# Positions in the GluN2C-Containing NMDAR Regulate Alcohol Sensitivity and Ion Channel Gating

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# POSITIONS IN THE GLUN2C-CONTAINING NMDAR REGULATE ALCOHOL SENSITIVITY AND ION CHANNEL GATING

by

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## ABSTRACT

POSITIONS IN THE GLUN2C-CONTAINING NMDAR REGULATE

ALCOHOL SENSITIVITY AND ION CHANNEL GATING

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The N-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate-gated ion channel, has been shown to be a major target of ethanol in the central nervous system (CNS). Previous studies have identified positions in the third and fourth membrane-associated (M) domains of the NMDAR GluN1 and GluN2A subunits that influence ethanol sensitivity. Among the alcohol sensitive sites, a methionine residue is highly conserved in all GluN1 and GluN2 subunits. We proposed the methionine position (Met-821) in the M4 domain of the GluN2C subunit can regulate ethanol sensitivity and ion channel gating.

14 mutations were made at the methionine position, 7 substitutions yielded functional receptors, which can influence ethanol sensitivity, glutamate potency and desensitization compared to wild type NMDAR containing GluN2C subunit. The other 7 mutations showed small spontaneous currents with apparent ethanol inhibition.

The predicted structure of the NMDAR indicates that alcohol sensitive positions in the M3-M4 intersubunit interfaces between the two subunit types interactively regulate ethanol sensitivity and ion channel gating. We proposed that the Met-821 position interact with the Gly-638 or Phe-639 position in the GluN1 M3 domain to regulate ethanol sensitivity and ion channel gating. Dual tryptophan mutants G638W/M821W and F639W/M821W showed small spontaneous currents with apparent ethanol inhibition. To test the interaction between these two pairs of positions, cysteine mutations were made at Gly-638, Phe-639, and Met-821. Dual cysteine mutants G638C/M821C and F639C/M821C yielded functional receptors. G638C/M821C showed significant interaction with respect to ethanol inhibition, suggesting these pair of positions interactively regulate ethanol sensitivity and ion channel gating.

DTT reducing experiments showed DTT-potentiated currents and increased deactivation time constant Tau in the dual cysteine mutant G638C/M821C.

In the present studies, we showed that the Met-821 position involved in regulating ethanol sensitivity and ion channel gating. We also showed Gly-638 and Met-821 positions in the M3-M4 intersubunit interfaces between GluN1 and GluN2C subunits interactively regulate ethanol sensitivity. The results we observed from GluN2C-containing NMDAR are different from the previous discoveries in the NMDAR containing GluN2A subunit. The difference may mainly lies in the sequence difference between GluN2A and GluN2C M4 domains and small hydrophobic environment formed near the methionine position.

## DEDICATION

*To my parents,*

*Zhiying and Changze*

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## ABBREVIATIONS

| | |
|---|---|
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ANOVA | analysis of variance |
| AP5 | D-2-amino-5-phosphono-pentanoic acid |
| ATD | amino-terminal domain |
| BAPTA | α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| CaMKII | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| CNS | central nervous system |
| CTD | carboxyl-terminal domain |
| DCKA | 5,7-dichlorokynurenic acid |
| DTT | dithiothreitol |
| $E_{max}$ | maximum excitatory effect |
| $EC_{50}$ | 50% excitatory concentration |
| EPSC | excitatory postsynaptic current |
| EPSP | excitatory postsynaptic potential |
| GABA | γ-Aminobutyric acid |
| GFP | green fluorescent protein |
| HEK | human embryonic kidney |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| 5-HT | 5-hydroxytryptamin |

| | |
|---|---|
| $IC_{50}$ | 50% inhibitory concentration |
| $I_p$ | glutamate-activated peak current |
| $I_{ss}$ | glutamate-activated steady-state current |
| LBD | ligand-binding domain |
| LTD | long-term depression |
| LTP | long-term potentiation |
| MD | membrane-associated domain |
| MOT | mean open time |
| MTS | cysteine-specific methanethiosulfonate |
| MTSEA | MTS-ethylammonium |
| MTSET | MTS-ethyltrimethylammonium |
| NMDA | N-methyl-D-aspartic acid |
| $P_o$ | open probability |
| PKA | protein kinase A |
| PKC | protein kinase C |
| SCAM | substituted cysteine accessibility method |

## DECLARATION

I declare that I myself carried out all the work in this thesis except where referenced and that it has not been submitted for any previous higher degree.

# PUBLICATIONS

## Papers

Ren H, Zhao YL, **Wu M**, Peoples RW., A Novel Alcohol-Sensitive Position in the N-Methyl-D-Aspartate Receptor GluN2A Subunit M3 Domain Regulates Agonist Affinity and Ion Channel Gating. Mol Pharmacol. , 2013. **84**(4): p. 501-510.

**Wu M** & Peoples RW. A Novel Alcohol-sensitive Position In The M4 Domain Of The GluN2C-containing NMDAR. **(In preparation)**

**Wu M** & Peoples RW. Cysteine Cross-linking Between Positions in the M3 and M4 Domains at the Intersubunit Interface of The GluN2C-containing NMDAR Interactively Regulate Alcohol Action. **(In preparation)**

## Abstracts

**Wu M**, Zhao YL, Peoples RW. A Novel Alcohol-sensitive Position In The M4 Domain Of The NMDA Receptor GluN2C Subunit. The Research Society on Alcoholism 2013, Orlando, FL

Zhao YL, **Wu M**, Peoples RW. Sites of alcohol action at the GluN1/GluN2B NMDA receptor M3-M4 domain intersubunit interfaces" Society for Neuroscience 2013, San Diego, CA

# Chapter 1. Introduction

## The NMDA receptor: a general introduction

The N-methyl-D-aspartate (NMDA) receptor, belonging to the ionotropic glutamate receptor family, is highly expressed in the central nervous system (CNS)[2, 3]. The NMDARs are sensitive to the specific agonist N-methyl-D-aspartate (NMDA)[4, 5], which distinguishes NMDARs from other L-glutamate-activated receptors (AMPA, kainite, and delta)[6]. As one of the most important neurotransmitter-activated ion channels in the brain, NMDAR plays critical roles in multiple aspects of brain function, including generating rhythms for breathing and locomotion activities, controlling synaptic plasticity underlying learning and memory processes, and regulating higher cognitive brain functions, such as cognition, attention, and fear[7-10].

