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Abstract

Background

Alcohol alters synaptic transmission in the brain. The *N*-methyl-D-aspartate (NMDA) receptor (NMDAR), a subtype of glutamate-gated ion channel, is an important synaptic target of alcohol in the brain. We and others have previously identified 4 alcohol-sensitive positions in the third and fourth membrane-associated (M) domains, designated $M3_{1-2}$ and $M4_{1-2}$, of the GluN1, GluN2A, and GluN2B NMDAR subunits. In the present study, we tested whether the corresponding positions in the GluN2C subunit also regulate alcohol sensitivity and ion channel gating.

Methods

We performed alanine- and tryptophan-scanning mutagenesis in the GluN2C subunit followed by expression in HEK 293 cells and electrophysiological patch-clamp recording.

Results

Alanine substitution at the $M3_1$ (F634) and $M4_{1-2}$ (M821 and M823) positions did not alter ethanol (EtOH) sensitivity, whereas substitution of alanine at the $M3_2$ position (F635) yielded nonfunctional receptors. Tryptophan substitution at the $M3_{1-2}$ positions did not change EtOH sensitivity, whereas tryptophan substitution at the $M4_1$ position increased, and at the $M4_2$ position decreased, EtOH sensitivity. The increased EtOH sensitivity of the tryptophan mutant at $M4_1$ is in marked contrast to previous results observed in the GluN2A and GluN2B subunits. In addition, this mutant exhibited increased desensitization, but to a much lesser extent compared to the corresponding mutations in GluN2A and GluN2B. A series of mutations at $M4_1$ altered EtOH sensitivity, glutamate potency, and desensitization. Seven amino acid substitutions (of 15 tested) at this position yielded nonfunctional receptors. Among the remaining mutants at $M4_1$, EtOH sensitivity was not significantly correlated with hydrophobicity, molecular volume, or polarity of the substituent, or with glutamate EC_{50} values, but was correlated with maximal steady-state-to-peak current ratio, a measure of desensitization.

Conclusions

The identity and characteristics of alcohol-sensitive positions in the GluN2C subunit differ from those previously reported for GluN2A and GluN2B subunits, despite the high homology among these subunits.

Ethanol (EtOH) is a widely abused drug that acts on multiple pre- and postsynaptic targets in the brain to alter synaptic transmission (Abrahao et al., **2017**; Harrison et al., **2017**; McCool, **2011**). Among the most important targets of EtOH are *N*-methyl-D-aspartate receptors (NMDAR), glutamate-gated ion channels that are essential for multiple aspects of brain function, including forms of synaptic plasticity underlying learning and memory, motor function, cognition, attention, and reward (Bliss and Collingridge, **1993**; Dingledine et al., **1999**; Paoletti and Neyton, **2007**; Traynelis et al., **2010**). The major type of NMDAR in the adult central nervous system is a heterotetramer containing 2 GluN1 subunits and 2 GluN2 subunits, of which there are 4 types, GluN2A-D (Honer et al., **1998**; Kutsuwada et al., **1992**; Laube et al., **1998**). NMDARs are inhibited by EtOH at relevant concentrations and play a crucial role in the effects of EtOH in the brain (Krystal et al., **2003**; Vengeliene et al., **2008**; Woodward, **2000**). Although multiple molecular mechanisms can modulate NMDAR EtOH sensitivity (Ron, **2004**), the molecular mechanism by which EtOH directly acts on NMDAR appears to involve regulation of ion channel gating (Wright et al., **1996**) via interactions with specific amino acids in the membrane-associated (M) domains (Honse et al., **2004**; Ren et al., **2003b**, **2007**; Ronald et al., **2001**; Smothers and Woodward, **2006**). In the GluN1/GluN2A and GluN1/GluN2B NMDAR, these putative sites of EtOH action consist of small clusters of residues at the intersubunit interfaces of the M3 and M4 domains (Ren et al., **2012**; Zhao et al., **2015**, **2016**); some of these positions also interact with side chains in other M domains (Xu et al., **2015**). Mutations at key positions in these clusters in both the GluN1 and GluN2 subunits can strongly regulate ion channel gating (Ren

et al., **2003a**, **2007**, **2008**, **2012**, **2013**; Smothers and Woodward, **2016**; Zhao et al., **2016**), although the changes in gating differ considerably among positions and do not appear to underlie the changes in EtOH sensitivity. For example, in the GluN2A subunit M3 domain, although substitution of tryptophan at either of the positions significantly decreases EtOH sensitivity, tryptophan substitution at F636 decreases desensitization and increases mean open time (Ren et al., **2013**), whereas tryptophan substitution at F637 does not alter desensitization but decreases mean open time (Ren et al., **2007**).

