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Different Sites of Alcohol Action in the NMDA Receptor GluN2A and GluN2B Subunits

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Abstract: The NMDA receptor is a major target of alcohol action in the CNS, and recent behavioral and cellular studies have pointed to the importance of the GluN2B subunit in alcohol action. We and others have previously characterized four amino acid positions in the third and fourth membrane-associated (M) domains of the NMDA receptor GluN2A subunit that influence both ion channel gating and alcohol sensitivity. In this study, we found that substitution mutations at two of the four corresponding positions in the GluN2B subunit, F637 and G826, influence ethanol sensitivity and ion channel gating. Because position 826 contains a glycine residue in the native protein, we focused our attention on GluN2B(F637). Substitution mutations at GluN2B(F637) significantly altered ethanol IC_{50} values, glutamate EC_{50} values for peak (I_p) and steady-state (I_{ss}) current, and steady-state to peak current ratios ($I_{ss}:I_p$). Changes in apparent glutamate affinity were not due to agonist trapping in desensitized states, as glutamate I_{ss} EC_{50} values were not correlated with $I_{ss}:I_p$ values. Ethanol sensitivity was correlated with values of both I_p and I_{ss} glutamate EC_{50} , but not with $I_{ss}:I_p$. Values of ethanol IC_{50} , glutamate EC_{50} , and $I_{ss}:I_p$ for mutants at GluN2B(F637) were highly correlated with the corresponding values for mutants at GluN2A(F636), consistent with similar functional roles of this position in both subunits. These results demonstrate that GluN2B(Phe637) regulates ethanol action and ion channel function of NMDA receptors. However, despite highly conserved M domain sequences, ethanol's actions on GluN2A and GluN2B subunits differ.

Keywords: Alcohol, Ethanol, Glutamate, NMDA receptors

1. Introduction

Alcohol addiction, characterized by uncontrolled consumption of alcoholic beverages despite the negative consequences, is thought to be associated with disorganized motor function, cognition, and aberrant learning and memory processes (Koob, 2003 and Weiss and Porrino, 2002), all of which involve NMDA receptors. Following the initial findings that alcohol inhibits the NMDA receptor (Hoffman et al., 1989, Lima-Landman and Albuquerque, 1989 and Lovinger et al., 1989), a large number of studies have established that the NMDA receptor is a major target of alcohol action in the brain that plays a role in several ethanol-associated phenomena such as craving, tolerance, dependence, withdrawal, and relapse (Chandrasekar, 2013, Gass and Olive, 2008, Holmes et al., 2013, Krishnan-Sarin et al., 2015, Krupitsky et al., 2007, Krystal et al., 2003, Vengeliene et al., 2005, Vengeliene et al., 2008 and Woodward, 1999). Alcohol inhibits NMDA receptors by altering ion channel gating, specifically by decreasing channel opening frequency and mean open time (Lima-Landman and Albuquerque, 1989 and Wright et al., 1996), but does so

via an interaction with a novel modulatory site (Chu et al., 1995, Gonzales and Woodward, 1990, Göthert and Fink, 1989 and Peoples and Weight, 1992). The NMDA receptor is a heterotetramer formed from two obligatory GluN1 subunits and two GluN2 subunits, of which four different isoforms are expressed in the brain (GluN2A-GluN2D; (Dingledine et al., 1999)). Previous studies in our and other laboratories have identified and characterized a number of ethanol-sensitive positions in the M3 and M4 domain of the GluN1 and GluN2A subunit (Honse et al., 2004, Ren et al., 2003a, Ren et al., 2003b, Ren et al., 2007, Ren et al., 2012, Ren et al., 2013, Ronald et al., 2001, Smothers and Woodward, 2006 and Xu et al., 2012). Although the GluN2A subunit-containing NMDA receptor predominates in the mammalian brain, the GluN2B subunit has comparable ethanol sensitivity (Allgaier, 2002, Kuner et al., 1993, Masood et al., 1994, Popp et al., 1998 and Smothers et al., 2001) and plays an important role in alcohol action (Boyce-Rustay and Holmes, 2005, Izumi et al., 2005, Kash et al., 2008, Kash et al., 2009, Nagy, 2004, Wang et al., 2007 and Wills and Winder, 2013). However, the identity of ethanol-sensitive positions in the GluN2B subunit, and whether these are important for ion channel gating, is unknown. Because the M3 and M4 domain sequences of the GluN2A and GluN2B subunit are highly homologous, we tested whether positions in the GluN2B M domains corresponding to ethanol-sensitive positions in the GluN2A subunit regulate GluN2B subunit ethanol sensitivity. Despite the high homology between the GluN2A and GluN2B subunit M domains, we found that mutations at only two of four cognate positions altered alcohol sensitivity in the GluN2B subunit, and one of these is occupied by a glycine residue in the native protein. Although ethanol may interact with main chain carbonyl groups at positions containing glycine residues (Dwyer, 1999), such as 826, substitution of the glycine at this position could disrupt functional aspects of the alpha-helix. Although the characteristics of the remaining position, F637, were highly analogous to those of its cognate position in the GluN2A subunit, the differences at the other positions suggest that there are important differences in the action of ethanol on the GluN2A and GluN2B subunits.

2. Materials and methods

Chemicals. Ethanol (95%, prepared from grain) was obtained from Pharmco-Aaper (Brookfield, CT) and all other chemicals were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Chemicals used to make recording solutions were the highest purity available.

Site-directed mutagenesis, cell culture and transfection. Site-directed mutagenesis in plasmids containing GluN2B subunit cDNA was performed using the QuickChange kit (Stratagene La Jolla, CA, USA), and all mutations were verified by DNA sequencing. Transformed human embryonic kidney (tsA 201) cells were seeded in 35-mm poly-d-lysine coated dishes, and cultured in minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum to 70–95% confluence. Cells were then transfected with cDNA for the GluN1-1a, wild type or mutant GluN2B subunits and green fluorescent protein (pGreen Lantern; Invitrogen, Carlsbad, CA) at a 2:2:1 ratio using calcium phosphate transfection kit (Invitrogen). After transfection, 200 μM *dl*-2-amino-5-phosphonovaleric acid (APV) and 100 μM ketamine were added into culture medium to protect cells from receptor-mediated excitotoxicity. Cells were recorded within 48 h following transfection. Antagonists were removed before recording by extensive washing.