## NMDA receptor development and distribution

NMDARs are heterotetramers[1, 11-13], and functional receptors are assembled from two GluN1 subunits with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits[14-16]. The GluN1 subunit, as the key element of tetrameric NMDARs, is present and widespread from an early developmental stage[17-19]. In contrast, GluN2 subunits show distinct expression patterns during the developmental process. In the rat CNS, only

GluN2B and GluN2D subunits are expressed during the prenatal stage, but shortly after birth, GluN2A and GluN2C subunits quickly predominate while the GluN2B and GluN2D subunits decline to adult levels, resulting in a limited distribution[20]. GluN2B expression levels peak in the hippocampus and cortex during the third postnatal week and then decline to the low adult levels, while GluN2A expression continues to increase in the hippocampus and cortex, and peak throughout the brain during the third postnatal week before declining to adult levels[21-24]. GluN2C expression is very low in the cerebellum and forebrain at P7, but dramatically increases in the cerebellum at P12, where its expression peaks throughout granule cells during the third postnatal week and where it continuous to be expressed at high levels in the adult as the predominant subunit[23, 25]. In summary, the GluN1 and GluN2A subunits are ubiquitous in the adult brain; the GluN2B subunit is mainly in the forebrain; the GluN2C subunit predominates in the cerebellum and various select nuclei; and the GluN2D subunit is limited to the diencephalon and the midbrain[20, 25-28].

**NMDA receptor physiology**

The NMDARs are integral membrane proteins formed by four subunits, at least two GluN1 subunits with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits[14-16]. Each individual subunit contains four distinct domains: the extracellular amino-terminal domain (ATD), which is involved in subunit assembly, trafficking and modulation; the ligand-binding domain (LBD),

which participates in agonist or competitive antagonist binding and channel activation; the membrane-associated domains (MD), which form the membrane-spanning part of the receptor, and an intracellular carboxyl-terminal domain (CTD), which is mainly responsible for receptor localization and signaling regulation. Although each GluN subunit shares highly homologous sequence and architecture, NMDAR kinetics, regulation, and interaction with multiple intracellular proteins varies depending upon subunit composition[28, 29]. First, at resting membrane potentials, most subtypes of NMDARs undergo rapid channel block by extracellular $Mg^{2+}$, which is only relieved with simultaneous depolarization and synaptic release of glutamate[30-32]. GluN2C–containing NMDARs are ~ 10 fold less sensitive to $Mg^{2+}$ block compared to receptors containing GluN2A and GluN2B NMDARs[14, 33]. Second, NMDARs are highly permeable to both monovalent cations and $Ca^{2+}$ ions. NMDAR activation results in an influx of $Ca^{2+}$ as well as the influx of $Na^{+}$ and efflux of $K^{+}$[34-37]. Ca2+ entry has been demonstrated to be the key trigger for many important physiological activities including long-term potentiation (LTP) [7, 38, 39]and long-term depression (LTD)[40-42], where the relative magnitude of the rise in intracellular Ca2+ concentration and its temporal and spatial character determines which type of synaptic plasticity is induced[43-46]. Third, NMDAR activation requires coincident binding of both glutamate and the co-agonist glycine[47, 48]. Glutamate is the endogenous agonist of GluN2 subunits[49, 50], while glycine and D-serine act as the agonist at the GluN1

subunit[51, 52]. In addition to glutamate, D- and L-aspartate are also endogenous agonists for the GluN2 subunits[53-56]. The GluN2 subunits show various glutamate potencies and efficacies. The GluN2A and GluN2B subunits have lower potency compared to the GluN2C and GluN2D subunits[14, 57, 58], but higher efficacy as indicated by higher open probability ($P_o$) during the time when the receptor is fully occupied by agonist (intraburst $P_o$). Fourth, NMDAR desensitization is defined as a reduced response in the sustained presence of agonist. There are at least three different processes of NMDAR apparent desensitization: true desensitization, in which NMDAR responses are diminished in the continued presence of glutamate in a time-dependent manner[28, 59]; glycine-dependent apparent desensitization, in which binding of glutamate decreases the affinity for glycine, so that NMDAR responses decay in the presence of low concentrations of glycine[60, 61]; calcium inactivation, in which intracellular $Ca^{2+}$ causes a decay in NMDAR-mediated current through an interaction with the GluN1 subunit cytoplasmic domain[61-63]. All forms of apparent and real desensitization are prominent in GluN2A-containing NMDARs, but are not observable in GluN2C-containing NMDARs[14, 64-66]. Fifth, NMDAR deactivation contributes to the EPSC time course and is also dependent on subunit composition. The time constants of deactivation in NMDARs containing GluN2C or GluN2D subunits are much higher than those in GluN2A- and GluN2B- containing NMDARs.

## The NMDA receptor pharmacology

The complicated architecture of the NMDAR provides agonists and antagonists with several distinct binding sites. The glycine binding site in the GluN1 subunit ligand-binding domain (LBD) is activated by glycine or D-serine, and blocked by antagonists such as 7-chlorokynurenic acid and its analog 5,7-dichlorokynurenic acid (5,7-DCKA)[67-69]. Glutamate, NMDA, aspartate, and other agonists activate the glutamate binding site in GluN2 subunits and the classical competitive antagonists of this site are (*R*)-2-amino-5-phosphonopentanoate (AP5 or APV)and its analogs, such as AP7[47]. Extracellular $Mg^{2+}$ blocks the NMDAR channel pore at negative membrane potentials. Other open channel blockers, such as MK-801 and ketamine, block the ion channel in a manner that is not voltage-dependent, although recovery from block is accelerated by the outward movement of ions at positive membrane potentials[70]. Zinc inhibits NMDAR via dual actions: at millimolar concentrations, it produces ion channel block[71], and at nanomolar concentrations, it binds to the GluN2A ATD[72-74] of GluN2A-containing NMDAR to cause a rapid decay in current[75, 76]. Polyamines such as spermine and spermidine can inhibit or potentiate the NDMARs activities, at high and low concentrations respectively. At low micromolar concentrations, polyamines promote channel opening and at high concentrations, they block the channel[77-79].

