and current amplitude for each of the GluN2A TM4 mutants (e.g., M817C, V820C, F821C, L824C) expressed with either of the GluN1 TM1 mutants (V566C, S569C) in the absence or presence of the F639C mutation. As shown in Fig. 3A and consistent with results shown in Fig. 1, the F639C mutation significantly reduced ethanol inhibition when coexpressed with the wild-type GluN2A subunit (F_{1,100} = 15.97; P = 0.0001). However, this action was blunt when coexpression of either of the GluN1 TM1 V566C or S569C mutants, as there was a significant difference in ethanol inhibition between F639C and the combined V566C/F639C (F_{1,100} = 12.02; P = 0.001) or S569C:F639C mutants (F_{1,101} = 4.42; P = 0.038).

Alone, the TM4 M817C mutation did not alter ethanol inhibition (Fig. 3A), but did blunt the ability of F639C to reduce inhibition (F_{1,138} = 2.47; P = 0.12). This effect was modulated by the GluN1 TM1 mutation, as F639C significantly reduced inhibition of the double S569C:M817C mutant (F_{1,137} = 4.6; P = 0.034), but did not reduce ethanol inhibition when combined with the V566C:M817C mutant. The V566C:M817C mutant itself showed a trend toward reduced ethanol inhibition as compared with M817C alone, but this did not quite reach statistical significance (F_{1,130} = 3.24; P = 0.07).

A significant three-way interaction was observed among TM1 V566C, TM3 F639C, and TM4 V820C (F_{1,134} = 6.45; P < 0.012; Fig. 3A) with a strong trend toward a similar three-way interaction among TM1 S569C, TM3 F639C, and the TM4 V820C (F_{1,137} = 3.40; P = 0.07). Alone, V820C significantly enhanced ethanol inhibition as compared with wild-type receptors (F_{1,134} = 6.72; P = 0.011). In the presence of TM4 V820C, F639C significantly reduced the degree of ethanol inhibition (F_{1,134} = 13.95; P = 0.0003), whereas inhibition was enhanced by F639C when V820C and the V566C mutation were combined (V566C:V820C versus V566C:F639C:V820C, F_{1,134} = 6.47; P = 0.012). Ethanol inhibition of the V566C:V820C mutant was itself significantly lower than that of the V820C mutant (F_{1,134} = 18.25; P = 0.00004) and that for the wild-type receptor (F_{1,85} = 8.18; P = 0.007). Although the ethanol inhibition of the S569C:V820C mutant was not different from wild-type, it was different from the V820C mutant (F_{1,137} = 5.89; P = 0.02). Although adding F639C to S569C:V820C produced a trend toward greater inhibition by ethanol, this

![Fig. 2. Sites of cysteine substitutions in TM1, TM3, and TM4 residues in GluN1 and GluN2A subunits. (A) Cartoon shows structure of GluN1 (red) and GluN2A (white) TM domains and location of cysteine mutants used in the study. Valine (V) 566 and serine (S) 569 are in the TM1 of the GluN1 subunit; phenylalanine (F) 639 is in TM3 of GluN1; and methionine (M) 817 and leucine (L) 824 span the positions of residues examined in the TM4 of the GluN2A subunit. Valine (V) 820 and phenylalanine (F) 821 are not shown for sake of clarity. (B) Sequence alignment of TM1, TM3, and TM4 amino acids in the GluN1/GluN2A TM domains and location of cysteine mutants used in the study. Valine (V) 566 and serine (S) 569 are in the TM1 of the GluN1 subunit; phenylalanine (F) 639 is in TM3 of GluN1; and methionine (M) 817 and leucine (L) 824 span the positions of residues examined in the TM4 of the GluN2A subunit. (C) Agonist-evoked currents (10 μM glutamate/glycine) in wild-type and cysteine-substituted mutants in the absence (bottom blue trace) and presence (top red trace) of 100 mM ethanol. Scale bars: x-axis, 1 second; y-axis (wild-type 100 pA; F639C-100 pA; V566C:F639C-500 pA; F639C/F821C-50 pA; V566C/V820C-100 pA; L824C-50 pA).]
Fig. 3. Effects of TM1, TM3, and TM4 cysteine substitutions on ethanol inhibition and amplitude of GluN1/GluN2A receptors. Panels show inhibition of steady-state currents by 100 mM ethanol (A) and mean control steady-state current amplitude (B) for each TM4 mutant tested (from top to bottom, WT, M817C, V820C, F821C, L824C). In each graph, values represent the mean (±S.E.M.) of the indicated measurement in the absence (−F639C) and presence (+F639C) of the TM3 F639C mutation and are plotted as a function of the GluN1 TM1 cysteine substitution (V566C, open squares; S569C, open triangles). Note slightly different y-axis scales for ethanol inhibition of V820C, F821C, and L824C mutants as compared with the wild-type and M817C mutants. ^Value significantly different (P < 0.05) from wild-type control (left red circle; top panel); #value significantly different (P < 0.05) from corresponding F639C lacking TM4 mutant (left side; each panel); *value significantly different (P < 0.05) from same mutant lacking the F639C mutation (−F639C versus +F639C, each panel). See Results for full details of statistical analysis.
effect did not reach statistical significance \((F_{1,127} = 1.43; P = 0.23)\).

