HHS Public Access

Author manuscript

Alcohol Clin Exp Res. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:

Alcohol Clin Exp Res. 2019 June ; 43(6): 1180–1190. doi:10.1111/acer.14042.

Positions in the NMDA Receptor GluN2C Subunit M3 and M4 Domains Regulate Alcohol Sensitivity and Receptor Kinetics

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Abstract

Background: Alcohol alters synaptic transmission in the brain. The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate-gated ion channel, is an important synaptic target of alcohol in the brain. We and others have previously identified four 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 NMDA receptor 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 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 ethanol sensitivity, whereas tryptophan substitution at the $M4_1$ position increased, and at the $M4_2$ position decreased, ethanol sensitivity. The increased ethanol 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 ethanol sensitivity, glutamate potency, and desensitization. Seven amino acid substitutions (of 15 tested) at this position yielded nonfunctional receptors. Among the remaining mutants at $M4_1$, ethanol 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.

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Keywords

NMDA receptor; alcohol sensitivity; ion channel gating; desensitization

Introduction

Ethanol is a widely-abused drug that acts on multiple pre- and postsynaptic targets in the brain to alter synaptic transmission (McCool, 2011; Abrahao *et al.*, 2017; Harrison *et al.*, 2017). Among the most important targets of ethanol 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 CNS is a heterotetramer containing two GluN1 subunits and two GluN2 subunits, of which there are four types, GluN2A-D (Kutsuwada *et al.*, 1992; Honer *et al.*, 1998; Laube *et al.*, 1998). NMDARs are inhibited by ethanol at relevant concentrations and play a crucial role in the effects of ethanol in the brain (Woodward, 2000; Krystal *et al.*, 2003; Vengeliene *et al.*, 2008). Although multiple molecular mechanisms can modulate NMDAR ethanol sensitivity (Ron, 2004), the molecular mechanism by which ethanol 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 (Ronald *et al.*, 2001; Ren *et al.*, 2003b; Honse *et al.*, 2004; Smothers and Woodward, 2006; Ren *et al.*, 2007). In the GluN1/GluN2A and GluN1/GluN2B NMDAR, these putative sites of ethanol 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; Zhao *et al.*, 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; Ren *et al.*, 2007; Ren *et al.*, 2008; Ren *et al.*, 2012; Ren *et al.*, 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 ethanol sensitivity. For example, in the GluN2A subunit M3 domain, although substitution of tryptophan at either of the positions significantly decreases ethanol 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; Monyer *et al.*, 1994; Wenzel *et al.*, 1997; Karavanova *et al.*, 2007), and lesser amounts in the thalamus, olfactory bulb, globus pallidus, and hippocampus (Monyer *et al.*, 1994; Wenzel *et al.*, 1997; Ravikrishnan *et al.*, 2018). 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 (Ravikrishnan *et al.*, 2018; Alsaad *et al.*, 2019; Verkhratsky and Chvatal, 2019). Compared to NMDA receptors containing GluN2A and

GluN2B subunits, GluN2C-containing NMDA receptors have a shorter mean open time and much lower open probability (Dravid *et al.*, 2008), lower single-channel conductance (Stern *et al.*, 1992; Dravid *et al.*, 2008), and lower sensitivity to Mg^{2+} block (Monyer *et al.*, 1992), with little to no desensitization and glutamate deactivation similar to that of GluN2B (Monyer *et al.*, 1992; Krupp *et al.*, 1996; Vicini *et al.*, 1998). The GluN2C-containing NMDA receptor also shows differences in alcohol sensitivity. GluN2C-containing NMDA receptors are less sensitive to ethanol compared to GluN2A- and GluN2B-containing NMDA receptors (Masood *et al.*, 1994; Chu *et al.*, 1995; Mirshahi and Woodward, 1995), but the basis for the lower ethanol 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

Ethanol (95%, prepared from grain) was obtained from Aaper Alcohol & Chemical Co. (Shelbyville, KY), and all other drugs were obtained from Sigma-Aldrich. 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 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 (GFP) using the calcium phosphate transfection kit (Invitrogen). Magnesium chloride ($MgCl_2$), 10 mM, was added to the culture medium to prevent excitotoxic cell death. $MgCl_2$ was removed before use in experiments by extensive washing. Cells were used in experiments 24 – 48 hr 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 – 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 ethanol were prepared fresh daily and applied to cells using a stepper motor-driven rapid solution exchange apparatus (Warner Instruments, Inc.) and 600 μm inner diameter square glass tubing. In concentration-response experiments, the order of application of the various concentrations of ethanol 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).

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 (Zimmerman *et al.*, 1968; Hopp and Woods, 1981).

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 one-way analysis of variance (ANOVA) followed by the Dunnett 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 NMDA receptor subunits (Figure 1) (Ren *et al.*, 2012; Xu *et al.*, 2015; Zhao *et al.*, 2015; Zhao *et al.*, 2016). To facilitate comparisons among the subunit types, we designate the two positions in the M3 domain corresponding to F636 and F637 in the GluN2A subunit as $M3_1$ and $M3_2$, respectively, and the two 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 three of the four positions did not exhibit any grossly apparent changes in characteristics such as desensitization (Figure 2A), but no current could be detected in response to maximal concentrations of glutamate in the GluN2C(F635A) mutant. Consequently, ethanol inhibition could not be determined in GluN2C(F635A) mutant subunits, but ethanol IC_{50} values were unchanged in the remaining alanine mutants

relative to the wild-type subunit (Figure 2B, C). In contrast, tryptophan mutation at F635, as at each of the remaining positions, yielded functional receptors (Figure 2A). Desensitization of glutamate-activated current appeared to be increased in the GluN2C(M821W) mutant relative to the wild-type subunit. Ethanol 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: 1450 ± 128 mM; $P < 0.0001$; Figure 2B, C).