GluN2C subunits differ from GluN2A and GluN2B subunits in multiple respects. Unlike the GluN2A and GluN2B subunits, the GluN2C subunit has a limited distribution, with the greatest abundance in the cerebellum (Farrant et al., **1994**; Karavanova et al., **2007**; Monyer et al., **1994**; Wenzel et al., **1997**), and lesser amounts in the thalamus, olfactory bulb, globus pallidus, and hippocampus (Monyer et al., **1994**; Ravikrishnan et al., **2018**; Wenzel et al., **1997**). In thalamus, globus pallidus, and substantia nigra, GluN2C subunits appear to be expressed primarily in interneurons, whereas in cortex, hippocampus, and amygdala, they are expressed primarily in glial cells (Alsaad et al., **2019**; Ravikrishnan et al., **2018**; Verkhratsky and Chvátal, **2019**). Compared to NMDARs containing GluN2A and GluN2B subunits, GluN2C-containing NMDARs have a shorter mean open time and much lower open probability (Dravid et al., **2008**), lower single-channel conductance (Dravid et al., **2008**; Stern et al., **1992**), and lower sensitivity to Mg^{2+} block (Monyer et al., **1992**), with little to no desensitization and glutamate deactivation similar to that of GluN2B (Krupp et al., **1996**; Monyer et al., **1992**; Vicini et al., **1998**). The GluN2C-containing NMDAR also shows differences in alcohol sensitivity. GluN2C-containing NMDARs are less sensitive to EtOH compared to GluN2A- and GluN2B-containing NMDARs (Chu et al., **1995**; Masood et al., **1994**; Mirshahi and Woodward, **1995**), but the basis for the lower EtOH sensitivity of GluN2C subunits is not known. Smothers and Woodward (**2016**) have recently shown that substitution of tryptophan in the fourth membrane-associated domain of the GluN2C subunit at a position corresponding to one previously shown to decrease alcohol inhibition in the GluN2A subunit (Honse et al., **2004**; Salous et al., **2009**) greatly decreases alcohol inhibition, but apart from this observation little is known about the action of alcohol in the M domains of the GluN2C subunit. In the present study, we studied the molecular determinants of alcohol inhibition of GluN2C-containing NMDAR by introducing mutations in the GluN2C subunit at positions corresponding to those shown to modulate alcohol action in the GluN2A and GluN2B subunits. Despite high homology in the M domains among the GluN2 subunits, we report that mutations at these positions in the GluN2C subunit differentially modulate alcohol action compared to the GluN2A and GluN2B subunits.

Materials and Methods

Materials

EtOH (95%, prepared from grain) was obtained from Aaper Alcohol & Chemical Co. (Shelbyville, KY), and all other drugs were obtained from Sigma-Aldrich (St. Louis, MO). Chemicals used to make recording solutions were the highest purity available.

Molecular Biology, Cell Culture, and Transfection

Site-directed mutagenesis in plasmids containing rat GluN1 or GluN2C subunit cDNA was performed using the QuikChange II kit (Agilent Technologies, Santa Clara, CA), and all mutations were verified by double-strand DNA sequencing. TSA201 cells, a transformed human kidney 293 cell line, were maintained in flasks containing serum-supplemented Dulbecco's minimum Eagle's medium in a humidified 5% CO_2 incubator. For recordings, cells were plated onto fibronectin-coated 35-mm dishes at high density (approximately 5×10^5 cells per dish) and transfected with plasmids containing cDNA for GluN1, GluN2C, and green fluorescent protein using the calcium phosphate transfection kit (Invitrogen, Waltham, MA). Magnesium chloride, 10 mM, was added to the culture medium to prevent excitotoxic cell death. Magnesium chloride was removed before use in experiments by extensive washing. Cells were used in experiments 24 to 48 hours after transfection.

Electrophysiological Recording

Whole-cell patch-clamp recording was performed at room temperature using an Axon 200B amplifier (Molecular Devices, Sunnyvale, CA). Patch pipettes (1 to 3 MΩ) were pulled from thin-wall borosilicate glass and filled with internal solution containing 140 mM CsCl, 2 mM Mg_4ATP, 10 mM BAPTA, and 10 mM HEPES (pH 7.2). The recording solution contained 150 mM NaCl, 5 mM KCl, 0.2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, and 10 mM sucrose. The ratio of added HEPES-free acid and sodium salt was calculated to result in a solution pH of 7.4 (Buffer Calculator, R. Beynon, University of Liverpool); pH was adjusted as necessary using HCl or NaOH. Solutions of agonists and EtOH were prepared fresh daily and applied to cells using a stepper motor-driven rapid solution exchange apparatus (Warner Instruments, Inc., Hamden, CT) and 600-μm-inner diameter square glass tubing. In concentration–response experiments, the order of application of the various concentrations of EtOH was randomized for each cell to eliminate time-dependent effects. Data were filtered at 2 kHz (8-pole Bessel) and acquired at 5 kHz on a computer using a DigiData interface and pClamp software (Molecular Devices, San Jose, CA).