Significant three-way interactions were also observed among TM1 V566C, TM3 F639C, and TM4 F821C \((F_{1,128} = 5.20; P = 0.02)\) and among TM1 S569C, TM3 F639C, and TM4 F821C \((F_{1,120} = 8.05; P = 0.005)\; \text{Fig. 3A}\). In this study, the F821C mutation that had no effect on ethanol inhibition on its own prevented the reduction in ethanol inhibition by F639C. This effect was TM1 residue dependent as inhibition was reduced by F639C in the presence of S569C and F821C \((F_{1,120} = 11.63; P = 0.001)\). A trend for a similar effect was also observed for the V566C mutation, but the pairwise comparison did not reach statistical significance \((V566C:F821C \text{ versus } V566C:F639C: F821C, F_{1,128} = 3.02; P < 0.085)\).

In the final set of triple mutants tested, there was a significant three-way interaction among TM1 S569C, TM3 F639C, and TM4 L824C \((F_{1,122} = 7.11; P = 0.009)\), but not among TM1 V566C, TM3 F639C, and TM4 L824C \((F_{1,131} = 2.14; P = 0.146)\). Alone, the L824C mutant had a greater degree of ethanol inhibition than the wild-type receptor \((F_{1,122} = 19.78; P = 0.00002)\), and inhibition of the L824C receptor was reduced by adding either F639C \((F_{1,122} = 21.51; P = 0.000008)\), or V639C \((F_{1,122} = 15.81; P = 0.00003)\), or the S696C mutation \((F_{1,122} = 9.77; P = 0.002)\). Coexpressing F639C with the V566C:L824C mutant produced a nonsignificant \((F_{1,131} = 2.82; P = 0.095)\) decrease in ethanol inhibition, whereas a slight but non-significant increase was observed for the S569C:F639C:L824C combination.

**Cysteine Mutants and Receptor Function.** To determine whether the changes in ethanol inhibition in cysteine-substituted mutants were correlated with changes in receptor function, we analyzed the effects of mutations on mean steady-state currents (Fig. 3B), macroscopic desensitization (defined as the ratio of the steady state to peak amplitude; Fig. 4A), and the off-rate of current decay (Fig. 4B) for each mutant combination. These measures were taken from control recordings of each receptor combination used in the ethanol inhibition studies. There were few significant interactions between mutants on current amplitude most likely due to cell-dependent differences in protein expression. However, a three-way interaction among TM1 S569C, TM3 F639C, and TM4 M817C was noted \((F_{1,109} = 4.21; P = 0.043)\), and pairwise comparisons showed that the amplitude of S569C:M817C was significantly smaller than that of S569C alone \((F_{1,109} = 4.17; P = 0.044)\) and the wild-type receptor \((F_{1,24} = 9.30; P = 0.006)\), but not that of the M817C mutant \((F_{1,109} = 3.378; P = 0.069)\). In addition, there was a significant difference in amplitude between the wild-type receptor and the L824C mutant \((F_{1,108} = 4.63; P = 0.034)\).