Electrophysiological recording. Whole-cell patch-clamp recordings were performed at room temperature using an Axopatch 1D or 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Gigaohm seals were formed in whole-cell recording using patch pipettes with tip resistances of 2–4 $\text{M}\Omega$, and series resistances of 1–5 $\text{M}\Omega$ were compensated by 80%. Cells were voltage-clamped at -50 mV and superfused in an external recording solution containing (in mM): NaCl, 150; KCl, 5; CaCl_2 , 0.2; HEPES, 10; glucose, 10; and sucrose, 10; osmolality was adjusted to 340 mmol/kg with 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); final pH was adjusted, if necessary, using HCl or NaOH. Low Ca^{2+} was used to minimize NMDA receptor inactivation, and EDTA, 10 μM , was added to the recording solution to eliminate the fast

component of apparent desensitization due to high-affinity Zn^{2+} inhibition (Low et al., 2000, Ren et al., 2003b and Erreger and Traynelis, 2005). Recording solutions were prepared fresh daily and applied to cells using a stepper motor-driven solution exchange apparatus (Warner Instruments, Hamden, CT, USA) and three-barrel square glass tubing of internal diameter 600 μm . The intracellular recording solution (patch-pipette) contained (in mM) CsCl, 140; Mg_4ATP , 2; BAPTA, 10; and HEPES, 10 (pH 7.2). In order to increase the speed of the solution exchange in glutamate concentration-response experiments, cells were lifted off the surface of the dish after gaining a gigaohm seal. 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 of amino acids were calculated using Spartan Pro (Wavefunction, Inc., Irvine, CA) following structural optimization using the AM1 semi-empirical parameters. Values used for amino acid hydrophathy, hydrophilicity, and polarity were reported previously (Ren et al., 2003b).

Molecular modeling. Molecular modeling was performed on a Dell T3500 workstation with Discovery Studio 3.1 software (Accelrys, now Biovia; San Diego, CA). A model of a truncated version of the NMDA receptor was built based on the crystal structure of the rat NMDA heterotetramer that included both the ligand-binding and channel domains (Karakas and Furukawa, 2014). We constructed a model confined to the channel structure of the protein and excluded the ligand-binding domain. The 4PE5 PDB file was used as a source of atomic coordinates and the sequences of rat GluNR1 and GluNR2B or GluNR2A subunits were aligned to the 4PE5 sequence. The original crystal structure lacks a number of key loops or segments, so these were built in piecemeal fashion using insert loop commands and, in some cases, by grafting a desired loop or segment to a new location. A model with the GluNR2B subunit was constructed first, and the version of the tetramer with the GluNR2A subunit was created from this model by mutating individual amino acids that differ in the two sequences. The structures were subjected to the same limited energy minimization regimen to resolve any major steric bumps.

Data analysis. In concentration-response experiments, IC_{50} or EC_{50} and n (slope factor) were calculated using the equation: $y = E_{max}/1 + (IC_{50} \text{ or } EC_{50}/x)^n$, where y is the percent inhibition (for IC_{50}) or the measured current amplitude (for EC_{50}), x is concentration, 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. Linear relations of mean values of log IC_{50} , log EC_{50} , or maximal steady-state to peak current ratio ($I_{SS}:I_P$) for the various mutants in GluN2A and GluN2B subunit were made using linear regression analysis. Values of log IC_{50} , log EC_{50} , and maximal steady-state to peak current ratio ($I_{SS}:I_P$) for GluN2A mutants are from previous studies (Ren et al., 2003a, Ren et al., 2007, Ren et al., 2013 and Honse et al., 2004). Time constants (τ) of deactivation were determined from fits of the current decay following the removal of glutamate (in the continued presence of glycine) to an exponential function using Clampfit (Axon Instruments). In most cells, deactivation was best fitted using a bi-exponential function; in these cases, the weighted time constant is reported. For cells in which deactivation was adequately fitted by a single exponential function, this value is reported. All values are reported as the mean \pm SE.

3. Results

3.1. Effects of mutations at positions in GluN2B subunit M domains corresponding to alcohol-sensitive positions in GluN2A subunits

The M3 and M4 domains are highly conserved among GluN1, GluN2A and GluN2B subunits (Fig. 1A). Previous studies from this laboratory have identified and characterized a number of ethanol-sensitive positions in these domains of the GluN2A subunit (Fig. 1B) (Honse et al., 2004, Ren et al., 2003b, Ren et al., 2007 and Ren et al., 2013). In this study, we initially constructed tryptophan and alanine substitutions at positions that correspond to ethanol-sensitive residues in the GluN2A subunit: Phe637, Phe638, Met824, and Gly826. Concentration-response experiments for ethanol inhibition showed that tryptophan or alanine mutations at Phe637 significantly changed

ethanol IC₅₀ compared with the wild-type receptor, but that neither substitution altered ethanol sensitivity at F638 or M824. Tryptophan mutagenesis at GluN2B(Gly826) exhibited significantly decreased ethanol sensitivity compared with the wild-type receptor (Fig. 2A–B; ANOVA and Dunnett's test; $p < 0.01$ or $p < 0.05$). The effects of these substitutions in GluN2B on alcohol sensitivity thus substantially differ from the corresponding mutations in the GluN2A subunit (Fig. 2C–D). Because the native residue at position 826 is glycine, and thus lacks a side chain, we chose to focus our investigation on GluN2B(F637). In order to study the role of GluN2B(Phe637) on ethanol action in detail, we made a panel of mutants at this position and tested ethanol sensitivities using whole-cell patch-clamp recording. All mutants we constructed yielded functional receptors that were inhibited by ethanol in a concentration-dependent manner (Fig. 3). Concentration-response curves for ethanol inhibition were essentially parallel to each other, as their slope factors did not differ significantly.