## The NMDA receptor and alcohol addiction

Ethanol is one of the most widely abused drugs in the world. Chronic alcohol exposure can cause multiple aspects of changes in brain morphology, function and behavior. Studies have shown that alcohol addiction not only can cause brain shrinkage and loss of neurons, but is associated with aberrant learning and memory processes[80-83]. It acts on multiple target proteins in the central nervous system at high millimolar concentrations[2, 3]. Among those targets, the NMDARs play a crucial role for the inhibitory effect of ethanol in the brain[84-90]. One of the earliest studies from Lovinger *et al* (1989). showed that acute ethanol exposure can inhibit NMDA-activated current in hippocampal neurons[91]. Later, ethanol inhibition of NMDA receptor activity was also demonstrated by measuring NMDA receptor-mediated excitatory postsynaptic potentials/currents (EPSPs/EPSCs) in slices from many different brain regions, such as cortex[92, 93], amygdala[94], nucleus accumbens[95, 96], hippocampus[91], and dorsal striatum[97, 98]. Similar inhibitory effects of ethanol were also found in HEK cells and *Xenopus* oocytes, expressing recombinant NMDA receptors. Single-channel recordings provided evidence that ethanol decreases the open channel probability and mean open time of NMDA receptors in cultured cortical neurons[92]. Although all of this evidence indicates that ethanol rapidly inhibits NMDA receptor function *in vivo* and *in vitro*, the precise molecular mechanisms of ethanol inhibition on the NMDA receptors have been difficult to determine. By using electrophysiological techniques,

rapidly-inhibited NMDA-evoked currents were detected in response to acute ethanol exposure in a concentration-dependent manner[99, 100], which suggested ethanol directly interacts with NMDA receptors. The questions that remain concern where this small molecule binds in NMDA receptors and how it changes NMDA receptor channel kinetics.

Based on the evidence that alcohol and NMDARs antagonists produce similar inhibitory effects *in vivo* and *in vitro*, studies mainly focused on finding alcohol molecule binding sites in the agonist site or other modulatory sites. However, the effect of ethanol on NMDARs of cultured mouse hippocampal neurons only showed decreased $E_{max}$ values of the NMDARs concentration-response curve without affecting $EC_{50}$ values, which indicates that ethanol inhibit NMDARs activity in a non-competitive manner[86, 99, 101-103]. Whether the co-agonist (glycine) site can mediate ethanol's effects was initially controversial[86, 102-107]. Wright *et al*. (1996) showed that ethanol inhibition of NMDA channels does not involve substantial changes in fast closed state kinetics or changes in open channel conductance, and thus is not attributable to block of the open channel[92]. Peoples *et al*. (1997) showed there is no effect on ethanol inhibition of NMDA-activated currents even in the presence of different concentrations of $Mg^{2+}$ in the cultured cortical neurons[103]. These two evidence indicate that alcohol molecule binding sites are not in the channel pore. Taken together, there is no evidence that alcohol binding sites are in the

extracellular structure of the NMDARs and suggests ethanol may affect NMDARs activities via effects on lipids or other intracellular proteins, such as protein kinase C (PKC). Snell *et al*. (1994) demonstrated the involvement of PKC in ethanol-induced inhibition of NMDARs in cerebellar granule cells[108]. In contrast, Peoples and Stewart (2002) showed C-terminal truncation mutant did not abolish the effect of ethanol on the NMDARs[99]. This result against the possibility that the alcohol inhibition on NMDARs is mediated via intracellular part of the receptors. Results from mutagenesis studies with alcohol- and anesthetic-sensitive γ-aminobutyric acid A ($GABA_A$) and glycine receptors showed that mutation at a serine residue in the second transmembrane (TM2) domain or an alanine residue in the third transmembrane (TM3) domain greatly affected the potentiation of $GABA_A$ and glycine channel function by ethanol and volatile anesthetics[109]. Larger amino acids in the ethanol sensitive positions produce inhibition, while smaller amino acids producing enhanced potentiation. To find the alcohol action sites in the membrane-associated domain, Peoples and Woodward groups started a series of scanning studies in the M domain. Our lab first identified Met-823, a site of alcohol action in the M4 domain of the GluN2A subunit, which can not only alter ethanol sensitivity of GluN2A-containing NMDAR, but can regulate glutamate potency, apparent desensitization, mean open time, and peak current density[110, 111]. The Woodward lab found Phe-639, a site in the M3 domain of the GluN1 subunit, which also can alter ethanol sensitivity[112]. Above evidence confirmed the

hypothesis that alcohol molecule can act on the sites in the membrane-associated domains. In the following studies, a series of ethanol sensitive resides are recognized in both GluN1 and GluN2A subunit, Gly-638 in the M3 domain of the GluN1 subunit, the cognate positions Phe-636, Phe-637, Met-823, Ala825 in GluN2A subunit[110, 111, 113-115] (Figure 1). Taken together, these ethanol sensitive sites are hydrophobic amino acids; are not involved in lining the ion channel lumen; and influence ion channel gating properties.