The panels in Fig. 4 show values for the steady-state to peak (SS:Pk) ratio and current off-rate for each receptor combination tested. There were no significant three-way interactions among TM1, TM3, and TM4 mutants for SS:Pk for any of the combinations examined, but there were subunit-dependent changes in this measure of macroscopic desensitization. In some cases, the change in degree of desensitization appeared negatively correlated with the ethanol inhibition of the receptor, although this was not consistently observed. The F639C mutation itself significantly enhanced the SS:Pk ratio as compared with wild-type receptor \((F_{1,34} = 13.64; P = 0.001)\), and this was maintained in the presence of the TM1 V566C mutation \((V566C \text{ versus } V566C:F639C, F_{1,34} = 4.49; P = 0.041)\). In contrast, there was a significant two-way interaction between F639C and TM1 S569C \((F_{1,34} = 7.99; P = 0.008)\) as S569C blocked the increase in the SS:Pk ratio \((F_{1,58} = 9.44; P = 0.003)\). Pairwise comparisons showed that F639C increased the off-rate of current decay \((F_{1,58} = 6.26; P = 0.015)\), but not in the presence of V566C or S569C.

For the TM4 M817C mutant, F639C did not significantly enhance the SS:Pk ratio in receptors with wild-type or mutant \((V566C, S569C)\) TM1 residues. In contrast, the decay off-rate of TM4 M817C was increased with the addition of F639C \((F_{1,92} = 29.66; P = 4.3 \times 10^{-7})\), and a smaller, but significant F639C-induced increase was also observed in the S569C:F639C:M817C mutant \((F_{1,90} = 4.18; P = 0.044)\). No significant increase in off-rate was observed when F639C was added to the V566C:M817C mutant.

The TM4 V820C mutant showed a significant increase in SS:Pk ratio as compared with the wild-type receptor \((F_{1,110} = 9.74; P = 0.002)\), and adding F639C caused a further enhancement in this measure \((F_{1,110} = 16.13; P = 0.0001)\). F639C did not affect the SS:Pk ratio when added to the V566C:V820C mutant, whereas it reduced the ratio when added to the S569C:V820C mutant \((F_{1,115} = 4.45; P = 0.037)\). F639C also enhanced the decay off-rate of V820C receptors \((F_{1,90} = 8.74; P = 0.004)\), but not that of V566C:V820C or S569C:V820C combinations.

Like V820C receptors, the SS:Pk ratio of the TM4 F821C receptor was significantly greater than that of the wild-type receptor \((F_{1,106} = 18.84; P = 0.00003)\). Adding F639C further enhanced this ratio \((F_{1,106} = 15.85; P = 0.0001)\), and this enhancement was maintained in the triple V566C:F639C:F821C mutant \((F_{1,106} = 5.08; P = 0.026)\). No significant enhancement in SS:Pk was observed in S569C:F639C:F821C receptors as compared with the S569C:F821C combination. There was a significant three-way interaction for the decay off-rate among S569C, F639C, and F821C \((F_{1,84} = 15.28; P = 0.0002)\), where F639C induced a highly significant increase in the presence of the S569C:F821C combination \((F_{1,84} = 39.32; P = 1.49 \times 10^{-6})\), but not when combined with F821C or V566:F821C.

The SS:Pk ratio for the TM4 L824C mutant was significantly higher than that of the wild-type \((F_{1,101} = 7.40; P = 0.008)\), and this was not altered in the presence of F639C, V566C:F639C, or S569C:F639C. Alone, TM4 L824C had minimal effects on the current off-rate, and no increase was observed in F639C:L824C or V566C:F639C:L824C mutants. A small but statistically significant increase in the decay off-rate was seen for the S569C:F639C:L824C mutant \((F_{1,97} = 4.16; P = 0.04)\).