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 ethanol sensitivity in GluN2A (Ren *et al.*, 2003a; Ren *et al.*, 2003b; Honse *et al.*, 2004) but has no effect on ethanol sensitivity in GluN2B (Zhao *et al.*, 2015). To determine the role of the characteristics of the substituent at this position on ethanol 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 (Figure 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 ethanol in a concentration-dependent manner. Ethanol IC_{50} values varied significantly among the mutants, ranging from 140 to 250 mM (ANOVA, $p < 0.0001$; Figure 3). Among the mutants at position 821, ethanol sensitivity was increased by substitution of leucine, serine, or tryptophan, and decreased by substitution of cysteine.

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 (Figure 4). Of the seven functional mutations at M821, EC_{50} values for glutamate-activated peak current were altered in five ($P < 0.001$; ANOVA), EC_{50} values for glutamate-activated steady-state current were altered in two ($P < 0.001$; ANOVA), and the steady-state to peak current ratio (I_{ss}:I_p) was altered in five ($P < 0.0001$; ANOVA; Figure 5A). 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 ($R^2 = 0.0918$, $P > 0.05$; Figure 5B).

Relation of Ethanol 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 ethanol 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 ethanol 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 ($R^2 = 0.0810$; $P > 0.05$) (Figure 6).

Relation of ethanol sensitivity to glutamate potency and desensitization among mutants at GluN2C(M821).

It is possible that the observed variation in ethanol sensitivity among mutants at GluN2C(M821) could be attributable to changes in receptor kinetics. To test this possibility, we asked whether ethanol 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, ethanol 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 ($R^2 = 0.376$, $P > 0.05$; Figure 7).

Comparison of ethanol-sensitive positions in the GluN2A, GluN2B, and GluN2C Subunits.

Several previous studies from this laboratory have used scanning mutagenesis to identify ethanol-sensitive positions in the M3 and M4 domains of the GluN2A and GluN2B subunits (Ren *et al.*, 2003b; Honse *et al.*, 2004; Ren *et al.*, 2007; Ren *et al.*, 2013; Zhao *et al.*, 2015). Comparison of the ethanol sensitivity of tryptophan substitution mutants at these positions among the GluN2A-C subunits revealed a number of striking differences as well as similarities (Figure 8). At the $M3_1$ position, tryptophan substitution decreased ethanol sensitivity in both the GluN2A and GluN2B subunits, but had no effect in the GluN2C subunit (Figure 8A). At the $M3_2$ and $M4_1$ positions, ethanol sensitivity was decreased by tryptophan substitution in GluN2A, unchanged in GluN2B, and increased in GluN2C. At the $M4_2$ position, tryptophan substitution decreased ethanol sensitivity in all three GluN2 subunits.

If ethanol sensitivity at the corresponding position in two 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, ethanol sensitivity was not correlated (Figure 8B).

Discussion

Previous work from this laboratory has demonstrated the existence of four alcohol-sensitive positions in the M3 and M4 domains of the NMDA receptor GluN2A subunit (Ren *et al.*, 2003b; Honse *et al.*, 2004; Ren *et al.*, 2007; Ren *et al.*, 2013); two 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; Ren *et al.*, 2007; Ren *et al.*, 2008; Ren *et al.*, 2013; Zhao *et al.*, 2016). Similar positions have been demonstrated in the GluN1 subunit (Ronald *et al.*, 2001; Smothers and Woodward, 2006; Ren *et al.*, 2012; Xu *et al.*, 2015). A recent study from the Woodward laboratory has shown that the $M4_2$ position in the GluN2C subunit strongly regulates ethanol sensitivity (Smothers and Woodward, 2016). We confirm and extend this finding, and additionally show that the

side chains at two of the remaining positions in the GluN2C subunit influence alcohol sensitivity, and at least one of the positions regulates ion channel gating.

In previous studies, the four positions that regulate alcohol sensitivity in the GluN2A subunit (Ren *et al.*, 2003b; Honse *et al.*, 2004; Smothers and Woodward, 2006; Ren *et al.*, 2007; Ren *et al.*, 2012; Ren *et al.*, 2013) do not all modulate alcohol sensitivity in the GluN1 (Ronald *et al.*, 2001; Smothers and Woodward, 2006; Ren *et al.*, 2012) and GluN2B (Zhao *et al.*, 2015) subunits. In the GluN1 subunit, the $M3_1$ and $M3_2$ positions strongly regulate alcohol sensitivity (Ronald *et al.*, 2001; Smothers and Woodward, 2006; Ren *et al.*, 2012) 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 (Ronald *et al.*, 2001; Ren *et al.*, 2003b; Honse *et al.*, 2004; Smothers and Woodward, 2006; Ren *et al.*, 2007; Ren *et al.*, 2012; Zhao *et al.*, 2015), but not all (Ronald *et al.*, 2001; Ren *et al.*, 2003b; Ren *et al.*; 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 three 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 six-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 ethanol. 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 (Figure 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 ethanol sensitivity among the wild-type 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 ethanol 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 ethanol 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 Iss:Ip 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, ethanol 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 ethanol sensitivity and measures such as molecular volume have been previously observed in other alcohol-sensitive ion channels including $GABA_A$ and glycine receptors (Mihic *et al.*, 1997; Wick *et al.*, 1998; Yamakura *et al.*, 1999; Kash *et al.*, 2003), as well as in the NMDA receptor 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; Ren *et al.*, 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 ethanol molecule does not directly interact with the cavity formed by this position, but other interpretations are also plausible. For example, it is possible that ethanol 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 ethanol sensitivity is consistent with this interpretation, although the observation that these substitutions also differentially affected receptor kinetics raises the possibility that the changes in ethanol sensitivity are secondary to changes in receptor kinetics. However, ethanol sensitivity among the mutants at GluN2C(M821) was not dependent upon the measures of receptor kinetics tested: ethanol 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:

This study was supported by grants RO1 AA015203–01A1 and AA015203–06A1 from the NIAAA, National Institutes of Health, and by a Way-Klingler Fellowship Award from Marquette University to R.W.P. The authors have no conflicts of interest to disclose.

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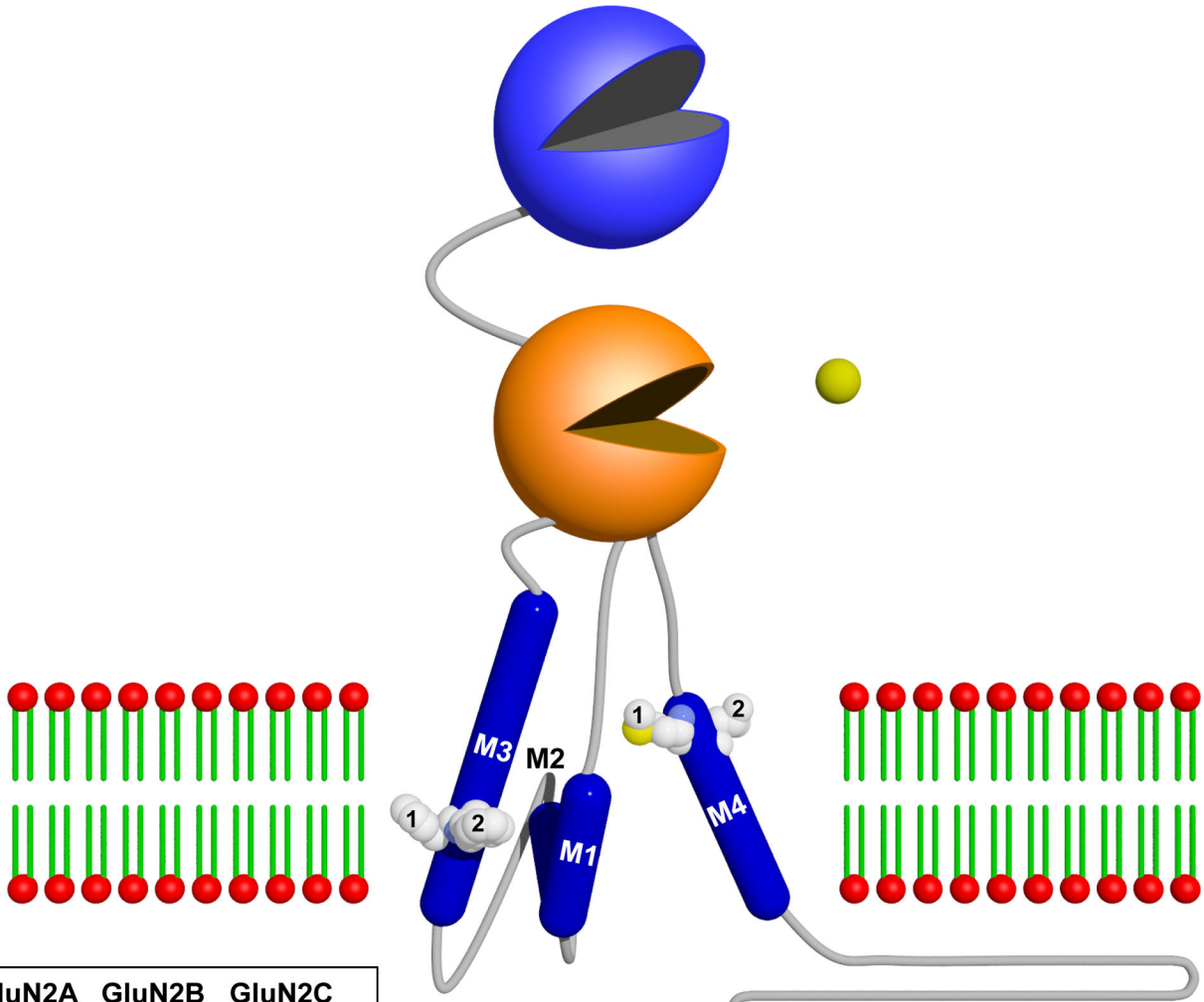

	GluN2A	GluN2B	GluN2C
$M3_1$	Phe	Phe	Phe
$M3_2$	Phe	Phe	Phe
$M4_1$	Met	Met	Met
$M4_2$	Ala	Gly	Leu

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 four 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) for the GluN2B subunit. *Inset*, Residues at the alcohol-sensitive positions are highly conserved among the GluN2A-C subunits.