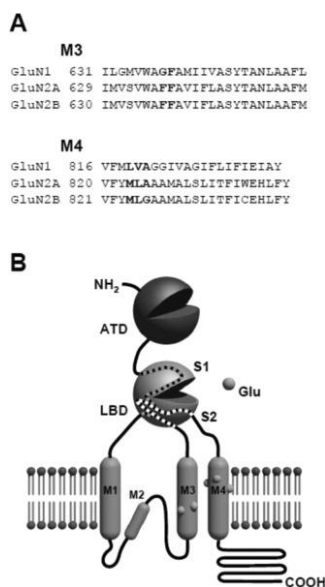


Fig. 1. Positions in the GluN2B subunit M3 and M4 domains corresponding to alcohol-sensitive positions in the GluN2A subunit. **A.** Partial sequences of the M3 and M4 domains in GluN2A and GluN2B subunits. Ethanol-sensitive positions in GluN2A and their cognate positions in GluN2B are indicated in bold. GluN2B(Phe637) is underlined. **B.** Topological model of the GluN2B subunit showing the amino-terminal domain (ATD), ligand binding domain (LBD) composed of lobes S1–S2, and membrane-associated domains (M1–M4) with the residues corresponding to the alcohol-sensitive positions in GluN2A shown as spheres.

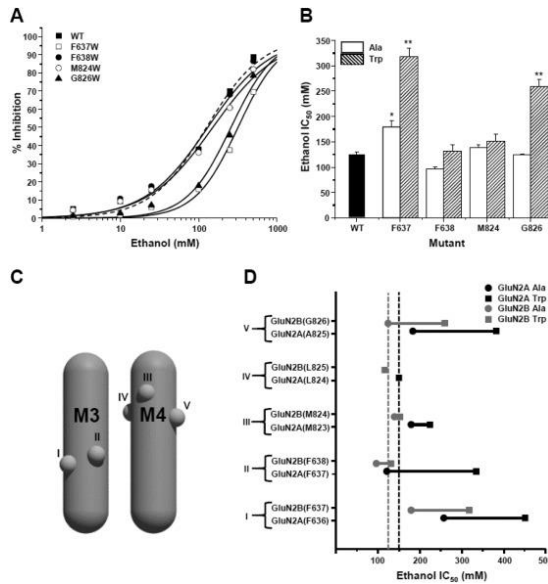


Fig. 2. Ethanol sensitivity of tryptophan and alanine substitution mutations at GluN2B M3 and M4 residues corresponding to GluN2A ethanol-sensitive positions. **A.** Concentration-response curves show ethanol inhibition of glutamate-activated currents in the presence of 50 μ M glycine in cells expressing either wild-type GluN1 and GluN2B subunits or wild-type GluN1 and GluN2B tryptophan mutants at Phe637, Phe638, Met824, or Gly826. One-letter amino acid codes are used. Error bars are not shown to increase clarity. Curves shown are the best fits to the equation given under "Materials and methods". The curve for the wild-type receptor is shown as a dashed line. **B.** Bar graphs show average IC₅₀ values for ethanol inhibition of glutamate-activated current in the presence of 50 μ M glycine in cells expressing wild-type GluN2B or GluN2B mutant subunits. IC₅₀ values that are significantly different from the wild-type receptor are indicated by asterisks (* P < 0.05; ** P < 0.01; ANOVA and Dunnett's test). Results are means \pm S.E of 5–7 cells. **C.** The M3 and M4 domains showing the approximate locations of the five positions tested. **D.** Comparison of ethanol IC₅₀ values of various alanine or tryptophan mutants to the wild-type value. The x-axis shows the ethanol IC₅₀ values of GluN2A subunit alanine (black circles) and tryptophan (black squares) substitutions and GluN2B subunit alanine (gray circles) and tryptophan (gray squares) substitutions at these positions. Ethanol IC₅₀ values of wild-type GluN2A-containing NMDA receptors (black dotted line) and GluN2B-containing NMDA receptors (gray dotted line) are shown. Ethanol IC₅₀ values for alanine and tryptophan substitutions at various positions in GluN2A subunit are from (Honse et al., 2004, Ren et al., 2003b, Ren et al., 2007 and Ren et al., 2013).

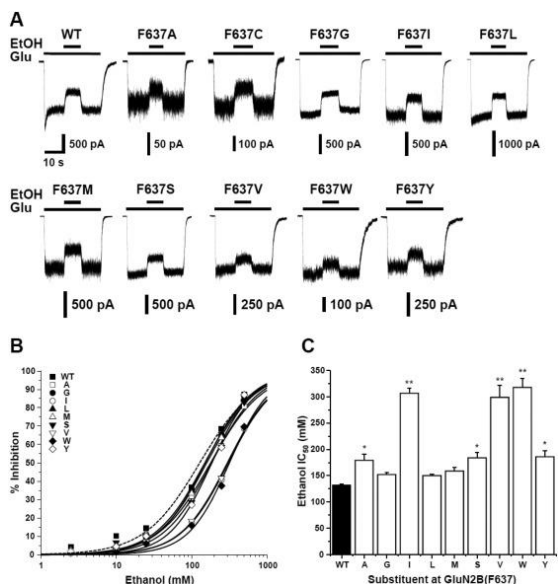


Fig. 3. Mutations at GluN2B(Phe637) alter ethanol sensitivity. **A.** Traces show currents activated by 10 μ M glutamate in the presence of 50 μ M glycine and their inhibition by 100 mM ethanol in cells expressing the GluN1 subunit with either wild-type GluN2B subunits or various GluN2B(Phe637) mutant subunits. One-letter amino acid codes are used. **B.** Concentration-response curves for ethanol inhibition of glutamate-activated currents in the presence of 50 μ M glycine in cells expressing various substitution mutations at GluN2B(Phe637). Results are the means \pm S.E of 5–7 cells. Error bars are not shown to increase clarity. The dashed line shows the fit for the wild-type receptor. All curves are best fits to the equation given under “Materials and methods”. **C.** Average IC₅₀ values for ethanol inhibition of glutamate-activated current in wild-type or GluN2B(Phe637) mutant receptors. Asterisks indicate significant differences from the IC₅₀ value of the wild-type receptor (* P < 0.05; ** P < 0.01; ANOVA and Dunnett's test). Results are the means \pm S.E of 5–7 cells.