Calculation of Physicochemical Properties of Amino Acids

Molecular (van der Waals) volumes and log octanol: water partition coefficients (LogP) of amino acids were calculated using Spartan '16 software (Wavefunction, Inc., Irvine, CA) following structural optimization using the AM1 semi-empirical parameters. Values used for amino acid hydrophilicity and polarity were reported previously (Hopp and Woods, **1981**; Zimmerman et al., **1968**).

Data Analysis

In concentration–response experiments, IC_{50} or EC_{50} and n (slope factor) were calculated using the equation $y = E_{max}/1 + (IC_{50}$ or $EC_{50}/x)^n$, where y is the measured current amplitude, x is concentration, n is the slope factor, and E_{max} is the maximal current amplitude. Statistical differences among concentration–response curves were determined by comparing log-transformed IC_{50} or EC_{50} values from fits to data obtained from individual cells using 1-way analysis of variance (ANOVA) followed by the Dunnett's test.

Results

Alcohol-Sensitive Positions in the GluN2C M3 and M4 Domains

In previous studies, we and others have identified clusters of alcohol-sensitive positions in the M3 and M4 domains of the GluN1, GluN2A, and GluN2B NMDAR subunits (Fig. **1**; Ren et al., **2012**; Xu et al., **2015**; Zhao et al., **2015**, **2016**). To facilitate comparisons among the subunit types, we designate the 2 positions in the M3 domain corresponding to F636 and F637 in the GluN2A subunit as $M3_1$ and $M3_2$, respectively, and the 2 positions in the M4 domain corresponding to M823 and A825 in the GluN2A subunit as $M4_1$ and $M4_2$, respectively. To test whether the corresponding positions in the GluN2C subunit similarly regulate alcohol sensitivity, we constructed alanine and tryptophan substitution mutants at the $M3_{1-2}$ residues, F634 and F635, and the $M4_{1-2}$ residues, M821 and L823, of the GluN2C subunit. Glutamate-activated currents in alanine substitution mutants at 3 of the 4 positions did not exhibit any grossly apparent changes in characteristics such as desensitization (Fig. **2**A), but no current could be detected in response to maximal concentrations of glutamate in the GluN2C(F635A) mutant. Consequently, EtOH inhibition could not be determined in GluN2C(F635A) mutant subunits, but EtOH IC_{50} values were unchanged in the remaining alanine mutants relative to the wild-type (WT) subunit (Fig. **2**B,C). In contrast, tryptophan mutation at F635, as at each of the remaining positions, yielded functional receptors (Fig. **2**A). Desensitization of glutamate-activated current appeared to be increased in the GluN2C(M821W) mutant relative to the WT subunit. EtOH sensitivity was significantly increased in the GluN2C(F635W) and GluN2C(M821W) subunits (IC_{50} values of 98.0 ± 24.9 and 138 ± 5.46 mM, respectively, vs. 207 ± 7.27 mM in the native

subunit; $p < 0.0001$ and 0.05), but was markedly decreased in the GluN2C(L823W) subunit (IC$_{50}$ value: 1,450 ± 128 mM; $p < 0.0001$; Fig. 2B,C).
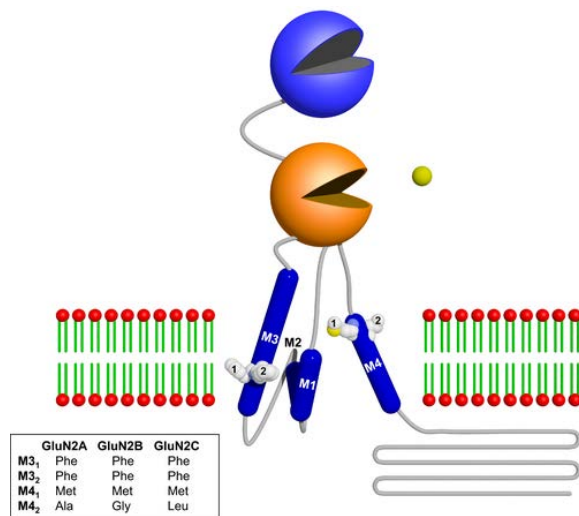

Figure 1 Topology of the GluN2C subunit showing the side chains corresponding to alcohol-sensitive positions in the GluN2A subunit. The diagram shows the extracellular N-terminal (**blue**) and ligand-binding (**orange**) domains, membrane-associated domains M1-M4 (**dark blue**), and the intracellular C-terminal domain (**gray**). Side chains corresponding to the 4 alcohol-sensitive positions, M3$_{1,2}$ and M4$_{1,2}$, in the GluN2A M3 and M4 domains are shown. Dimensions and orientation of the M domains and side chains are from Karakas and Furukawa (2014) for the GluN2B subunit. *Inset*, residues at the alcohol-sensitive positions are highly conserved among the GluN2A-C subunits.
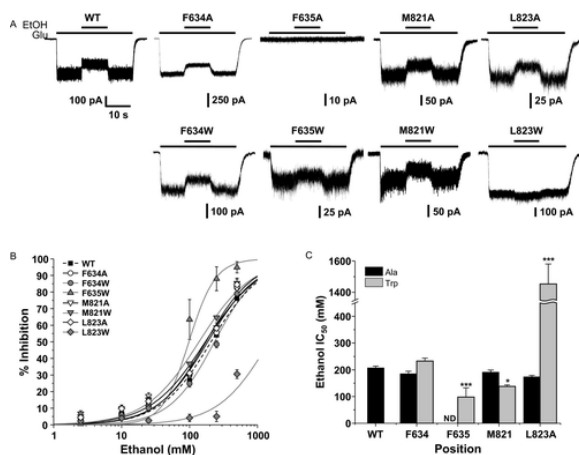