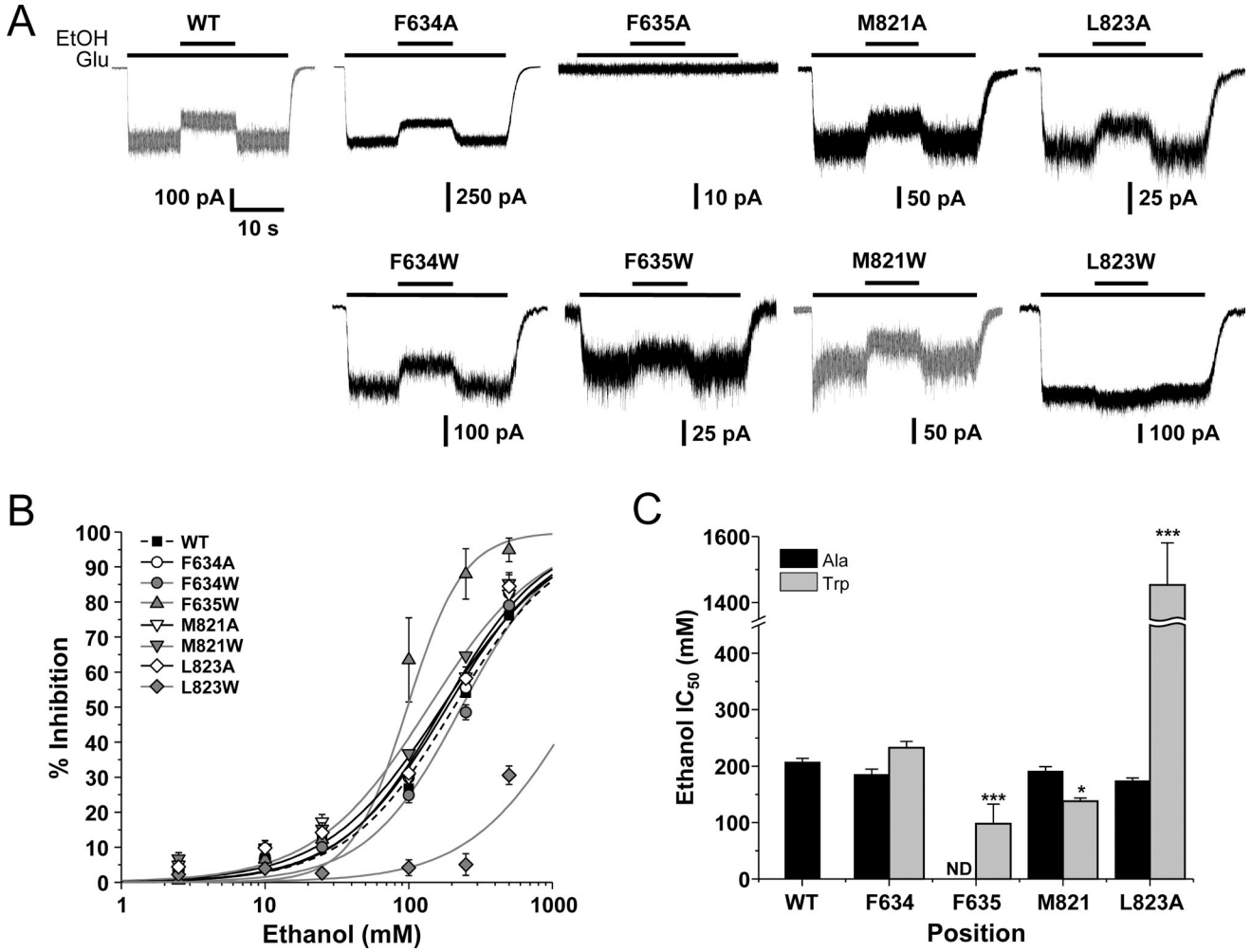

Figure 2. Alanine and tryptophan substitution mutations at the $M3_{1–2}$ and $M4_{1–2}$ positions in the GluN2C subunit can alter ethanol sensitivity.

A, Traces are currents activated by glutamate (Glu), 10 μM, in the presence of glycine, 50 μM, and their inhibition by ethanol (EtOH), 100 mM, in cells expressing the wild-type (WT) or mutant subunits as indicated. *B*, Concentration-response curves for ethanol inhibition of currents evoked by glutamate, 10 μM, in the presence of glycine, 50 μM, in cells expressing the wild-type 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 6–17 cells; error bars indicate S.E. values. *C*, Bar graphs show average IC_{50} values for ethanol inhibition of glutamate-activated current in the presence of 50 μM glycine in cells expressing wild-type or mutant GluN2C subunits. Ethanol 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 wild-type receptor are indicated by asterisks (*$P < 0.05$; ***$P < 0.001$; ANOVA and Dunnett's test). Results are means ± S.E of 6 – 17 cells.