Because alcohol molecule can act on multiple sites in NMDARs, it is likely that ethanol sensitive residues form a small environment to regulate ethanol sensitivity together. Dual mutations at Phe-637 and Met823 in GluN2A subunit can influence ethanol sensitivity and receptor kinetics, which suggests that these two positions are functionally linked because modulation of ethanol by dual mutants is not additive[116]. Based on the reported structure of the GluA2 glutamate receptor M domains[1], it is possible that sites of alcohol action is formed by groups of 4-6 residues clustered in small regions at the M3-M4 intersubunit interfaces between GluN1 and GluN2A subunit[114] (Figure 1). By using two-way ANOVA and mutant cycle analysis of log-transformed ethanol IC50 values, significant interactions affecting ethanol inhibition was observed at four pairs of positions in GluN1/GluN2A: Gly-638/Met-823, Phe-639/Leu-824, Met-818/Phe-636, and Leu-819/Phe-637[114]. Unlike the interaction between

Phe637 and Met823 in GluN2A subunit, these sites can alter ethanol action in a manner that all side chains of interacted residues are in close proximity[114].

Although accumulated evidence showed the important role of GluN1 and GluN2A subunit in the action of alcohol, it is largely unknown how other GluN2 subunits are involved in the modulation. The protein sequence of M3 and M4 domains is highly conserved through all GluN subunits. So, it is likely that GluN2C subunit also contain alcohol sensitive sites in the M3 and M4 domains. Unlike GluN2A and GluN2B subunit, GluN2C subunit has a limited distribution in cerebellum[20, 25-27]. The GluN2C subunit also can be found in thalamus[25], olfactory bulb[25], oligodendrocytes[117], and hippocampal interneurons[20]. The GluN2C subunit has its unique electrophysiologic and pharmacologic properties that differ from those of the GluN2A and GluN2B subunits. For example, the GluN2C-containing NMDA receptor has a lower open probability, being opens for only ~1% of the time during agonist activation[64], a lower single-channel conductance, shorter open time, lower sensitivity to Mg2+ block[20, 118], and higher affinity for the agonist and coagonist glutamate and glycine[57, 119]. In vivo studies have shown that GluN2C knockout mice have significant deficits in working memory and acquisition of conditioned fear[120], suggesting that GluN2C plays an important role in controlling cerebellum function. 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[121-123]. To fill the gap and better understand the function of GluN2C subunit, we will focus on studying the molecular mechanism of alcohol modulation of GluN2C-containing NMDAR.

# Chapter 2. Material and Methods

## Materials

Ethanol (95%, prepared from grain) will be obtained from Aaper Alcohol & Chemical Co. (Shelbyville, KY), and all other drugs will be obtained from Sigma. Chemicals used to make recording solutions were the highest purity available.

## Molecular Biology, Cell Culture, and Transfection

Site-directed mutagenesis in plasmids containing GluN1 or GluN2C subunit cDNA was performed using the QuikChange II kit (Agilent Technologies, Santa Clara, CA), and all mutants 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 35mm dishes at high-density (approximately $5 \times 10^5$ cells per dish) and transfected with GluN1, GluN2C, and green fluorescent protein (GFP) using the calcium phosphate transfection kit (Invitrogen). 10mM magnesium chloride ($MgCl_2$) 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 24-48h 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 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 containing 150 mm NaCl, 5 mmKCl, 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). 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 (Axon Instruments).

## Cysteine Cross-linking

Wild type and cysteine-substituted mutant receptors were treated with the reducing agent 10mM DTT for 3-5min. In the presence of 300 μM glutamate, steady-state currents were measured. The effect of DTT was calculated by the

equation [(($I_{after}/I_{initial}$) − 1) × 100], where $I_{initial}$ and $I_{after}$ are the stabilized current before and DTT treatment.

## Molecular Modeling

The model of the MD of the heteromeric GluN1/GluN2C NMDAR was generated using the GluA2 (PDB-3KG2) as a template[1]. We first manually aligned the GluN1 and GluN2C sequences with the respective sequences of GluA2 as described in Supplemental Fig2. [1] in Discovery Studio 2.5 (Accelrys, San Diego, CA). Then we mutated Gly-638, Phe-639 and M821 into cysteines.

## Data Analysis

In concentration-response experiments, $IC_{50}$ or $EC_{50}$ and *n* (slope factor) were calculated using the equation $\boldsymbol{y} = \boldsymbol{E}_{max}/1 + (IC_{50}$ or $EC_{50}/\boldsymbol{x})^{\boldsymbol{n}}$, where $\boldsymbol{y}$ is the measured current amplitude, $\boldsymbol{x}$ is concentration, $\boldsymbol{n}$ is the slope factor, and $\boldsymbol{E}_{max}$ 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.

Time constant ($\tau$) of deactivation were determined from fits of the current decay after the removal of glutamate (in the continued presence of glycine) to an

exponential function using Clampfit (Axon Instruments/Molecular Devices). Cells were adequately fitted by a single exponential function.

Significant interactions respect to ethanol sensitivity, steady state current and deactivation among mutants were determined by two-way ANOVA and by mutant cycle analysis[124]. Natural logarithm (ln) transformed values of WT and mutant IC50 or EC50 or time constant ($\tau$) values were used for computing interaction free energies by using the equation $\Delta\Delta \boldsymbol{G}_{INT}$ = RT[ln(WT) + ln(mut1,mut2) − ln(mut1) − ln(mut2)], with propagated errors reported in standard error (SEM). $\Delta\Delta G_{INT}$ ± error were analyzed using one-sample ***t*** test for statistical significance from zero energy, with degrees of freedom (df) = $N_{WT} + N_{MUT1} + N_{MUT2} + N_{MUT1,MUT2} - 4$, where $N_X$ = number of cells used for each combination of wild-type and mutant subunits.