Figure 2 Alanine and tryptophan substitution mutations at the M3$_{1-2}$ and M4$_{1-2}$ positions in the GluN2C subunit can alter ethanol (EtOH) sensitivity. (**A**) Traces are currents activated by glutamate (Glu), 10 μM, in the presence of glycine, 50 μM, and their inhibition by EtOH, 100 mM, in cells expressing the wild-type (WT) or mutant subunits as indicated. (**B**) Concentration–response curves for EtOH inhibition of currents evoked by Glu, 10 μM, in the presence of glycine, 50 μM, in cells expressing the WT and mutant subunits as indicated. Curves shown are the best fits to the equation given in the Materials and Methods. Data points are means of 6 to 17 cells; error bars indicate SE values. (**C**) Bar graphs show average IC$_{50}$ values for EtOH inhibition of Glu-activated current in the presence of 50 μM glycine in cells expressing WT or mutant GluN2C subunits. EtOH inhibition of the GluN2C(F635A) mutant subunit could not be determined (ND) because there was no detectable glutamate-activated current in cells expressing this subunit. IC$_{50}$ values that are significantly different from the value for the WT receptor are indicated by asterisks (*$p < 0.05$, ***$p < 0.001$; ANOVA and Dunnett's test). Results are means ± SE of 6 to 17 cells.

Effects of Mutations at GluN2C(M821) on Alcohol Sensitivity

Previous results from this laboratory have shown that tryptophan substitution at the position cognate to GluN2C(M821) in the GluN2A and GluN2B subunits increases desensitization in both subunits and decreases EtOH sensitivity in GluN2A (Honse et al., **2004**; Ren et al., **2003a**,**b**) but has no effect on EtOH sensitivity in GluN2B (Zhao et al., **2015**). To determine the role of the characteristics of the substituent at this position on EtOH sensitivity in the GluN2C subunit, we made additional substitutions at this position. Substitution at Met821 with alanine, cysteine, isoleucine, leucine, serine, threonine, or tryptophan yielded functional mutants (Fig. **3**), while substitution with asparagine, aspartate, arginine, glycine, phenylalanine, tyrosine, or valine produced mutants that did not exhibit glutamate-activated currents (results not shown). All of the functional mutants tested were inhibited by EtOH in a concentration-dependent manner. EtOH IC_{50} values varied significantly among the mutants, ranging from 140 to 250 mM (ANOVA, $p < 0.0001$; Fig. **3**). Among the mutants at position 821, EtOH sensitivity was increased by substitution of leucine, serine, or tryptophan, and decreased by substitution of cysteine.
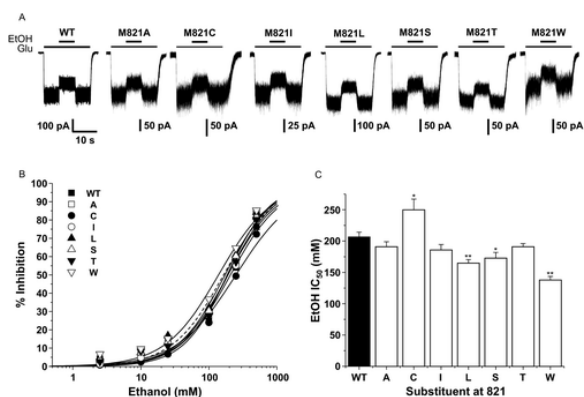

Figure 3 Substitution mutations at the $M4_1$ position (M821) in the GluN2C subunit can alter ethanol (EtOH) sensitivity. (**A**) Traces are currents activated by glutamate (Glu), 10 μM, in the presence of glycine, 50 μM, and their inhibition by EtOH, 100 mM, in cells expressing the wild-type (WT) or mutant subunits as indicated. (**B**) Concentration–response curves for EtOH inhibition of currents evoked by glutamate, 10 μM, in the presence of glycine, 50 μM, in cells expressing the WT and mutant subunits as indicated. Curves shown are the best fits to the equation given in the Materials and Methods. Data points are means of 6 to 10 cells; error bars are omitted to improve clarity. (**C**) Bar graphs show average IC_{50} values for EtOH inhibition of glutamate-activated current in the presence of 50 μM glycine in cells expressing WT or mutant GluN2C subunits. IC_{50} values that are significantly different from the value for the WT receptor are indicated by asterisks (*$p < 0.05$, **$p < 0.01$; ANOVA and Dunnett's test). Results are means ± SE of 6 to 10 cells.