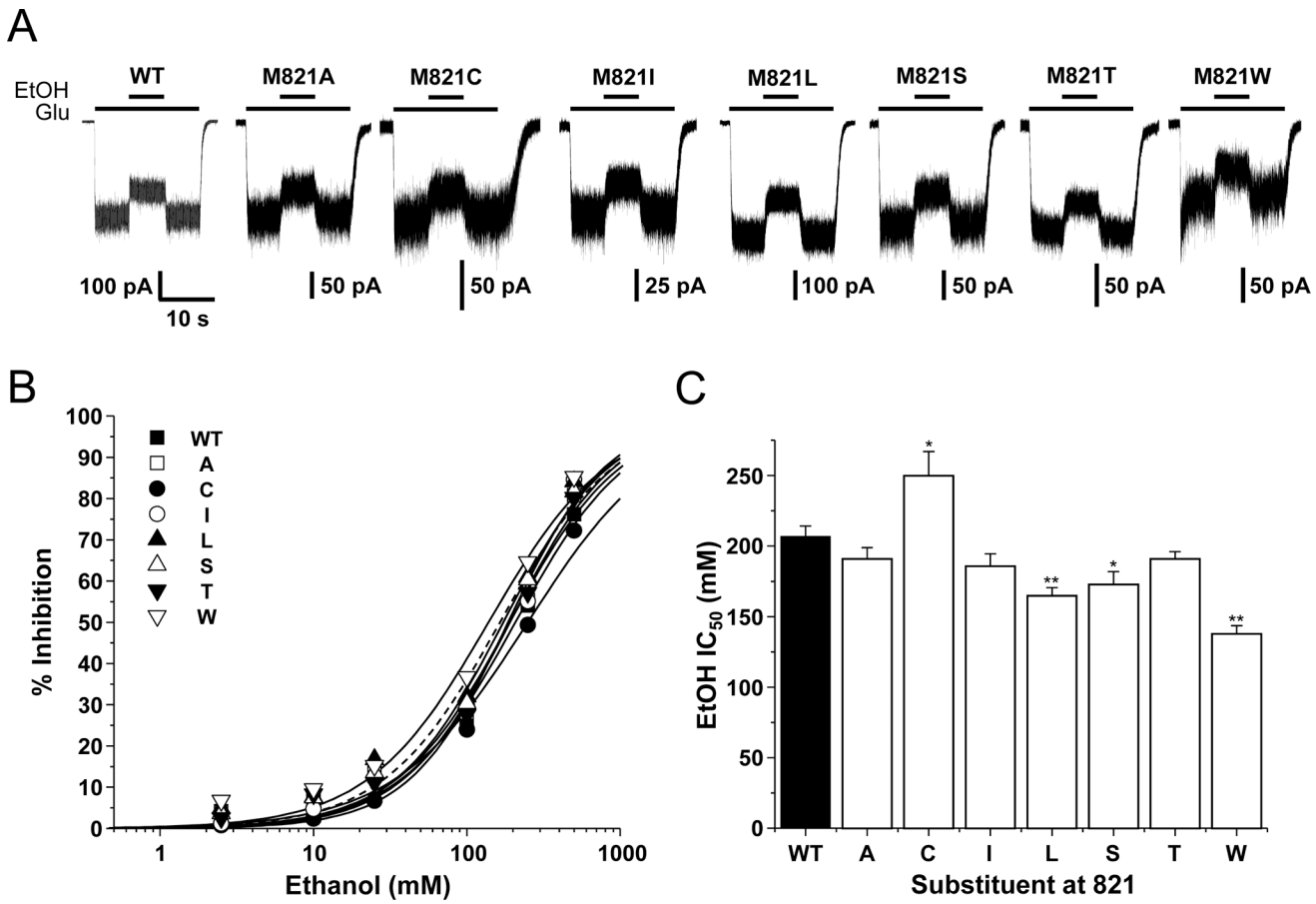

Figure 3. Substitution mutations at the $M4_1$ position (M821) in the GluN2C subunit can alter ethanol sensitivity.

A, Traces are currents activated by glutamate (Glu), 10 μM, in the presence of glycine, 50 μM, and their inhibition by ethanol (EtOH), 100 mM, in cells expressing the wild-type (WT) or mutant subunits as indicated. *B*, Concentration-response curves for ethanol inhibition of currents evoked by glutamate, 10 μM, in the presence of glycine, 50 μM, in cells expressing the wild-type 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 – 10 cells; error bars are omitted to improve clarity. *C*, Bar graphs show average IC_{50} values for ethanol inhibition of glutamate-activated current in the presence of 50 μM glycine in cells expressing wild-type or mutant GluN2C subunits. IC_{50} values that are significantly different from the value for the wild-type receptor are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ANOVA and Dunnett's test). Results are means ± S.E of 6 – 10 cells.