Six out of ten substitution mutants at GluN2B(Phe637) showed significantly decreased ethanol sensitivity compared to the wild-type (WT) receptor (ANOVA; p < 0.05 or p < 0.01), however, none of the mutants exhibited increased ethanol sensitivity. Interestingly, although isoleucine and leucine are isomeric amino acids, ethanol sensitivity was significantly changed by substitution of isoleucine (IC₅₀ value: 307 \pm 9.41 mM; ANOVA and Dunnett's test; p < 0.01), but not leucine (IC₅₀ value: 150 \pm 1.32 mM; ANOVA and Dunnett's test; p > 0.05).

3.2. Mutations at GluN2B(Phe637) of NMDA receptor alter NMDA receptor function

Previous studies showed that ethanol inhibits NMDA receptors by altering ion channel gating, primarily by decreasing mean open time of the channel, and studies from our laboratory reported that

mutations at ethanol-sensitive positions in the GluN2A subunit also strongly influence ion channel gating kinetics (Ren et al., 2003a, Ren et al., 2007, Ren et al., 2008, Ren et al., 2013 and Honse et al., 2004). In this study, we first compared glutamate peak and steady-state EC_{50} values between GluN2B wild-type and GluN2B(F637W) mutant subunits. The GluN2B(F637W) mutant showed significantly decreased glutamate EC_{50} values for peak ($1.1 \pm 0.17 \mu\text{M}$; ANOVA and Dunnett's test; $p < 0.01$) and steady-state current ($1.05 \pm 0.16 \mu\text{M}$; ANOVA and Dunnett's test; $p < 0.01$; Fig. 4A and B). In addition, the mutant receptor also showed significantly decreased apparent desensitization, as indicated by the $I_{ss}:I_p$ ratio for 300 μM glutamate-activated current (Fig. 4C; ANOVA and Dunnett's test; $p < 0.01$), and an increased time constant of deactivation (τ) following removal of agonist (F637W: $2170 \pm 2.84 \text{ ms}$; WT: $920 \pm 3.30 \text{ ms}$; ANOVA; $p < 0.0001$; Fig. 4D). To further determine whether mutations at GluN2B(Phe637) can influence NMDA receptor characteristics, we performed glutamate concentration-response experiments on all mutants tested for ethanol sensitivity (Fig. 5). Seven out of ten mutants showed significantly decreased glutamate $I_p EC_{50}$ values (ANOVA and Dunnett's test; $p < 0.01$; Fig. 6A), but only five mutants showed decreased glutamate $I_{ss} EC_{50}$ values (ANOVA and Dunnett's test; $p < 0.01$; Fig. 6B). Interestingly, there was again a difference between the isoleucine and leucine mutants, in that isoleucine substitution significantly altered glutamate $I_{ss} EC_{50}$ ($0.85 \pm 0.078 \mu\text{M}$; ANOVA and Dunnett's test; $p < 0.01$), whereas leucine substitution did not ($2.5 \pm 0.24 \mu\text{M}$; ANOVA and Dunnett's test; $p > 0.05$; Fig. 6B). Pronounced increases were observed for maximal $I_{ss}:I_p$ ratios in all mutants (ANOVA and Dunnett's test; $p < 0.01$; Fig. 6C).

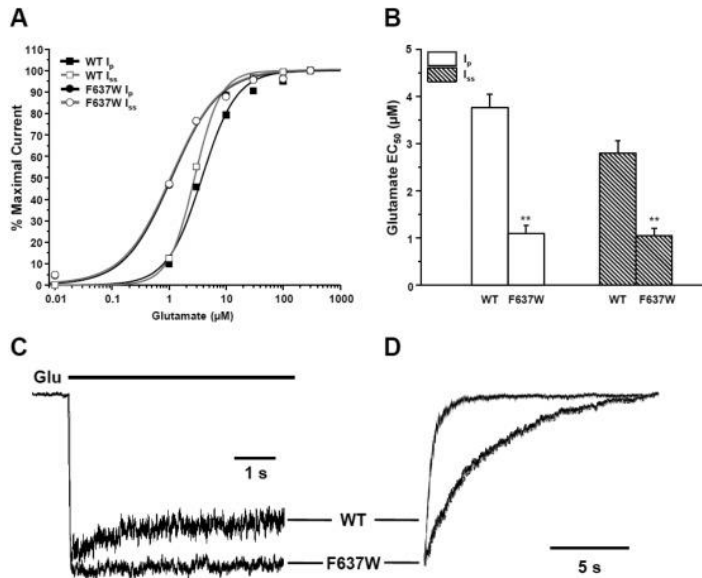


Fig. 4. Tryptophan substitution at GluN2B(Phe637) alters glutamate potency, desensitization, and deactivation. **A.** Glutamate concentration-response curves for glutamate-activated peak and steady-state currents in the presence of 50 μM glycine in lifted cells expressing GluN1/GluN2B or GluN1/GluN2B(F637W) receptors. Data points are the means ± S.E of 6 cells. Error bars are not shown to increase clarity. Curves shown are best fits to the equation in "Materials and methods" for peak (black) and steady-state (gray) currents. **B.** Average peak (open bars) and steady-state (cross-hatched bars) current EC₅₀ values recorded in lifted cells expressing either wild-type GluN1/GluN2B receptors or GluN1/GluN2B(F637W) receptors. Values that differed significantly from wild-type peak and steady-state current EC₅₀ values are indicated by asterisks (***P* < 0.01; ANOVA and Dunnett's test). Results are the means ± S.E of 6 cells. **C.** Normalized traces show the desensitization of 300 μM glutamate-activated current in the presence of 50 μM glycine in lifted cells expressing either wild-type GluN1/GluN2B or GluN1/GluN2B(F637W) receptors. **D.** Normalized traces show the difference in the deactivation of glutamate-activated current following removal of glutamate in lifted cells expressing either wild-type GluN1/GluN2B or GluN1/GluN2B(F637W) subunits.