Effects of Mutations at GluN2C(M821) on Glutamate Potency and Desensitization

At GluN2A(M823), the cognate site of GluN2C(M821), mutations not only affected alcohol sensitivity of the receptors, but also altered measures of receptor gating, such as glutamate potency and desensitization. To test whether mutations at GluN2C(M821) had similar effects on glutamate potency and desensitization, we performed concentration–response experiments for glutamate in the functional mutants using a rapid solution exchange apparatus in lifted cells (Fig. **4**). Of the 7 functional mutations at M821, EC_{50} values for glutamate-activated peak current were altered in 5 ($p < 0.001$; ANOVA), EC_{50} values for glutamate-activated steady-state current were altered in 2 ($p < 0.001$; ANOVA), and the steady-state-to-peak current ratio ($I_{ss}:I_p$) was altered in 5 ($p < 0.0001$; ANOVA; Fig. **5**A). As is evident from the discrepancy between the numbers of mutants in which steady-state current EC_{50} and $I_{ss}:I_p$ values were altered, apparent desensitization was affected even when steady-state EC_{50} values were unchanged, and correlation analysis revealed that these measures were not significantly correlated ($R^2 = 0.0918$, $p > 0.05$; Fig. **5**B).
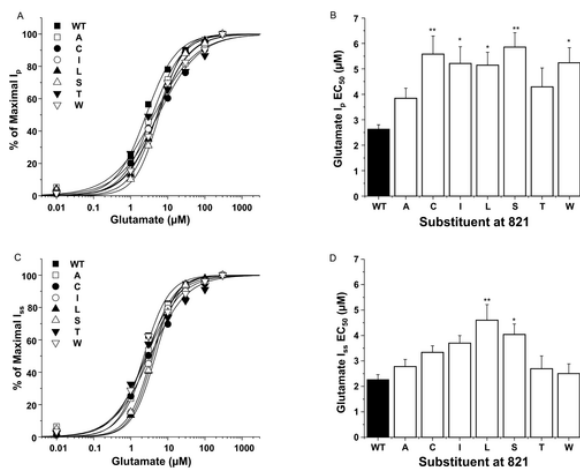
Figure 4 Substitution mutations at the M4₁ position (M821) in the GluN2C subunit can alter glutamate potency. (**A**, **C**) Concentration–response curves for activation of peak (**A**) and steady-state (**C**) currents evoked by various concentrations of glutamate in the presence of glycine, 50 μM, in cells expressing the wild-type (WT) and mutant subunits as indicated. Curves shown are the best fits to the equation given in the Materials and Methods. Data points are means of 6 to 7 cells; error bars are omitted to improve clarity. (**B**, **D**) Bar graphs show average EC_{50} values for glutamate activation of peak (**B**) and steady-state (**D**) current in the presence of 50 μM glycine in cells expressing WT or mutant GluN2C subunits. EC_{50} values that are significantly different from the value for the WT receptor are indicated by asterisks (*$p < 0.05$, **$p < 0.01$; ANOVA and Dunnett's test). Results are means ± SE of 6 to 7 cells.
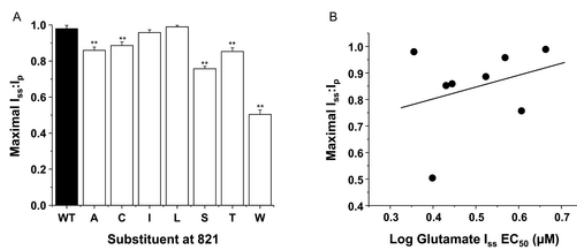

Figure 5 Substitution mutations at the M4₁ position (M821) in the GluN2C subunit can alter desensitization. (**A**) Bar graph shows average values of steady-state-to-peak current ratio (I_{ss}:I_p) for currents activated by glutamate, 300 μM, in the presence of glycine, 50 μM, in cells expressing the wild-type (WT) and mutant subunits as indicated. Values that are significantly different from the value for the WT receptor are indicated by asterisks (**$p < 0.01$; ANOVA and Dunnett's test). Results are means ± SE of 6 to 7 cells. (**B**) Graph plots maximal steady-state-to-peak current ratio (I_{ss}:I_p) against the EC_{50} for glutamate activation of steady-state current (I_{ss}). Maximal I_{ss}:I_p and glutamate I_{ss} EC_{50} values were not significantly correlated ($R^2 = 0.0918$, $p > 0.05$; ANOVA).