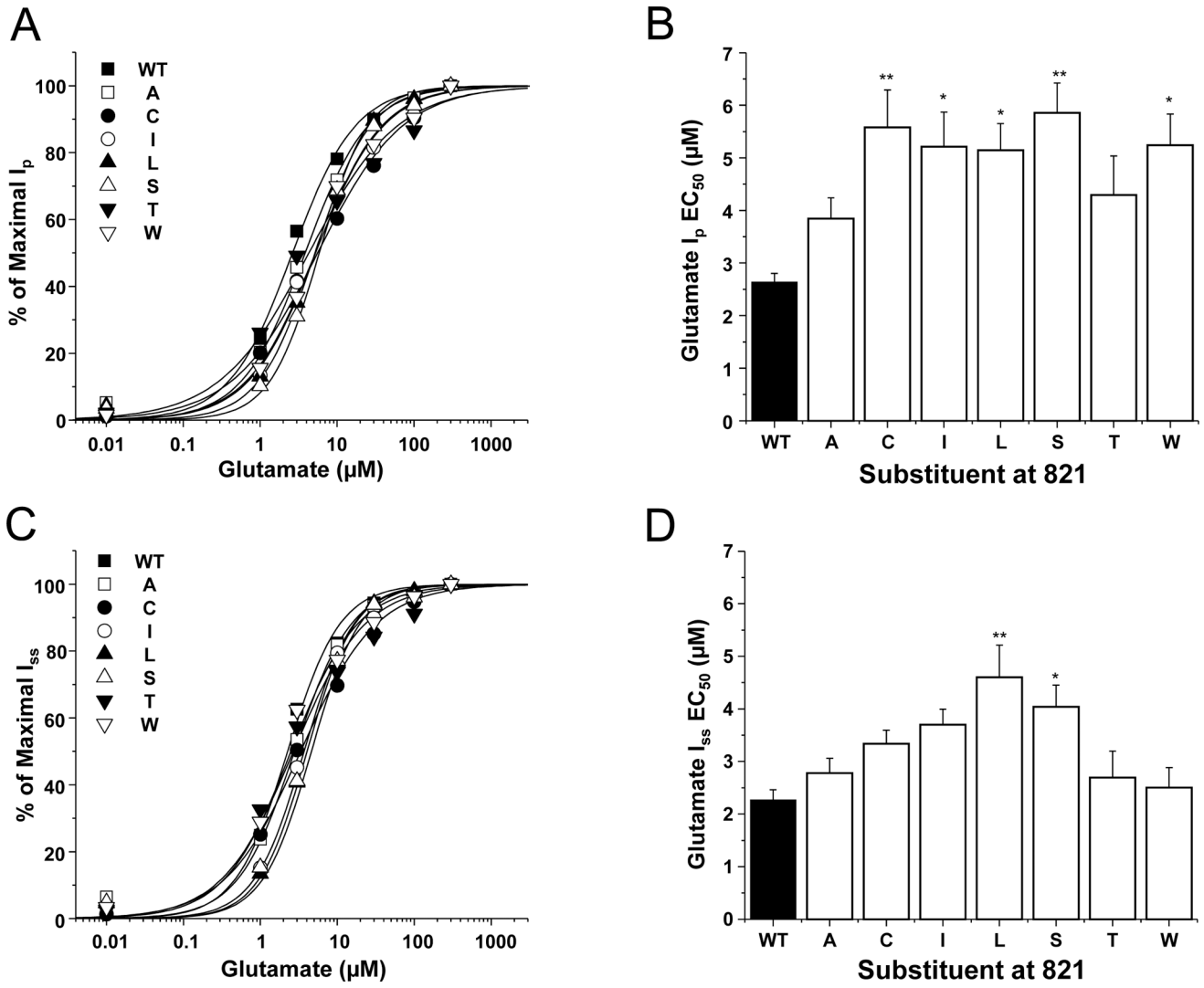

Figure 4. Substitution mutations at the $M4_1$ position (M821) in the GluN2C subunit can alter glutamate potency.

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 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 – 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 wild-type or mutant GluN2C subunits. EC_{50} values that are significantly different from the value for the wild-type receptor are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ANOVA and Dunnett's test). Results are means ± S.E of 6 – 7 cells.

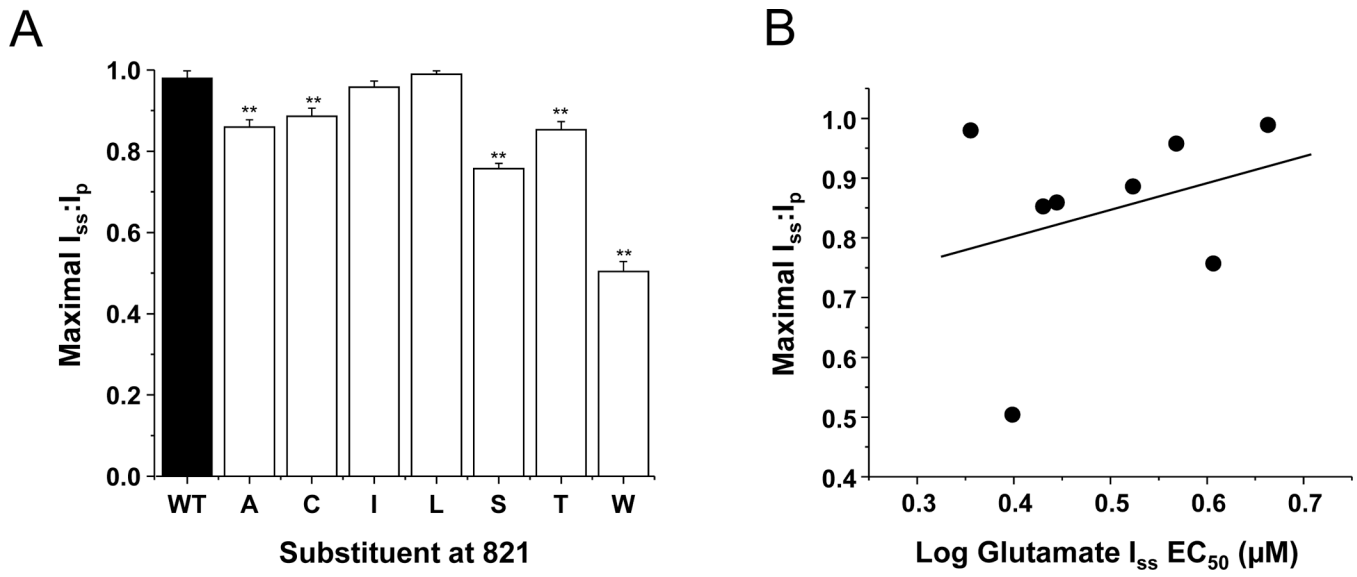

Figure 5. Substitution mutations at the $M4_1$ position (M821) in the GluN2C subunit can alter desensitization.