**Effects of Cysteine Substitutions at Homologous Sites.** In addition to the mutants described above, cysteines were introduced at pairs of positions within the GluN1 and GluN2 TM domains previously suggested to form sites of interaction for ethanol \((Ren \text{ et al.}, 2012)\). These pairs were glycine 638 (G638) in GluN1 TM3 and methionine 823 (M823) in GluN2A TM4; methionine 818 (M818) in GluN1 TM4 and phenylalanine 636 (F636) in GluN2A TM3; and leucine 819 (L819) in GluN1 TM4 and phenylalanine 637 (F637) in GluN2A TM4. Figure 5 shows the effects of these mutations on ethanol inhibition \((F_{1,45} = 4.75; P = 0.037)\), current amplitude \((F_{1,45} = 3.58; P = 0.067)\), and decay off-rate \((F_{1,45} = 4.39; P = 0.044)\). For each set of mutants tested, we used one-way analysis of variance with Tukey’s posthoc test to analyze for differences between the three possible combinations in each set. The mean control values for the wild-type GluN1/GluN2A receptors are included in the graphs for comparison, but were not used for analysis. There
Fig. 4. Effects of TM1, TM3, and TM4 domain cysteine substitutions on measures of receptor function of GluN1/GluN2A receptors. Panels show the steady-state to peak ratio (A) and current decay time (B) of control currents for each TM4 mutant tested (from top to bottom, WT, M817C, V820C, F821C, L824C). In each graph, values represent the mean (±S.E.M.) of the indicated measurement in the absence (-F639C) and presence (+F639C) of the TM3 F639C mutation and are plotted as a function of the GluN1 TM1 cysteine substitution (V566C, open squares; S569C, open triangles). Note slightly different y-axis scale for decay time for the F821C mutant. ^Value significantly different (P < 0.05) from wild-type control (left red circle; top panel); *value significantly different (P < 0.05) from same mutant lacking the F639C mutation (-F639C versus +F639C, each panel). See Results for full details of statistical analysis.
was a significant treatment effect for ethanol inhibition in the G638C:M832C group ($F_{2,21} = 5.88; P = 0.009$), and pairwise comparisons revealed a significant difference between G638C and G638C:M832C ($P < 0.01$). Analysis of mean current amplitudes of the G638C:M832C set also revealed a significant treatment effect ($F_{2,25} = 11.56; P = 0.0002$) with significant differences between G638C and G638C:M832C ($P < 0.001$) and M832C and G638C:M832C ($P < 0.05$). There was also a significant treatment effect on the SS:Pk ratio for G638C:M832 ($F_{2,28} = 8.60; P = 0.0012$) with pairwise differences between G638C and M832C ($P < 0.01$) and M832C and G638C:M832 ($P < 0.05$). No statistically significant difference was observed for decay off-rate for the G638C:M832C set of mutants. No significant difference in ethanol inhibition was observed for members of the M818C:F636C set, but there was a significant treatment effect for current amplitude ($F_{2,27} = 3.26; P = 0.05$) with F636C amplitude being reduced compared with M818C:F636C ($P < 0.05$). No significant treatment effects were observed for SS:Pk ratio or decay off-rates for the M818C:F636C. In the last set tested, there was no difference in ethanol inhibition, SS:Pk ratio, or decay off-rate for the L819C:F637C set, whereas a significant treatment effect was observed for mean amplitude ($F_{2,24} = 24.44; P = 0.0001$). Pairwise comparisons revealed that the amplitude of the L819C mutant was significantly different from that of F637C ($P < 0.0001$) and L819C:F637C ($P < 0.0001$).

**Test of Cysteine Cross-Linking.** Cysteine substitution of residues within GluN1 TM1, TM3 and GluN2A TM4 domains is predicted to generate disulfide bonds that may underlie the alterations in ethanol inhibition and receptor function noted in Figs. 3–5. To examine this possibility more directly, we measured currents from selected mutant receptors before and immediately after treatment with the sulphydryl-reducing agent DTT. For each receptor tested, two to three stable control currents were obtained before exposing the cell to 10 mM DTT for 1 minute in the presence of AP5, followed by immediate exposure to the same agonist solution used to generate control responses. This approach has been shown previously to reliably potentiate NMDA with cysteines substituted in extracellular domain and areas near the outer membrane surface (Furukawa et al., 2005; Talukder et al., 2010). Note that all recordings in this study were done in the presence of EDTA (10 $\mu$M) to chelate low nanomolar concentrations of zinc that are present in solutions and that tonically inhibit GluN2A-containing receptors. Because DTT is also an effective zinc chelator, these conditions should ensure that any effect of DTT on current amplitude is due to thiol reduction rather than relief of zinc inhibition (Paoletti et al., 1997). As a positive control for disulfide bond reduction, we used a mutant containing pairs of cysteines introduced into the extracellular domain of GluN1 (N521C:L777C) and GluN2A (E516C:L780C) previously reported to display a reproducible increase in current amplitude following DTT treatment (Furukawa et al., 2005; Borschel et al., 2011). As shown in Fig. 6, A and B, this mutant had a nearly 2-fold increase in amplitude following exposure to DTT (one-sample $t$ test; $t_{2,30,8,5} = 0.0221$), whereas no significant potentiation was observed for wild-type GluN1/GluN2A receptors. Of the other mutants tested, four showed a modest but statistically significant potentiation in current following DTT treatment, including the single cysteine