# Chapter 3. A Methionine (Met-821) Position in the M4 Domain of GluN2C Subunit Can Alter Ethanol Sensitivity.

## Introduction

Alcohol, one of the oldest and most widely abused drugs in the world, produces its effects primarily via actions on ion channels in the nervous system. The N-methyl-D-aspartate (NMDA) receptor, a subtype of ionotropic glutamate receptor family, has been demonstrated to be a major target in mediating the inhibitory effects of alcohols in the mammalian brain. Chronic ethanol exposure can result in up-regulation of NMDA receptor function and enhanced glutamate-mediated excitotoxicity[125-128]. The Lovinger and colleagues first showed that acute ethanol exposure can inhibit NMDA-activated currents in hippocampal neurons. In the following studies, ethanol inhibition on NMDARs activities also has been demonstrated by measuring NMDARs-mediated excitatory postsynaptic potentials/currents (EPSPs/EPSCs) in various slices from many different brain regions, such as cortex[92, 93], amygdala[94], nucleus accumbens[95, 96], hippocampus[91], and dorsal striatum[97, 98]. Similar inhibitory effects of ethanol were also observed in cell lines, like HEK293 cells, expressing recombinant NMDARs. In single-channel studies, mean open time and frequency of channel opening are decreased in the presence of ethanol[92].

The NMDAR is heterotetramer, composed of two GluN1 subunits with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits[14-16]. The GluN2 subunit contains four subtypes (A-D) that arise from separate genes, whereas the GluN1 subunit has eight isoforms that are generated by alternative splicing of a single gene[129]. The subunit compositions affects the physiological and pharmacological properties of the NMDARs[8]. All 32 combinations of GluN1/GluN2 subunits have been examined in the presence of 100mM ethanol. Generally, receptors containing GluN2A and GluN2B subunits are more sensitive to ethanol inhibition than receptors containing GluN2C and GluN2D subunits[47], suggesting subunit composition can influence ethanol sensitivity.

Because ethanol and NMDARs antagonists produce similar inhibitory effects on receptor activities *in vivo* and *in vitro*, an initial question was whether ethanol was a NMDAR antagonist binding in the agonist binding site. However, a number of studies showed the effect of ethanol only decreased $E_{max}$ values of the concentration-response curve without affecting $EC_{50}$ values which indicates that ethanol inhibit NMDARs activity in a non-competitive manner[86, 99, 101-103]. Peoples *et al*. (1996) showed ethanol inhibition on NMDARs did not involve substantial changes in fast closed state kinetics, changes in open channel conductance, or block of the open channel[92] and there was no effect on ethanol inhibition of NMDA-activated currents even in the presence of

different concentrations of $Mg^{2+}$ in cultured cortical neurons[103], which indicates that alcohol molecule binding sites are not in the channel pore. Taken together, there is no evidence that alcohol binding sites are in the extracellular structure of the NMDARs and suggests ethanol may affect NMDARs activities via effects on lipids or other intracellular proteins, such as protein kinase C (PKC). Snell *et al*. (1994) demonstrated the involvement of PKC in ethanol-induced inhibition of NMDARs in cerebellar granule cells[108]. In contrast, Peoples and Stewart (2002) showed C-terminal truncation mutant did not abolish the effect of ethanol on the NMDARs[99]. This result argues against the possibility that the alcohol inhibition on NMDARs is mediated via intracellular part of the receptors.

Results from mutagenesis studies with alcohol- and anesthetic-sensitive γ-aminobutyric acid A ($GABA_A$) and glycine receptors showed that mutation at a serine residue in the second transmembrane (TM2) domain or an alanine residue in the third transmembrane (TM3) domain greatly affected the potentiation of $GABA_A$ and glycine channel function by ethanol and volatile anesthetics[109]. Larger amino acids in the ethanol sensitive positions produce inhibition, while smaller amino acids producing enhanced potentiation. Although the sequence and structural homology between $GABA_A$ and glycine and glutamate receptors are extremely low, it is likely that ethanol sensitive sites exist in the M domains of the NMDARs.

The membrane-associated domains (MD) of the NMDARs are composed of four segments, including M1, M3, M4 spanning the membrane[130-133] and M2 forming a reentrant loop[134]. Using the substituted cysteine accessibility method (SCAM)[135], cysteine substitution mutants were generated to identify channel-lining residues of the GluN1 subunit[136]. Within the M1 segment, all nine consecutive positions (W545-H553) were not accessible to the cysteine-specific methanethiosulfonate (MTS) reagents in the presence of glutamate, in contrast the preM1 segment, defined as the segment further N-terminal to the M1 segment, were accessible to both MTS-ethylammonium (MTSEA) and the larger MTS-ethyltrimethylammonium (MTSET). These reagents can covalently link their positively charged -S-$CH_2$-$CH_2$-$NH_3^+$ or -S-$CH_2$-$CH_2$-$N(CH_3)_3^+$ groups to the sulfhydryl groups of cysteines exposed to the water-accessible surface of the channel[134]. The overall pattern of accessibility for MTSEA and MTSET suggests that positions in preM1, but not in M1 itself, are exposed at the water-accessible surface of the extracellular vestibule[136]. The SCAM results also shows that the regions C-terminal to M3 and N-terminal to M4 are also the primary determinants of the extracellular vestibule[136]. The residues in the M3 and M4 domains are mainly involved in channel-lining.

The Woodward group first examined whether positions in the M domains alter ethanol sensitivity. Alanine substitutions at several residues within the preM1, M1, and M2 domains did not produce significant changes in ethanol sensitivity.

In contrast, alanine mutation at GluN1 (Phe-639)/GluN2A yielded significantly less sensitive to ethanol than wild type[137]. Tryptophan substitution at this position produces slightly more sensitivity to ethanol inhibition than wild type. These results may suggest that the physical or chemical properties of the amino acid substitution at this position may be an important determinant of ethanol sensitivity[137]. The Phe-639 residue is highly conserved through all GluN1 and GluN2 subunits, suggesting a key role for this residue. Coexpressing either GluN1 (F639A)/GluN2B or GluN1 (F639A)/GluN2C also produce significantly less sensitivity, which is consistent with the results observed from mutant combination GluN1 (F639A)/GluN2A[137]. Although the F639A mutation significantly reduced ethanol sensitivity of all mutant combinations NDMARs, it did not fully eliminate ethanol inhibition, indicating there may be other ethanol sensitive sites or the cognate sites in the GluN2 subunits also play a critical role in altering ethanol sensitivity.