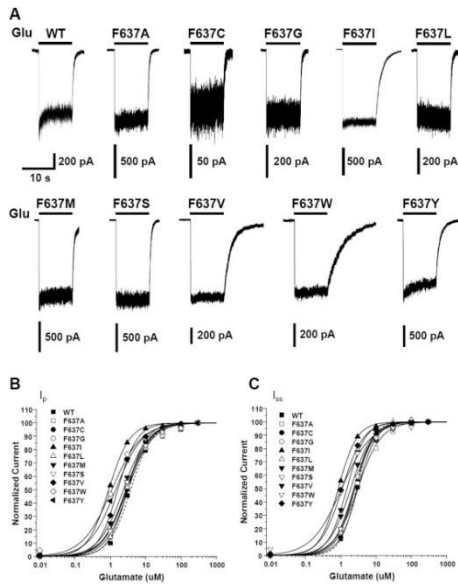


Fig. 5. Mutations at GluN2B(Phe637) alter glutamate EC_{50} . *A*. Traces show 300 μM glutamate-activated current in the presence of 50 μM glycine in lifted cells expressing wild-type receptors or receptors containing various substitutions at GluN2B(Phe637). One-letter amino acid codes are used. *B–C*. Concentration-response curves for glutamate-activated peak (*B*) and steady-state (*C*) current recorded from lifted cells expressing wild-type or mutant receptors. Results are the means ± S.E of 4–6 cells. Error bars are not shown to increase clarity. All curves shown are best fits to the equation described in “Materials and methods”. The dashed curves indicate the fits for the wild-type receptor.

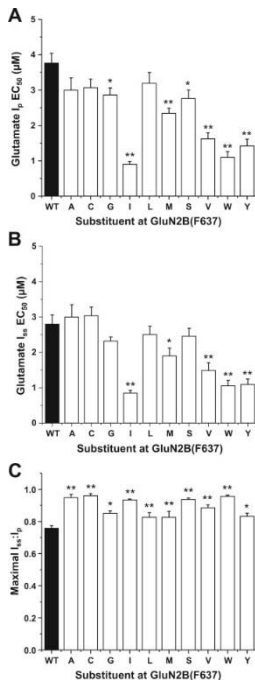


Fig. 6. Mutations at GluN2B(Phe637) change glutamate potency. Bar graphs show the average EC_{50} values for glutamate-activated peak (*A*) and steady-state (*B*) currents

recorded from cells expressing GluN1 and wild-type GluN2B or GluN2B(Phe637) mutant subunits. Asterisks indicate EC_{50} values that differ significantly from that of the wild-type GluN1/GluN2B subunit (** $P < 0.01$; ANOVA and Dunnett's test). Results are the means \pm S.E of 4–6 cells. C. The average values of maximal steady-state to peak current ratio ($I_{ss}:I_p$) in lifted cells co-expressing GluN1 and GluN2B wild-type subunits or GluN2B subunits containing various substitutions at F637. Currents were activated by 300 μ M glutamate in the presence of 50 μ M glycine. Asterisks indicate $I_{ss}:I_p$ values that are significantly different from the value for the wild-type GluN1/GluN2B subunit (** $P < 0.01$; ANOVA and Dunnett's test). Results are the means \pm S.E of 4–6 cells.

Figure options

3.3. Relation of GluN2B(Phe637) mutant peak current glutamate EC_{50} , steady-state EC_{50} , and maximal steady-state to peak current ratio

For a series of mutants at GluN2A(F636), glutamate EC_{50} values for steady-state current were highly correlated with those for peak current, but not with values of $I_{ss}:I_p$ (Ren et al., 2013). In the present study, we observed that peak current glutamate EC_{50} values were strongly correlated with steady-state current glutamate EC_{50} values ($R^2 = 0.95$, $P < 0.0001$; Fig. 7A). However, EC_{50} values for glutamate-activated steady-state current for mutants at GluN2B(F637) were not significantly correlated with maximal $I_{ss}:I_p$ values ($R^2 = 0.014$, $P > 0.05$; Fig. 7B).

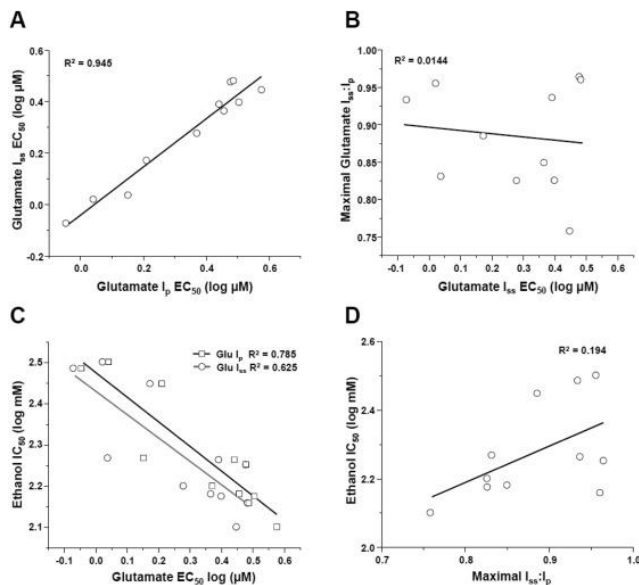


Fig. 7. Correlations among glutamate peak and steady-state current EC_{50} ethanol IC_{50} values. A. Graph plots values of glutamate log EC_{50} for steady-state current *versus* peak current in a series of mutants. Glutamate EC_{50} values for peak and steady-state

current were significantly correlated ($R^2 = 0.95$, $P < 0.0001$). The lines shown are the least squares fits to the data. *B.* Graph plots log maximal steady-state to peak current ratio ($I_{ss}:I_p$) versus either glutamate peak current log EC_{50} values or glutamate steady-state current log EC_{50} values for various GluN1/GluN2B(Phe637) mutant receptors. Log maximal $I_{ss}:I_p$ for glutamate was not correlated with either peak or steady-state glutamate EC_{50} ($p > 0.05$). The lines shown are the least squares fits to the data. *C–D.* Graphs plot log ethanol IC_{50} values in a series of mutants versus log values of glutamate EC_{50} for peak or steady-state current (*C*) or the maximal $I_{ss}:I_p$ values (*D*). Ethanol IC_{50} values were correlated with glutamate EC_{50} for peak ($R^2 = 0.75$, $p < 0.001$) and steady-state current ($R^2 = 0.59$, $p < 0.005$), but not with $I_{ss}:I_p$ values ($R^2 = 0.19$, $p > 0.05$). The lines shown are the least squares fits to the data.