![Fig. 5. Effects of cysteine substitutions at selected TM3 and TM4 residues of GluN1/GluN2A receptors on ethanol inhibition and receptor function. Data represent the mean (±S.E.M.) value for each mutant combination for (A) inhibition of steady-state currents by 100 mM ethanol; (B) steady-state current amplitude under control conditions; (C) steady-state to peak ratio for currents under control conditions; (D) current decay time under control conditions. Symbols: indicate value is significantly different from indicated mutant (*$P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001$).](image-url)
mutant L824C ($t_{2.369,7}, P = 0.0497$) and the double mutants V566C/L824C ($t_{4.229,6}, P = 0.0179$) and M818C/F636C ($t_{4.418,6}, P = 0.0045$).

To further examine whether cysteine-substituted NMDA receptors showed evidence of cross-linking, we used Western blotting to analyze the electrophoretic mobility of the GluN1 subunit under nonreducing and reducing conditions. Intersubunit cysteine cross-linking between GluN1 and GluN2 subunits should result in higher-molecular-weight bands than for the predicted monomers, and reduction of these bonds under strong reducing conditions should eliminate these higher-molecular-weight bands. Under nonreducing, denaturing conditions, a prominent band at the predicted molecular weight of GluN1 was observed in samples prepared from cells transfected with wild-type GluN1/GluN2A receptors. There was no evidence of significant cross-linking with the wild-type receptor, whereas for the GluN1(CC)/GluN2A(CC) positive control, higher-molecular-weight bands were obvious (Fig. 6C). Treatment with DTT prior to gel loading had little effect on GluN1 mobility in cells transfected with wild-type GluN1/GluN2A receptors, but largely eliminated the higher-molecular-weight bands of the GluN1(CC)/GluN2A(CC) mutant. There was clear evidence of higher-molecular-weight bands under nonreducing conditions for other cysteine-substituted mutants, including one, GluN1/GluN2A(L824C), where only a single additional cysteine was introduced. Treatment of samples with DTT prior to gel loading eliminated these higher-molecular-weight bands and enhanced the intensity of bands at the predicted molecular weight of GluN1. These experiments were repeated several times, and similar band patterns were observed (additional data not shown).

**Discussion**

In the present study, we show that covalent modification of the TM3 GluN1 F639 residue affects ethanol inhibition of NMDA receptors and that residues within GluN1 TM1 and GluN2 TM4 domains form novel intra- and intersubunit sites of action for ethanol. Replacing TM3 F639 with the smaller cysteine reduced ethanol inhibition consistent with the inverse relationship between molecular volume at F639 and ethanol inhibition (Ronald et al., 2001; Smothers and Woodward, 2006). Ethanol inhibition of GluN1(F639C) receptors was restored to normal following treatment with thiol-reactive agents that did not affect wild-type receptors. Data from the modeling and cysteine substitution studies identified discrete residues within the GluN1 TM1 and GluN2A TM4 domains that mimicked or blocked F639C’s effect on ethanol inhibition. Together, these results suggest that ethanol alters NMDAR function via interaction with multiple residues in a microdomain formed by GluN1 TM1/TM3 and GluN2A TM4 domains.