Based on the assumption that additional site or sites of alcohol action in one of the M domains exists, we used tryptophan scanning mutagenesis and found a highly conserved methionine residue in the M4 domain of GluN2A subunit can influence NMDARs alcohol sensitivity in a manner that is related both to desensitization of the ion channel and the physical and chemical properties of the substituent amino acid[111]. GluN2A(M823C), GluN2A(M823S), and GluN2A(M823W) produced the lowest sensitivity to ethanol, whereas

GluN2A(M823F), GluN2A(M823L), and GluN2A(M823Y) resulted in the highest sensitivity to ethanol[111]. In the studies of alcohol action of $GABA_A$ and glycine receptors, it has been shown that the physical and chemical properties of substituent amino acid play a determinant role of ethanol sensitivity. Linear regression analysis of alcohol sensitivity versus hydropathy, hydrophilicity, hydrogen bonding, molecular volume, and polarity of the substituent was performed and significant linear relationships were observed between ethanol $IC_{50}$ and both hydrophilicity and molecular volume[111].

It should be noted that either GluN1 (Phe-639) or GluN2A (Met-823) are not able to completely eliminate the inhibitory effects of alcohol, which indicates alcohol molecule acts on multiple sites of the NMDARs. In the following studies of scanning ethanol sensitive sites, a series positions of alcohol action are identified in both GluN1 and GluN2A subunit, Gly-638 in the M3 domain of the GluN1 subunit, the cognate positions Phe-636, Phe-637, Met-823, Ala825 in GluN2A subunit[110, 111, 113-115]. Taken together, these ethanol sensitive sites are hydrophobic amino acid; are not involved in lining the ion channel lumen; influence ion channel gating properties.

In previous studies of the role of GluN1 (Phe-639) in alcohol sensitivity modulation, similar significant reduced ethanol sensitivity were observed among the different GluN2 subunit containing NMDARs[137]. Although

GluN2C-containing NMDAR is much less sensitivity to ethanol compared to GluN2A- and GluN2B-containing NDMARs, the mutant combination GluN1 (Phe-639)/GluN2C is still markedly less than its wild type[137]. The methionine residue is highly conserved among all GluN2 subunits and has been shown to play a critical role in alcohol action modulation in GluN2A-containing NMDAR[111]. So, it is likely this methionine residue can influence ethanol sensitivity in the GluN2C-containing NMDAR. Figure 2 shows the model of the GluN2C subunit with the membrane-associated domains (M1-M4), and the presumed location of Met-821 residue in the M4 domain of GluN2C subunit.

## Results

### *Ethanol Inhibition by Various GluN2 Subunits at the Methionine Residue in M4.*

According to the previous studies in this laboratory, a methionine residue in M4 domain of the GluN2A subunit not only regulates ethanol sensitivity, but influences ion channel gating[110, 111]. To determine whether the effects of the methionine residue on ethanol sensitivity are consistent through the GluN2 subunits, GluN2A (M823W), GluN2B (M824W), and GluN2C (M821W) were coexpressed with GluN1 subunit. Surprisingly, ethanol sensitivity of GluN2 subunits with tryptophan substitution at the methionine residue exhibited

differently that determined for their respective wild type counterparts (Figure 3). As reported previously, GluN2A (M823W) showed significantly decreased ethanol sensitivity[111]; in contrast, GluN2C (M821W) showed significantly increased ethanol sensitivity. There was no significant difference between wild type GluN2B and GluN2B (M824W). The slope factors of the ethanol concentration-response curves did not differ significantly among the subunits.

***Mutations at Met-821 Can Alter Ethanol Sensitivity and Glutamate Activation.***

Since the GluN2C (Met-821) position involved in altering ethanol sensitivity, we made 14 mutations at the methionine position to determine how this position regulates ethanol sensitivity. There were 7 mutations yielded functional receptors and were tested inhibited by ethanol in a concentration-dependent manner. Ethanol $IC_{50}$ values among the mutants varied ranging from 138 to 215 mM (ANOVA, $p < 0.0001$).The slope factors of the ethanol concentration-response curves did not differ significantly among the various mutants. Expression of the mutant subunits GluN2C (M821L), GluN2C (M821S), GluN2C (M821W) with GluN1 subunits resulted in the highest sensitivity to ethanol. Tryptophan, the largest and most hydrophobic amino acid, unsurprisingly produced the greatest effect of alcohol action on the receptor with ethanol $IC_{50}$ value of 138 mM. Alanine, the smallest hydrophobic amino

acid that is unlikely to destabilize α-helical regions, is predicted to eliminate or diminish hydrophobic interactions present between larger side chains. However, GluN2C (M821A) did not alter ethanol sensitivity(Figure 4).

Surprisingly, a number of amino acid substitutions at this site yielded nonfunctional receptors. M821D, M821F, M821G, M821N, M821R, M821V, M821Y exhibited abnormal ion channel function. The abnormal functional mutants exhibited small spontaneous currents with apparent ethanol inhibition. This is the first time in this laboratory we observed ion channel behavior like these mutants(Figure 4).

***Ethanol Sensitivity is Independent of the Physical and Chemical Properties of the Substituent at GluN2C (Met-821).***

In the previous study showing that GluN2A (M823W) can alter ethanol sensitivity of the GluN2A-containing NMDAR, a linear relation was observed between ethanol $IC_{50}$ and hydrophilicity ($R^2$ = 0.522; $p < 0.05$) and molecular volume ($R^2$ = 0.683; $p < 0.005$) of the substituent[111]. If the observed changes in ethanol sensitivity among the GluN2C (Met-821) mutant subunits were due to a direct interaction of ethanol with this site, a significant linear relation of ethanol $IC_{50}$ with the physical and chemical properties of the amino acid substituent at this site is expected. To evaluate the relative contribution of the

physicochemical parameters of the amino acid at this site to alcohol sensitivity, linear regression analysis of ethanol $IC_{50}$ values versus hydropathy, hydrophilicity, molecular volume and polarity of the substituent were performed[111]. There was no significant linear relation observed between ethanol $IC_{50}$ values and hydropathy ($R^2$ = 0.007; $p$ > 0.05), hydrophilicity ($R^2$ = 0.09; $p$ > 0.05), molecular volume ($R^2$ = 0.205; $p$ > 0.05), or polarity ($R^2$ = 0.047; $p$ > 0.05) (Figure 5). Although we did not detect any correlation, we observed a positive trend between ethanol $IC_{50}$ values and hydrophilicity, and a negative trend between ethanol $IC_{50}$ values and molecular volume, which may due to missing plots. There were seven mutations that showed abnormal glutamate activation.