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 and mutant subunits as indicated. Values that are significantly different from the value for the wild-type receptor are indicated by asterisks (**$P < 0.01$; ANOVA and Dunnett's test). Results are means ± S.E. of 6 – 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).

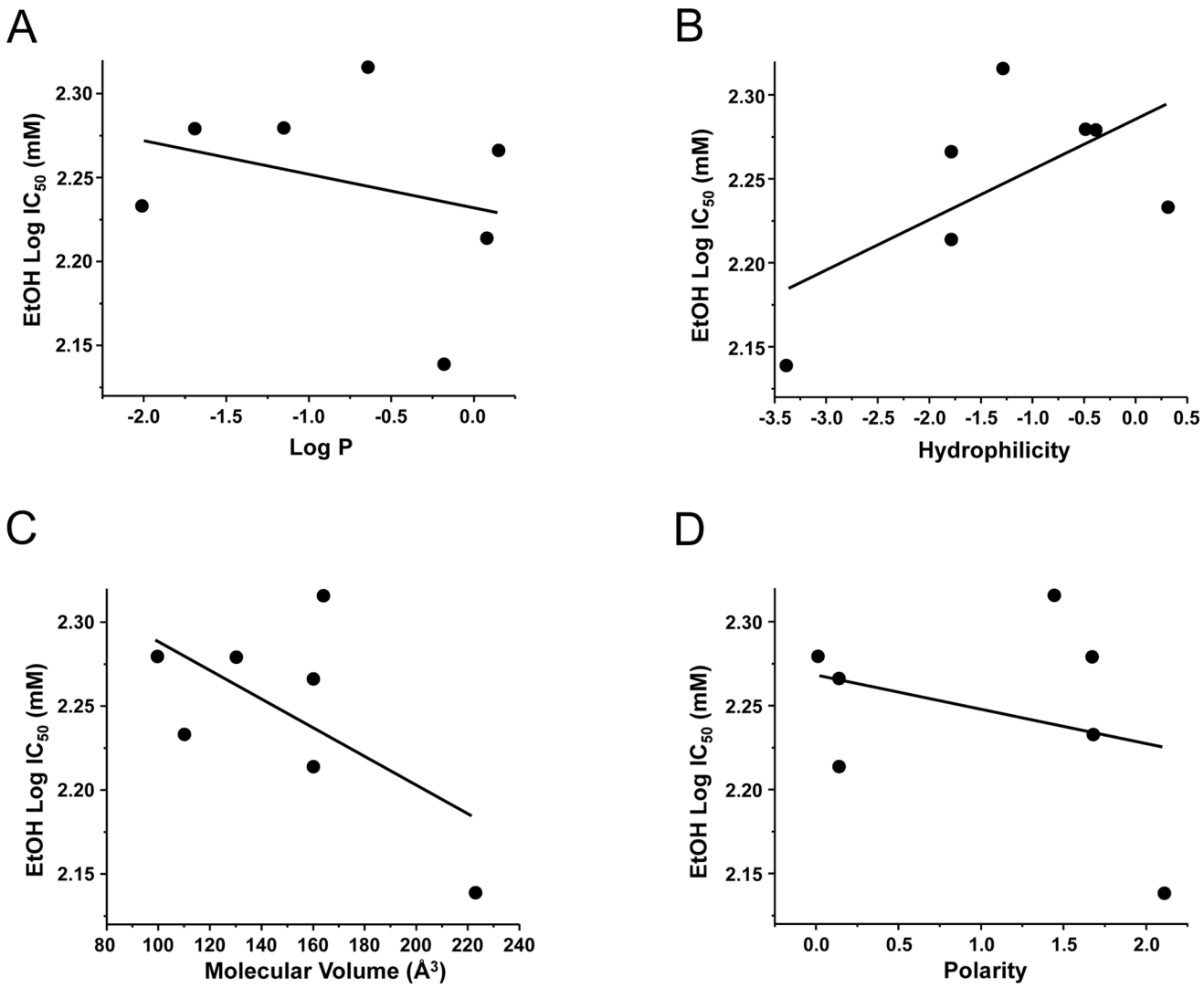

Figure 6. Ethanol sensitivity of GluN2C M4$_1$ mutant subunits is not related to the physicochemical parameters of the substituent.

The graphs plot log ethanol 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 ($P > 0.05$).