Relation of EtOH Sensitivity to the Physical and Chemical Properties of the Substituent at GluN2C(M821)

To evaluate the relative contribution of the physicochemical parameters of the amino acid at GluN2C(M821) to alcohol sensitivity, linear regression analyses of EtOH IC_{50} values versus Log P (the logarithm of the octanol:water partition coefficient), hydrophilicity, molecular volume, and polarity of the substituent were performed. No significant linear relations were observed between log EtOH IC_{50} values and Log P ($R^2 = 0.0917$; $p > 0.05$), hydrophilicity ($R^2 = 0.276$; $p > 0.05$), molecular volume ($R^2 = 0.249$; $p > 0.05$), or polarity ($R^2 = 0.0810$; $p > 0.05$) (Fig. 6).
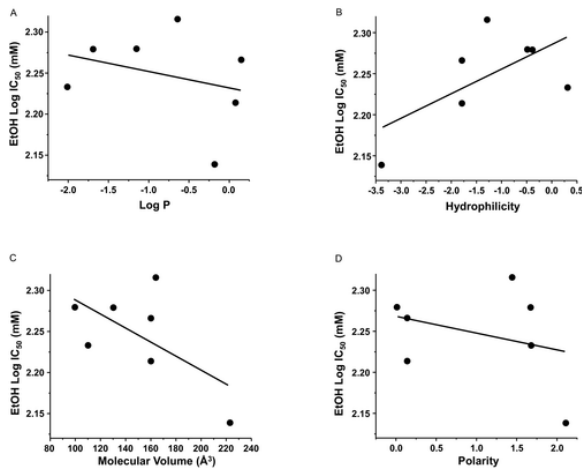
Figure 6 Ethanol (EtOH) sensitivity of GluN2C M4₁ mutant subunits is not related to the physicochemical parameters of the substituent. The graphs plot log EtOH IC_{50} values versus Log P (**A**), hydrophilicity (**B**), molecular volume (**C**), and polarity (**D**) for various GluN2C(M821) mutant subunits. No significant linear relations were obtained among any of the measures tested ($p > 0.05$).

Relation of EtOH Sensitivity to Glutamate Potency and Desensitization Among Mutants at GluN2C(M821)

It is possible that the observed variation in EtOH sensitivity among mutants at GluN2C(M821) could be attributable to changes in receptor kinetics. To test this possibility, we asked whether EtOH IC_{50} values among the mutants were correlated with glutamate potency or desensitization. Although there was significant variation in each measure of receptor kinetics among the mutants, EtOH IC_{50} values were not correlated with glutamate peak EC_{50} ($R^2 = 0.0614$, $p > 0.05$), steady-state EC_{50} ($R^2 = 0.00462$, $p > 0.05$), or maximal $I_{ss}:I_p$ ($R^2 = 0.376$, $p > 0.05$; Fig. **7**).
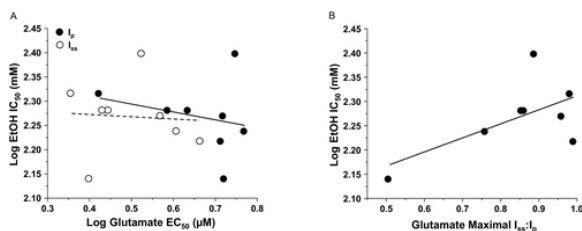

Figure 7 Ethanol (EtOH) sensitivity of GluN2C M4₁ mutant subunits is not related to glutamate potency or desensitization. The graphs plot log EtOH IC_{50} values versus the EC_{50} for glutamate activation of peak (I_p) or steady-state (I_{ss}) current (**A**) or maximal steady-state-to-peak current ratio ($I_{ss}:I_p$) (**B**) for various GluN2C(M821) mutant subunits. No significant linear relations were obtained among any of the measures tested ($p > 0.05$).

Comparison of EtOH-Sensitive Positions in the GluN2A, GluN2B, and GluN2C Subunits

Several previous studies from this laboratory have used scanning mutagenesis to identify EtOH-sensitive positions in the M3 and M4 domains of the GluN2A and GluN2B subunits (Honse et al., **2004**; Ren et al., **2003b**, **2007**, **2013**; Zhao et al., **2015**). Comparison of the EtOH sensitivity of tryptophan substitution mutants at these positions among the GluN2A-C subunits revealed a number of striking differences as well as similarities (Fig. **8**). At the M3₁position, tryptophan substitution decreased EtOH sensitivity in both the GluN2A and GluN2B subunits, but had no effect in the GluN2C subunit (Fig. **8**A). At the M3₂ and M4₁ positions, EtOH sensitivity was decreased by tryptophan substitution in GluN2A, unchanged in GluN2B, and increased in GluN2C. At the M4₂ position, tryptophan substitution decreased EtOH sensitivity in all 3 GluN2 subunits.
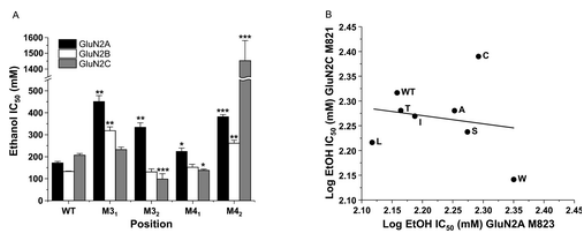
Figure 8 Ethanol (EtOH)-sensitive positions differ among GluN2A-C subunits. (**A**) Bar graph plots EtOH IC_{50} values for wild-type (WT) and tryptophan substitution mutant GluN2A-C subunits. EC_{50} values that are significantly different from the value for the corresponding WT receptor are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ANOVA and Dunnett's test). Data for GluN2A and GluN2B subunits are from Honse and colleagues ([2004](#)), Ren and colleagues ([2003b](#), [2007](#), [2013](#)), Zhao and colleagues ([2015](#)). (**B**) Graph plots log EtOH IC_{50} values for various substitution mutants at the $M4_1$ position in GluN2C versus those for various substitution mutants at the $M4_1$ position in GluN2A. EtOH sensitivity among GluN2A and GluN2C $M4_1$ mutants was not significantly linearly related ($p > 0.05$). Data for GluN2A subunits are from Ren and colleagues ([2003b](#)).