***Triheteromeric GluN1/GluN2A/GluN2C M821G NMDAR Can Restore Ion Channel Gating Function.***

Mutations at the methionine residue yielded abnormal functional mutants, including M821D, M821F, M821G, M821N, M821R, M821V, M821Y. It is likely that the $P_o$ of these mutants may be too low for detecting glutamate-activated currents. So, we coexpressed GluN1, GluN2A, GluN2C subunits to form triheteromeric NMDARs, in which GluN2A subunit increases the $P_o$ of the receptors and stabilize the conformation to induce channel gating. Because GluN2A subunit is supposed to contain a high affinity $Zn^{2+}$ binding site in the

ATD[138], whereas GluN2C subunit does not, diheteromeric GluN1/GluN2A NMDAR is highly sensitivity to $Zn^{2+}$ modulation. 200nm $Zn^{2+}$ is sufficient to cause almost 100% inhibition on the diheteromeric GluN1/GluN2A NMDAR[139]. Triheteromeric GluN1/GluN2A/GluN2C NMDAR restores half sensitivity to nanomolar $Zn^{2+}$ modulation[139]. We observed similar effects that 200 $Zn^{2+}$ inhibited almost all diheteromeric GluN1/GluN2A NMDAR and diheteromeric GluN1/GluN2C, GluN1/GluN2C M821G NMDARs was not sensitive to nanomolar concentration $Zn^{2+}$ inhibition and triheteromeric GluN1/GluN2A/GluN2C NMDAR restores half sensitivity to $Zn^{2+}$. We then coexpressed triheteromeric GluN1/GluN2A/GluN2C M821G NMDAR, which yielded functional receptor and Zn2+ produced half inhibition on the triheteromeric receptor(Figure 6). The functional triheteromeric GluN1/GluN2A/GluN2C M821G NMDAR seems to restore agonist sensitivity because a GluN2A subunit may induce receptor gating by inducing activation of the GluN2C (M821G) subunit.

## Discussion

The results of the present study demonstrate that mutations at the highly conserved methionine (Met-821) in the M4 domain of the GluN2C subunit can influence ethanol sensitivity of the GluN2C-containing NMDAR. As expected, the largest amino acid tryptophan produces largest change in the ethanol $IC_{50}$

values compared to wild type. In contrast, an alanine mutation at this position did not yield any significant change of ethanol sensitivity. These results are not in agreement with the hypothesis that alanine would alter ethanol sensitivity by eliminating or diminishing hydrophobic interactions present between larger side chains. We also observed a number of amino acid substitutions at this site yielding abnormal functional receptors, which did not exhibit glutamate-activated currents, but showed apparent ethanol inhibition in the presence of both glutamate and ethanol. Although it has been reported in the GluN2A (Met-823) paper that several nonfunctional receptors were yielded when methionine was replaced by other amino acid[111], this is the first observation that the channel did not open properly, but still responded to the inhibitory effects of ethanol.

Although diheteromeric GluN1/GluN2C (M821G) has abnormal function, triheteromeric GluN1/GluN2A/GluN2C (M821G) restored ion channel gating function. GluN2C-containing NMDAR has an extremely low open probability ($P_o$~1%)[64], and a lower single-channel conductance of approximately 35pS[20, 118]. In contrast, NMDAR containing GluN2A subunit has a 50-fold greater open probability ($P_o$~50%) compared to GluN2C-containing NMDAR[140]. The subunit-specific gating of NMDARs is controlled by the region formed by the ATD of the GluN2 subunit[140]. Moreover, single-channel analysis from mutations at GluN2A (M823) showed the substitutions at this

position can alter ion channel gating[110]. Taken together, it is likely that abnormal functional mutants have an extremely low open probability, which resulted in negligible glutamate-activated currents, but the coincidently opened channel still responded to ethanol and exhibited ethanol inhibition of these currents. Triheteromeric GluN1/GluN2A/GluN2C (M821G) NMDAR containing an ATD from the GluN2A subunit increases open probability and induces the channel to gate properly.

The functional mutations at Met-821 residue all produced glutamate-activated currents and ethanol-inhibited currents. The ethanol $IC_{50}$ values among the mutants are all different: M821L, M821S, and M821W mutants produced the highest sensitivity to ethanol. Taken together with our previous studies in GluN2A[111], this suggests that some physical or chemical properties of the amino acid substitution at this position may be an important determinant of ethanol sensitivity[109]. We thus expected a significant linear relation of ethanol $IC_{50}$ with the physical and chemical properties of the amino acid substituent at this site. However, the results showed no correlation between ethanol $IC_{50}$ values and any physicochemical parameters of the amino acid, suggesting ethanol sensitivity is not simply represented by physical and chemical properties of the substituent at GluN2C (Met-821).

It has been reported that alcohols and anesthetics bind to the sites between M2 and M3 domains of $GABA_A$ and glycine receptors and regulate channel function in a molecular volume dependent manner[109, 141-143]. Our lab reported a similar volume occupation effect at GluN2A (Met-823): a significant linear relation of ethanol sensitivity with molecular volume was observed when the value for the tryptophan mutant was excluded from the analysis[111]. All the evidence above suggested that the action of alcohol molecule involves filling a critical volume in a cavity formed in part by this site and the presence of a tryptophan, with the largest side chain, severely disrupts normal channel function and ethanol inhibition[111]. However, in the case of the GluN2C subunit, the increased ethanol sensitivity of the tryptophan mutant at Met-821 is in agreement with the volume occupation theory, and suggests that tryptophan does not disrupt channel function or the ability to interact with the alcohol molecule.