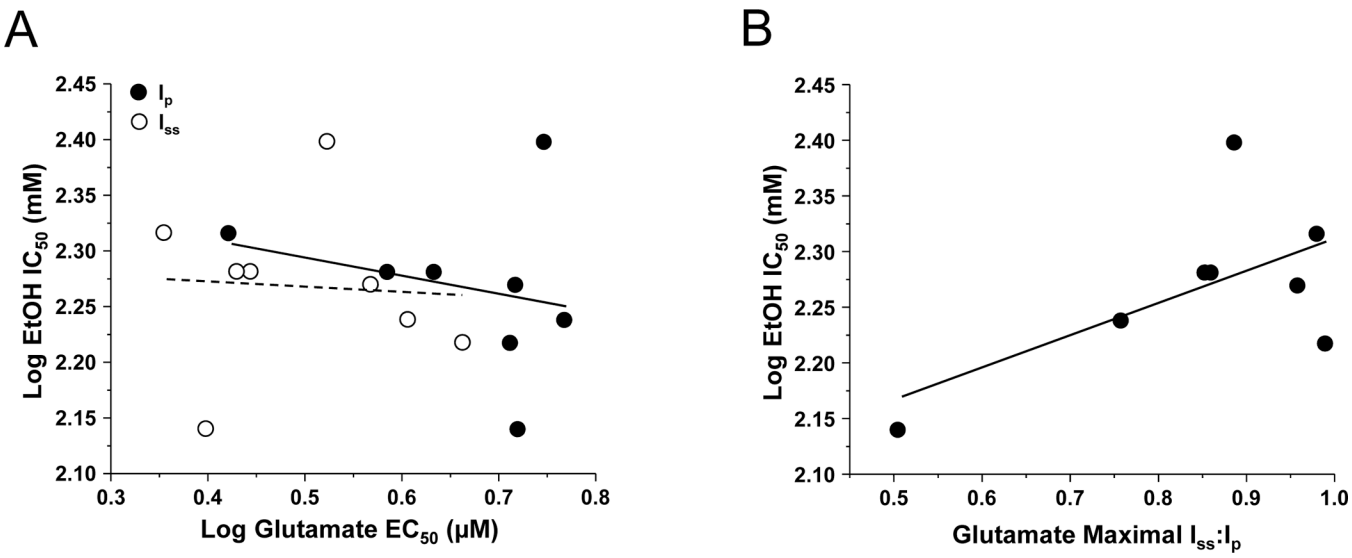

Figure 7. Ethanol sensitivity of GluN2C $M4_1$ mutant subunits is not related to glutamate potency or desensitization.

The graphs plot log ethanol 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 ($P > 0.05$).

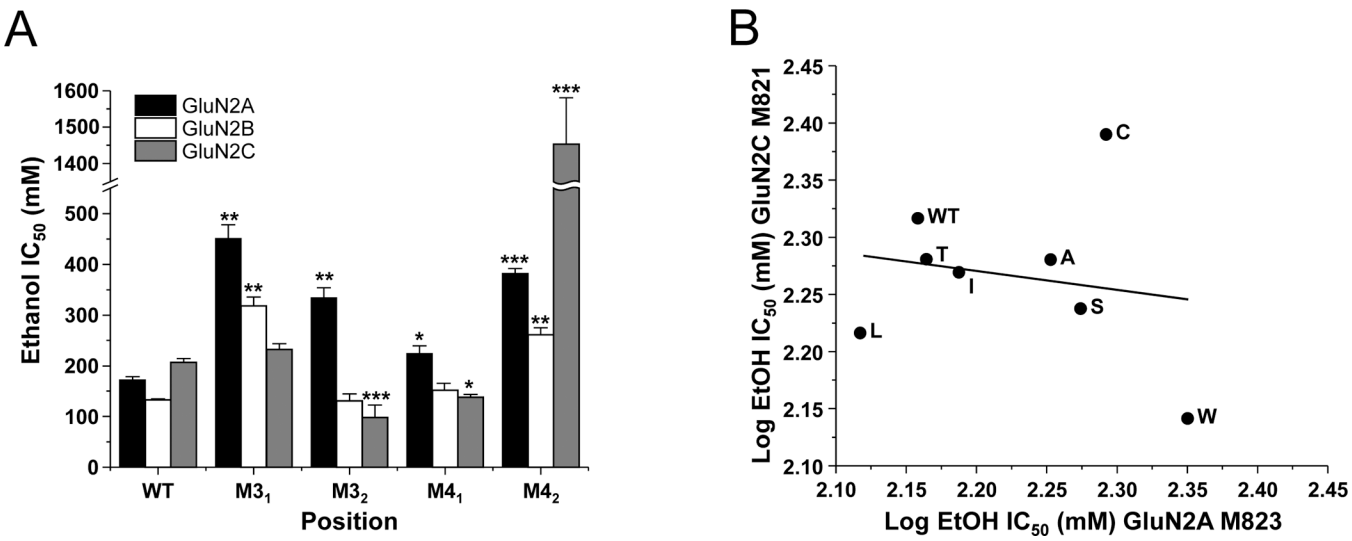

Figure 8. Ethanol sensitive positions differ among GluN2A-C subunits.

A, Bar graph plots ethanol 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 wild-type receptor are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ANOVA and Dunnett's test). Data for GluN2A and GluN2B subunits are from (Ren *et al.*, 2003b; Honse *et al.*, 2004; Ren *et al.*, 2007; Ren *et al.*, 2013; Zhao *et al.*, 2015). *B*, Graph plots log ethanol 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. Ethanol sensitivity among GluN2A and GluN2C $M4_1$ mutants was not significantly linearly related ($P > 0.05$). Data for GluN2A subunits are from (Ren *et al.*, 2003b).