If EtOH sensitivity at the corresponding position in 2 subunits is dependent upon similar factors, the effect of a series of substitution mutants at this position should be correlated. For a series of substitution mutants at the $M4_1$ position in the GluN2A and GluN2C subunits, however, EtOH sensitivity was not correlated (Fig. [8](#)*B*).

Discussion

Previous work from this laboratory has demonstrated the existence of 4 alcohol-sensitive positions in the M3 and M4 domains of the NMDAR GluN2A subunit (Honse et al., [**2004**](#); Ren et al., [**2003b**](#), [**2007**](#), [**2013**](#)); 2 cognate positions regulate alcohol sensitivity in the GluN2B subunit (Zhao et al., [**2015**](#)). The majority of these positions also regulate ion channel gating in GluN2A and GluN2B (Ren et al., [**2003a**](#), [**2007**](#), [**2008**](#), [**2013**](#); Zhao et al., [**2016**](#)). Similar positions have been demonstrated in the GluN1 subunit (Ren et al., [**2012**](#); Ronald et al., [**2001**](#); Smothers and Woodward, [**2006**](#); Xu et al., [**2015**](#)). A recent study from the Woodward laboratory has shown that the $M4_2$ position in the GluN2C subunit strongly regulates EtOH sensitivity (Smothers and Woodward, [**2016**](#)). We confirm and extend this finding and additionally show that the side chains at 2 of the remaining positions in the GluN2C subunit influence alcohol sensitivity and at least 1 of the positions regulates ion channel gating.

In previous studies, the 4 positions that regulate alcohol sensitivity in the GluN2A subunit (Honse et al., [**2004**](#); Ren et al., [**2003b**](#), [**2007**](#), [**2012**](#), [**2013**](#); Smothers and Woodward, [**2006**](#)) do not all modulate alcohol sensitivity in the GluN1 (Ren et al., [**2012**](#); Ronald et al., [**2001**](#); Smothers and Woodward, [**2006**](#)) and GluN2B (Zhao et al., [**2015**](#)) subunits. In the GluN1 subunit, the $M3_1$ and $M3_2$ positions strongly regulate alcohol sensitivity (Ren et al., [**2012**](#); Ronald et al., [**2001**](#); Smothers and Woodward, [**2006**](#)), whereas the M4 positions had much lesser effects (Smothers and Woodward, [**2006**](#)) or no effect (Ren et al., [**2012**](#)) on alcohol inhibition. In the GluN2B subunit, alcohol sensitivity was regulated only by the $M3_1$ and $M4_2$ positions (Zhao et al., [**2015**](#)). In addition, at alcohol-sensitive positions, alanine or tryptophan substitutions decreased alcohol sensitivity in most (Honse et al., [**2004**](#); Ren et al., [**2003b**](#), [**2007**](#), [**2012**](#); Ronald et al., [**2001**](#); Smothers and Woodward, [**2006**](#); Zhao et al., [**2015**](#)), but not all (Ren et al., [**2003b**](#), 2008; Ronald et al., [**2001**](#); Zhao et al., [**2015**](#)), instances. In the present study, GluN2C subunit alcohol sensitivity was not measurable in the alanine substitution mutant at F635 ($M3_2$) because it was not functional, but was unchanged by alanine substitution at the remaining 3 positions. Furthermore, tryptophan substitution had no effect at $M3_1$, increased alcohol sensitivity at $M3_2$ and $M4_1$, and markedly decreased alcohol sensitivity at $M4_2$. The over 6-fold decrease in alcohol IC_{50} for the GluN2C(L823W) subunit is the most pronounced change in alcohol sensitivity for a single-site mutant reported to date. This finding was consistent with the recent report of Smothers and Woodward ([**2016**](#)), who observed little to no inhibition of this mutant subunit by 100 mM EtOH. The explanation for the differential modulation of alcohol sensitivity by the M3-M4 residues among the different subunit types, despite the high homology in these domains (Fig. [**1**](#)), is unclear at