3.4. Determinants of ethanol sensitivity and receptor function among mutants at GluN2B(Phe637)

To determine the manner in which the physical–chemical properties of the substituent at GluN2B(F637) contribute to the changes in ethanol IC_{50} values, glutamate EC_{50} values, and $I_{ss}:I_p$ values, we tested for linear relations of these values to amino acid polarity, hydrophilicity, and molecular volume. However, no significant linear relations were detected among these measures (Table 1).

Table 1. Relation of NMDA receptor agonist potency and ethanol sensitivity to the physicochemical parameters of the substituent at GluN2B(Phe637).

	Glutamate I_p EC_{50}		Ethanol IC_{50}	
	R^2	P value	R^2	P value
Molecular volume	0.107	>0.05	0.073	>0.05
Hydrophilicity	0.081	>0.05	0.109	>0.05
Polarity	0.022	>0.05	0.034	>0.05

Because substitution mutations at GluN2B(Phe637) altered both ethanol sensitivity and NMDA receptor function, we asked whether the observed changes in ethanol sensitivity among the series of mutant subunits might have resulted from changes in agonist potency or ion channel gating kinetics. Plots of ethanol IC_{50} values against values of glutamate EC_{50} for peak current, steady-state current, or maximal $I_{ss}:I_p$ revealed that ethanol sensitivity of mutants was significantly negatively correlated with both peak ($R^2 = 0.75$, $p < 0.001$; Fig. 7C) and steady-state ($R^2 = 0.59$, $p < 0.005$) current EC_{50} values, but was not correlated with maximal $I_{ss}:I_p$ ($p > 0.05$; Fig. 7D).

3.5. Effects of mutations at GluN2B(Phe637) and GluN2A(Phe636) on ethanol sensitivity and ion channel function are similar

Because mutations at GluN2B(Phe637) and at its cognate position, GluN2A(Phe636), can both alter ethanol sensitivity and ion channel function (Ren et al., 2013), we evaluated whether the side chain at this position regulates the ion channel in a similar manner in the two subunits. We thus tested for correlations among mutants at GluN2B(Phe637) and GluN2A(F636) for glutamate I_p and I_{ss} EC_{50} , maximal $I_{ss}:I_p$, and ethanol IC_{50} values. For the two series of mutants at GluN2B(F637) and GluN2A(F636), we observed significant correlations for values of glutamate EC_{50} for peak ($R^2 = 0.84$, $p < 0.0001$; Fig. 8A) and steady-state ($R^2 = 0.76$, $p < 0.001$; Fig. 8B) current, maximal $I_{ss}:I_p$ ($R^2 = 0.49$, $p < 0.05$; Fig. 8C), and ethanol IC_{50} ($R^2 = 0.95$, $p < 0.0001$; Fig. 8D).

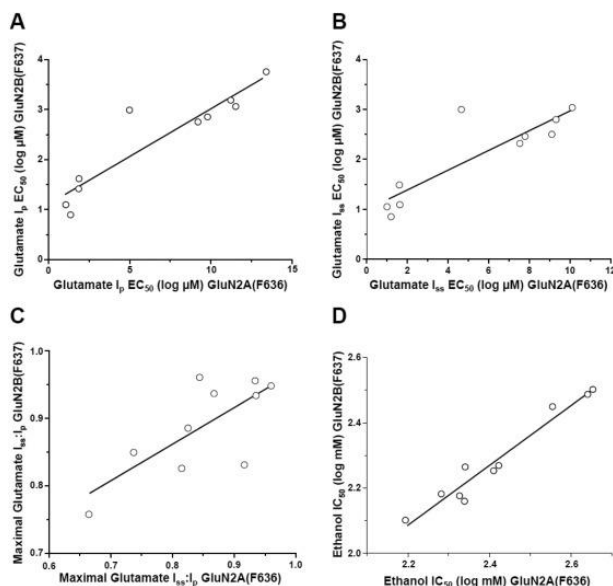


Fig. 8. Ethanol sensitivity and glutamate EC_{50} values of GluN2B(Phe637) mutants are correlated with these parameters in GluN2A(Phe636) mutants. A–B. Graphs plot log values of either I_p EC_{50} (A) or I_{ss} EC_{50} (B) for GluN2B(Phe637) mutants versus each of these two parameters for GluN2A(Phe636) mutants. Both peak and steady-state glutamate EC_{50} values were significantly correlated in the two subunit types ($R^2 = 0.84$, $P < 0.0001$ and $R^2 = 0.76$, $P < 0.001$, respectively). The lines shown are the least squares fits to the data. C. Values of maximal steady-state to peak current ratio ($I_{ss}:I_p$) of GluN2B(Phe637) and GluN2A(Phe636) mutant receptors were significantly correlated ($R^2 = 0.49$, $P < 0.05$). The line shown is the least squares fits to the data. D, Log ethanol IC_{50} values for mutants at GluN2B(Phe637) and GluN2A(Phe636) were significantly correlated ($R^2 = 0.95$, $P < 0.0001$). The line shown

is the least squares fit to the data. Ethanol IC_{50} values, glutamate EC_{50} values, and maximal $I_{ss}:I_p$ values of GluN2A(Phe636) mutants are from a previous study in this laboratory (Ren et al., 2013).