Treatment of GluN1(F639C) receptors with alcohol-like MTS reagents increased ethanol inhibition back to wild-type levels, suggesting that molecular volume at this site is an important determinant of ethanol inhibition. Approximately 65 cubic angstroms of molecular volume is lost when phenylalanine is replaced by cysteine (Vilpoux et al., 2009), whereas covalently linking propyl or pentyl-MTS to cysteine increases volume by approximately 102 and 140 cubic angstroms, respectively. Similarly, in previous studies, replacing GluN1 F639 with larger residues such as tryptophan (Ronald et al., 2001) or tyrosine (Smothers and Woodward, 2006) enhances or does not...
change ethanol inhibition, suggesting that TM3 639 accommodates large side chain volumes with little change in receptor function or ethanol inhibition. Despite this finding, others have reported reduced ethanol inhibition for an F639 tryptophan-substituted GluN1/GluN2A mutant (Ren et al., 2012), suggesting that other conditions (e.g., agonist concentration, method of ethanol exposure, etc.) may also be important in determining ethanol’s effect on receptor function. The effect of MTS modification of ethanol-sensitive sites is also dependent on the receptor type studied. For example, MTS treatment of cysteine-substituted glycine and GABA<sub>A</sub> receptors reduced potentiation of channel function by ethanol or certain anesthetics (Mascia et al., 2000; Borghese et al., 2014), suggesting differences in the structure or characteristics of alcohol-sensitive domains between ion channel subtypes.

In the present study, ethanol inhibition of F639C receptors was affected by cysteine substitutions at sites within GluN1 TM1 and GluN2A TM4 domains. These regions lie in close proximity and form a microdomain with the TM4 of a GluN2 subunit interacting with the TM1 and TM3 domains of the adjacent GluN1 subunit (Sobolevsky et al., 2009; Karakas and Furukawa, 2014; Lee et al., 2014). Although a functional role for these intersubunit associations is not yet clear, TM4 domains from neighboring subunits may coordinate movement of TM3 domains during agonist-evoked gating permitting reproducible transitions into the open state (Talukder and Wollmuth, 2011). Ethanol-induced disruption of this interaction could alter receptor gating and reduce channel function. By itself, the F639C mutant showed reduced ethanol inhibition and increases in the SS:Pk ratio and off-rate, suggesting enhanced gating and agonist sensitivity. Similarly, F639A receptors showed enhanced responses to glycine site partial agonists (Ronald et al., 2001) and have a small but significant left shift in the glycine, but not glutamate concentration response relationship (Ogata et al., 2006; Smothers and Woodward, 2006), suggesting enhanced gating. In the present study, ethanol inhibition of F639C receptors was restored to normal by intrasubunit GluN1 TM1 cysteine mutants that alone showed normal ethanol inhibition. TM1 mutants also reduced F639C’s effect on the SS:Pk ratio and off-rate decay rate, although this was mutation specific. Like MTS reagents, these effects may result from increased molecular volume around the F639C residue due to cysteine cross-linking of TM1 and TM3 residues.

Cysteine substitution in GluN2A TM4 domains generally did not affect the ability of F639C to reduce ethanol inhibition. However, the interaction between F639C and TM4 mutants was dependent on TM1 V566C or S569C. These TM1 mutations reversed the effects of F639C on ethanol inhibition of the V820C mutant and restored the F639C-mediated reduction in ethanol inhibition of the F821C mutant. Particularly interesting was the finding that the F639C-mediated reduction in ethanol sensitivity was mimicked by the V566C/V820C mutant that itself had the lowest degree of inhibition of any mutant tested. These results suggest a selective interaction between ethanol and sites within a microdomain formed by intra- and intersubunit associations of TM domains. Alteration of this microdomain by cross-linking may bidirectionally influence ethanol inhibition in a residue-dependent manner. Whereas previous studies focused on a role for TM3 and TM4 residues in ethanol action (Ronald et al., 2001; Ren et al., 2003, 2012; Honse et al., 2004; Smothers and Woodward, 2006; Xu et al., 2012), the present study is the first to show that TM1 residues may also contribute. Residues in extracellular regions link GluN1 TM1 to ligand-binding domains that interact with similar linker sites in GluN2A TM4 and may be involved in early steps of receptor activation that lead to TM3 domain movement (Talukder et al., 2010; Kazi et al., 2013). As TM3 is critically involved in channel gating (Jones et al., 2002; Chang and Kuo, 2008), ethanol perturbation of TM1 residues that interact with TM3 may alter the transitions between closed and open states of the receptor.