present, but may result from differences in the adjacent residues interacting with these side chains among the subunit types, perhaps involving subtle differences in structure (Zhao et al., **2015**). These structural differences may also contribute to the observed differences in EtOH sensitivity among the WT GluN2 subunits, such as the lower sensitivity of the GluN2C subunit compared to GluN2A or GluN2B (Masood et al., **1994**; Mirshahi and Woodward, **1995**). In the present study, substitution of alanine for leucine at $M4_2$, which resulted in a GluN2C subunit with the same residues at the alcohol-sensitive positions as the GluN2A subunit, appeared to slightly increase alcohol sensitivity, but the change was not significant. Additional differences among the GluN2A and GluN2C subunits at other, interacting positions may also be required to account for the differences in EtOH sensitivity.

A striking difference among the GluN2A-C subunits was observed for mutations at the highly conserved methionine at $M4_1$ (821 in GluN2C). For tryptophan substitution mutants at this position, alcohol sensitivity was decreased in GluN2A (Ren et al., **2003b**), unchanged in GluN2B (Zhao et al., **2015**), and increased in GluN2C. Interestingly, this disparity occurred despite similar changes in ion channel gating, such as increased desensitization, among the GluN2 subunit mutants (Ren et al., **2003a**; Zhao et al., **2015**). In contrast, alanine mutation at this position did not change EtOH sensitivity in any of the GluN2 subunits tested (Ren et al., **2003b**; Zhao et al., **2015**). These results suggest that any interactions formed by the native methionine side chain that regulate alcohol sensitivity are preserved in the alanine mutants. This does not necessarily extend to interactions regulating ion channel gating, however, as alanine mutation at the $M4_1$ position in the GluN2A and GluN2C subunits altered ion channel gating (Ren et al., **2003a**). The $M4_1$ position in the GluN2C subunit appeared to have the most stringent requirements for receptor function, as a greater number of amino acid substitutions at this position yielded nonfunctional receptors compared to the GluN2A and GluN2B subunits (Ren et al., **2003b**; Zhao et al., **2015**). An additional distinction among the GluN2 subunit types regarding gating was observed in the relation between glutamate potency and desensitization. In the GluN2A subunit, mutations at the $M4_1$ position can increase potency of glutamate for activation of steady-state current via agonist trapping at the binding site by increasing desensitization (Ren et al., **2003a**). Although mutations at $M4_1$ in the GluN2B and GluN2C subunits could increase desensitization, no relation was observed between steady-state current glutamate potency and I_{ss}:I_p values in either subunit (Zhao et al., **2015**).

The differences in the identity and characteristics of alcohol-sensitive amino acid positions among the GluN2 subunits may reflect differences in the interaction of alcohol with the putative-binding cavities bounded by these positions, as well as in the mechanism of alcohol modulation of ion channel function. In the present study, EtOH IC_{50} for mutants at the $M4_1$ position in GluN2C was not significantly related to any physicochemical measure of the substituent side chain. Correlations between EtOH sensitivity and measures such as molecular volume have been previously observed in other alcohol-sensitive ion channels including $GABA_A$ and glycine receptors (Kash et al., **2003**; Mihic et al., **1997**; Wick et al., **1998**; Yamakura et al., **1999**), as well as in the NMDAR GluN1 subunit at the $M3_2$ position (Smothers and Woodward, **2006**) and GluN2A subunit at the $M3_2$ and $M4_1$ positions (Ren et al., **2003b**, **2007**), and have been taken as evidence for alcohol binding in the vicinity of the side chain. The lack of such a relation in the present study could be interpreted as an indication that the EtOH molecule does not directly interact with the cavity formed by this position, but other interpretations are also plausible. For example, it is possible that EtOH interacts with the side chains at these positions in a manner that is more specific than simple volume occupation of a cavity and that would not be accurately represented by any of the physical–chemical scales used. The binding cavity may thus be sufficiently large to accommodate any of the hydrophobic amino acid side chains and an alcohol molecule without altering its conformation. The observation that the isomeric amino acids isoleucine and leucine, which have the same molecular volume and hydrophobicity but different structures, produce distinctly different effects on EtOH sensitivity is consistent with this interpretation, although the observation that these substitutions also differentially affected receptor kinetics raises the possibility that the changes in EtOH sensitivity are secondary to changes in receptor kinetics.

However, EtOH sensitivity among the mutants at GluN2C(M821) was not dependent upon the measures of receptor kinetics tested: EtOH IC_{50} values were not related to values of glutamate potency for activation of either peak or steady-state current or to a measure of desensitization, steady-state-to-peak current (I_{ss}:I_p) ratio. Additional experiments will be required to distinguish among these and other possible explanations.

Acknowledgments

Conflict of Interest

The authors have no conflicts of interest to disclose.

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