4. Discussion

We and others have used alanine- and tryptophan-scanning to identify alcohol-sensitive positions in the GluN1 and GluN2A subunits (Ren et al., 2003b, Ren et al., 2007, Ren et al., 2012, Ronald et al., 2001, Salous et al., 2009 and Smothers and Woodward, 2006). Previous studies in this laboratory identified several ethanol-sensitive positions in both the M3 and M4 domains of the GluN2A subunit: Phe636, Phe637, Met823, and Ala825 (Honse et al., 2004, Ren et al., 2003b, Ren et al., 2007 and Ren et al., 2013). In the present study, alanine and tryptophan substitutions were initially introduced into the cognate positions in the GluN2B subunit M3 and M4 domains, which are Phe637, Phe638, Met824, and Gly826. The results of the present study show, however, that the role of these positions in the GluN2A and GluN2B subunits in determining ethanol sensitivity differs. For example, tryptophan mutagenesis at GluN2A(Phe637) increases ethanol IC_{50} by over two-fold relative to the wild-type value (Ren et al., 2007), whereas in the GluN2B subunit, tryptophan substitution at the cognate position, Phe638, had no effect on ethanol sensitivity. Of the four positions in the GluN2B subunit we tested in this study, only Phe637 showed alterations in ethanol sensitivity following both tryptophan and alanine mutagenesis. We also found no changes in glutamate EC_{50} , maximal $I_{ss}:I_p$, or deactivation time constant in alanine or tryptophan substitution mutants at Phe638 or Gly826 (*results not shown*). Interestingly, alanine or tryptophan substitution at Met824 altered glutamate steady-state EC_{50} and maximal $I_{ss}:I_p$ values (*results not shown*), as was observed for the cognate position Met823 in GluN2A (Ren et al., 2003a), but did not alter ethanol sensitivity. It should be noted, however, that the effect of mutations at GluN2A(M823) on ethanol sensitivity is significant, but relatively small, so that a subtle difference at this position in the GluN2B could account for its lack of regulation of ethanol sensitivity. The tryptophan mutant at Phe637 showed increases in both glutamate EC_{50} and time constant of deactivation, and both tryptophan and alanine mutants at this position showed decreased macroscopic desensitization.

Although the GluN2A and GluN2B subunit share highly conserved M domain sequences, our observations demonstrate that ethanol's actions on the two subunits differ considerably. Our laboratory has reported that two positions in the GluN2A M3 and M4 domains can functionally interact to regulate ethanol sensitivity (Ren et al., 2008), and Smothers and Woodward (2006) identified a pair of positions in the GluN1 subunit that can functionally interact with respect to ethanol inhibition. Positions at the intersubunit M3-M4 domain interfaces interact to regulate ethanol sensitivity and ion channel function (Ren et al., 2012), and can also form three-way interactions with M1 domain residues (Xu et al., 2015). Thus, multiple ethanol-sensitive positions appear to form sites of ethanol action. Therefore, the differences in ethanol action between GluN2A and GluN2B subunits identified in this study may arise because positions in the GluN2B subunit interact with other positions in GluN2B or GluN1 in a manner that differs from the cognate positions in the GluN2A subunit. One possible candidate for such an interacting position is an isoleucine at position 571 in the M1-M2 linker of the GluN2A subunit, which corresponds to a valine at 572 in the GluN2B subunit (Fig. 9). Our energy-minimized models of this region place GluN2A(I571) sufficiently close to a substituted tryptophan at 637 to result in a steric interaction between these side chains, which may explain the ability of the substituted tryptophan to decrease the sensitivity of the receptor to ethanol. In contrast, the considerable distance between GluN2B(VaI572) and position 638 could easily accommodate a substituted tryptophan at 638 without steric interaction, so that mutations at 638 would not alter ethanol action. An alternative possibility is that differences among subunits may arise due to regulation of ethanol sensitivity by distant parts of the protein. Many studies from this and other laboratories have established that positions in the M domains are critically important for the action of ethanol (Honse et al., 2004, Ren et al., 2003a, Ren et al., 2003b, Ren et al., 2007, Ren et al., 2012, Ren et al., 2013, Ronald et al., 2001, Smothers and Woodward, 2006, Smothers et al., 2013 and Xu et al., 2012). For example, a recent study from this laboratory found that mutating only two of ten ethanol-sensitive positions in M3 and M4 could increase the NMDA receptor ethanol IC₅₀ value to over 1 M, rendering it insensitive to physiological concentrations of ethanol (Ren et al., 2012). However, although the NMDA receptor C-terminal and N-

terminal domains are not required for the action of ethanol (Peoples and Stewart, 2000 and Smothers et al., 2013), both regions can influence ethanol sensitivity (Alvestad et al., 2003, Anders et al., 1999, Anders et al., 2000, Peoples and Stewart, 2000 and Smothers et al., 2013), leaving open the possibility that these or other regions could contribute to the differences in alcohol action observed in GluN2A and GluN2B M domain mutants.

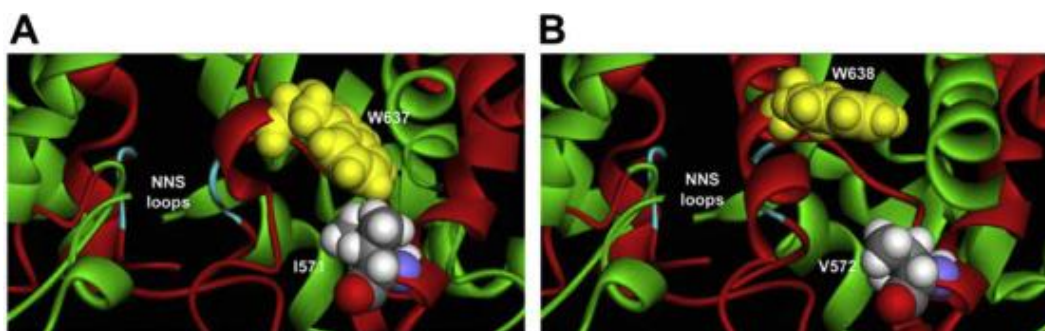


Fig. 9. Tryptophan substitution at a position in M3 differs in the GluN2A and GluN2B subunits. Molecular models of the NMDA receptor M domains showing tryptophan substitution at 637 in GluN2A (A) or 638 in GluN2B (B). Note the difference in the potentially interacting isoleucine 571 in GluN2A vs. valine 572 in GluN2B. The ribbon for GluN1 is colored green and for GluN2A/2B is colored red. The proteins are superimposed so that the perspective is the same for both. The models were subjected to equal amounts of energy minimization.