The Peoples laboratory first showed that residues in GluN2A TM4 could also reduce ethanol inhibition of NMDARs (Ren et al., 2003; Honse et al., 2004). More recently, they used tryptophan substitutions and mutant cycle analysis and identified four pairs of ethanol-sensitive residues between GluN1 and GluN2A subunits (Ren et al., 2012). Tryptophan mutants at GluN1 TM3 sites (G638 and F639) and GluN2A TM4 sites (F636, F637, and M823) all reduced ethanol sensitivity when expressed with their wild-type counterparts, whereas all but one double-substituted pair (L819W/F636W) showed wild-type ethanol inhibition. In the present study, substituting cysteines at these sites produced no significant interactions on ethanol inhibition for two of these pairs (M818C/F636C; L819C/F637C), whereas opposite effects were observed for the other two combinations. Thus, GluN1 TM3 G636C/GluN2A TM4 M823C receptors had enhanced ethanol inhibition, whereas receptors with cysteines located one position away had reduced ethanol inhibition. It is not surprising that cysteine and tryptophan would yield different results given their disparate physicochemical properties, but shifting the location of the cysteine by one position in each subunit might be predicted to produce similar results on ethanol inhibition. However, structural (Sobolevsky et al., 2009; Karakas and Furukawa, 2014; Lee et al., 2014) and homology (Xu et al., 2012) models reveal that these positions interact differently with other TM domains. F639L/E824 residues lie in a pocket formed by GluN1 TM1, TM3, and GluN2A TM4 domains, whereas G638/M823 face the backside of the TM3/4 helices and do not strongly interact with GluN1 TM1. In addition, although GluN2A TM3 F636 and F637 occupy similar positions in the TM3 domain as GluN1 F639, their TM4 partners are on the opposite face of the GluN1 TM4 helix away from their TM3 counterparts. In addition to these sites, tryptophan substitution of the nearby alanine 825 in the GluN2A TM4 (A825) sharply reduces ethanol inhibition (Ren et al., 2003; Honse et al., 2004). A825 faces away from all other TM domains and may instead lie along a lipid-accessible pathway that allows ethanol entry to channel gating domains. Replacing A825 with tryptophan might impede this access and reduce ethanol sensitivity. In support of this, preliminary single-channel studies show that A825W has little effect on rate constants for transitions between closed and open states (G. K. Popescu and J. J. Woodward, unpublished observation).

Despite the intriguing results obtained in the present study, there are several limitations that should be addressed. First, all experiments used a single concentration of agonist and ethanol, and thus, we cannot rule out that the effects of the mutations might vary under different conditions. However, in most studies (Mirshahi and Woodward, 1995; Peoples et al., 1997), ethanol inhibition of NMDARs is not influenced by agonist concentrations consistent with the finding that ethanol is an allosteric, noncompetitive antagonist of NMDARs (but see Woodward and Gonzales, 1990). In addition, although 100 mM
ethanol is rarely experienced by nonalcohol-dependent humans, mutations that reduce inhibition by this concentration cause similar reductions at lower, more behaviorally relevant levels (Ronald et al., 2001; Ren et al., 2012). Secondly, as F639C faces other TM domains and does not participate directly in ion permeation, no clear physiologic effect on channel function could be predicted for cysteine-substituted mutants. Thus, it is possible that changes in ethanol inhibition in these mutants could have arisen from alterations in side chain interactions rather than through disulfide bond formation or MTS modification. Nonetheless, with these limitations in mind, we conclude that residues within a discrete area bounded by TM1, TM3, and TM4 domains may be critical sites of interaction for ethanol on the NMDA receptor.

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Participated in research design: Woodward, Smothers, Xu.
Conducted experiments: Smothers, Xu.
Contributed new reagents or analytic tools: Smothers.
Performed data analysis: Xu, Smothers, Woodward.
Wrote or contributed to the writing of the manuscript: Woodward, Smothers, Xu.

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