Previous studies have found a relation between molecular volume of the side chain at sites of alcohol action in GABA_A and glycine receptors (Kash et al., 2003, Mihic et al., 1997, Wick et al., 1998 and Yamakura et al., 1999), in the M4 domain of the GluN2A subunit (Ren et al., 2003b), and at the cognate position to GluN2B(Phe637) in the M3 domain of the NMDA receptor GluN1 subunit (Smothers and Woodward, 2006) and GluN2A subunit (Ren et al., 2007). Although substitution mutations at GluN2B(Phe637) could significantly alter ethanol sensitivity, ethanol IC₅₀ was not related to side chain molecular volume among the GluN2B(Phe637) mutants tested. One explanation for this may be that the side chain at 637 may not directly project into the interior of the ethanol-sensitive site, but may rather form part of the outer boundary of this site. The reason for the discrepancy between the role of molecular volume at this position in the GluN2A and GluN2B subunits, however, is not clear at present, but serves as additional evidence that the sites of alcohol action on the two subunit types differ. The results of the present study also did not show any relation between ethanol sensitivity and the hydrophilicity of

the substituent at GluN2B(Phe637). Although such a relation was observed among mutants at GluN2A(Met823), indicating a possible role for hydrophobic binding (Ren et al., 2003b), similar relations were not observed for other positions (Ren et al., 2007 and Salous et al., 2009). The lack of a clear role for molecular volume and hydrophobicity in the present study was particularly apparent when comparing the leucine and isoleucine mutants: there was a striking difference in ethanol sensitivity between the two mutants, even though they have identical physicochemical characteristics. Thus, GluN2B(Phe637) can regulate receptor ethanol sensitivity in a complex manner that relies on the interaction between side chains at this position and other positions. Further studies will be needed to define these interactions.

All GluN2B(Phe637) mutants tested in this study showed decreased peak glutamate EC_{50} values and increased maximal $I_{ss}:I_p$ values, and only three showed decreased steady-state glutamate EC_{50} values. Because Phe637 is in the M3 domain, which has an important role in NMDA receptor ion channel gating, and is at a considerable distance from the ligand binding domain (Low et al., 2003, Sobolevsky et al., 2007, Sobolevsky et al., 2009 and Yuan et al., 2005), the changes in glutamate EC_{50} values among mutants at this position appear to result from modifications in ion channel gating. Although we did not determine direct measures of ion channel gating, such as mean open time, in this study, the differences in macroscopic desensitization indicate alterations in ion channel gating among the mutants. The observed differences in current amplitude among mutants at GluN2B(F637) may also result from altered ion channel gating. The isoleucine and leucine mutants also differed from each other with respect to glutamate EC_{50} , such that isoleucine, but not leucine, substitution increased peak and steady-state glutamate EC_{50} . This indicates that not only ethanol sensitivity of the NMDA receptor, but also ion channel gating, is sensitive to subtle structural alterations in the side chain at 637. A previous study showed that tryptophan substitution at GluN2A(Met823) significantly altered both glutamate I_{ss} EC_{50} and desensitization (Ren et al., 2003a), and that the changes in glutamate steady-state EC_{50} values among mutants at this position were due to agonist trapping in desensitized states. For a series of amino acid substitutions at Phe636 in the GluN2A subunit, which is the cognate position of GluN2B(Phe637), $I_{ss}:I_p$ values had an inverse

relationship with peak glutamate EC₅₀ values, but were not correlated with glutamate steady-state EC₅₀ values (Ren et al., 2013). In GluN2B(Phe637) mutants, however, glutamate peak and steady-state EC₅₀ values were highly correlated, but neither were correlated with maximal I_{ss}:I_p values, indicating that agonist potency can be influenced by the substituent at this position in a manner that is independent of changes in desensitization. The mechanism for this appears to be due to changes in ion channel gating, such as increases in mean open time, that secondarily affect agonist binding. Thus, although GluN2A(Phe636) and GluN2B(Phe637) are cognate positions, and mutations at both positions affect ethanol sensitivity in a remarkably similar manner, their roles in regulation of ion channel function differ.

Previous studies in our laboratory showed that ethanol sensitivity of the NMDA receptor was inversely correlated with glutamate EC₅₀. In these studies, we observed that mutations at GluN2A(F636) and GluN2A(F637) that had higher agonist potency exhibited lower ethanol sensitivity (Ren et al., 2007 and Ren et al., 2013). Similar inverse correlations between ethanol sensitivity and glutamate peak, steady-state EC₅₀, or apparent desensitization were observed in the current study at GluN2B(Phe637). Ethanol can influence desensitization in a number of ion channels (Dopico and Lovinger, 2009 and Moykkynen et al., 2003). It is possible that changes in ethanol sensitivity observed in the present study are secondary to alterations of ion channel function in GluN2B subunit-containing NMDA receptors, but this possibility is not consistent with results of previous studies in which ethanol inhibition of NMDA receptors did not involve changes in desensitization (Peoples et al., 1997, Ren et al., 2003b and Woodward, 2000). Furthermore, studies in cells expressing GluN1/GluN2A(F636W) NMDA receptors showed that ethanol sensitivity was not altered when ethanol was either pre-applied for 10 s before receptor was activated or when ethanol was applied during steady-state current (Ren et al., 2013). In the present study, we found that steady-state and peak current inhibition in mutants at GluN2B(Phe637) do not differ (*results not shown*). It thus appears most likely that similar factors may influence both ion channel kinetics and ethanol sensitivity in parallel.

5. Conclusions

Although the GluN2A and GluN2B subunit M3 and M4 domains are highly homologous, substitution mutations at only two of four positions tested affect alcohol sensitivity in both subunits. Furthermore, as GluN2B(826) is occupied by a glycine in the native protein, the side chain at this position is unlikely to participate in alcohol inhibition, although the main chain carbonyl group may do so (Dwyer, 1999). The characteristics of the remaining position, GluN2B(637), were highly analogous to those of its cognate position in the GluN2A subunit, suggesting that there are both important similarities and differences in the action of ethanol on the GluN2A and GluN2B subunits.

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