In the study of GluN1(Phe639), it has been shown that F639W can alter ethanol sensitivity in a non GluN2 subunit dependent way such that any GluN2 subunit coexpressed with GluN1(F639W) showed reduced inhibitory effects of 100 mM ethanol compared to their respective wild type counterparts[137]. Although like the phenylalanine residue, the methionine residue in the M4 domain is highly conserved through all GluN2 subunits, comparison between wild type and tryptophan mutants among GluN2 subunits showed that the methionine

position regulates ethanol sensitivity in a GluN2 subunit dependent manner. GluN2A (M823W) showed significantly decreased ethanol sensitivity, whereas GluN2C (M821W) significantly increased ethanol sensitivity, and there was no significant difference in ethanol sensitivity between the wild type GluN2B and GluN2B (M824W). These results are consistent with our previous evidence that alcohol action involves multiple adjacent residues that form a small environment to regulate ethanol sensitivity together[114]. In addition to the Met-823 residue, our lab found a phenylalanine residue in the M3 domain of the GluN2A subunit, the cognate site of GluN1 (Phe-639). Dual mutations at Phe-637 and Met823 in GluN2A subunit can influence ethanol sensitivity and receptor kinetics, which suggests that these two positions are functionally linked because modulation of ethanol by dual mutants is not additive[116]. Functional interactions not only occur between the residues within M3 and M4 domains in the same subunit, but direct interactions can also be observed between residues in the M3 and M4 domains from different subunits. By using two-way ANOVA and mutant cycle analysis of log-transformed ethanol IC50 values, significant interactions affecting ethanol inhibition were observed between multiple intersubunit M3/M4 domain pairs, including GluN1 (G638W)/GluN2A(M823W) [114]. Unlike the interaction between Phe637 and Met823 in the GluN2A subunit, the side chains of these interacting residues are in close proximity[114].

Considering the important role of GluN2C (Met-821) in regulation of alcohol action, we studied its role in altering ion channel gating and its possible interactions with other residues to affect ethanol sensitivity in the following studies.

# Chapter 4. A Methionine In the M4 Domain of the GluN2C Subunit Can Alter Ion Channel Gating.

## Introduction

Ionotropic glutamate receptors, including NMDA, AMPA, kainate, and delta receptors, are integral membrane proteins composed of four large subunits, which form a central ion channel pore. High similarity in sequence alignment among the ionotropic glutamate receptors family suggests that these receptors share a similar architecture. A glutamate receptor subunit contains four discrete semiautonomous domains: the amino-terminal domain (ATD), the ligand-binding domain (LBD), the membrane-associated domain (MD), and the carboxyl-terminal domain (CTD)[1].

The ATDs share sequence homology and structurally similarities with the LBD[1]. To study the function of the ATDs, numerous mutant subunits have been constructed including those lacking the entire ATD[73, 144-151]. Truncations in the ATD do not affect receptor assembly, and there are functional similarities between the truncated mutants and wild type receptor, suggesting a nonessential, regulatory role of the ATD for core function[10]. Evidence that the ATD can influence open probability, deactivation, desensitization, and subunit-specific assembly is consistent with regulatory

roles of the ATD[72, 140, 152-155]. Moreover, the ATD also harbors binding sites for divalent cations, such as $Zn^{2+}$[72], and subunit-selective negative allosteric modulators, such as ifenprodil[156]. The ATD has a clamshell-like structure, composed of two lobes-R1 and R2, tethered together by loops[150]. Binding of $Zn^{2+}$ to GluN2A or ifenprodil to GluN2B subunits seems to stabilize a closed-cleft conformation of the ATD[150].

The LBD also has a clamshell-like architecture containing two lobes-S1 and S2, which are structurally similar to the ATD[157]. The S1 was identified by the region of extracellular N-terminal domain preceding the PreM1 domain, and S2 is the loop between the M3 and M4 domains[157-160]. Between the S1 and S2 segment, there is an agonist binding pocket[1, 161, 162]. The activation of glutamate receptors involves simultaneous binding of agonists, such as glutamate, NMDA, and the coagonists, such as glycine or D-serine, to the GluN2 and GluN1 subunits, which leads to conformational change to enclose the agonists in the binding sites[157, 161, 163]. This conformational event triggers the subsequent transition of the channel pore into an open state[10, 164, 165]. In the open state, both the LBD and MD are in an unstabilized state, and stability can be restored by LBD reopening to allow agonist dissociation[10, 164, 165]. In the agonist-bound state, the LBD dimer interface will go through a rearrangement, allowing the receptor to enter a desensitized state[166-168]. Other forms of apparent NMDAR desensitization are related to glycine[60, 61]

or intracellular $Ca^{2+}$[61-63], and real or apparent ion channel desensitization can influence the amplitude, duration, and following frequency of NMDA-mediated synaptic events[61].

The MD is connected to the LBD by three short peptide linkers[1]. The MD of the NMDARs is composed of four segments, M1, M3, and M4 which span the membrane[130-133] and M2 which forms a reentrant loop[134]. The transmembrane helices M2 and M3 from each of the four subunits contribute to formation of the channel pore. In the previous chapter, we have discussed SCAM results of the MD. According to the cysteine substitution channel-lining experiments, the overall pattern of accessibility for MTSEA and MTSET suggests that preM1, but not M1 itself, are exposed at the water-accessible surface of the extracellular vestibule and the regions C-terminal to M3 and N-terminal to M4 are also the primary determinants of the extracellular vestibule[136].

Unlike other domains, the CTD is the most diverse one, varying in sequence and length among the glutamate receptor family[10]. The CTD mainly involves in regulatory effects on receptor localization and function. The CTD of GluN1 subunit contains a number of regulatory protein binding sites, including sites for Ca2+/calmodulin[169], scaffold proteins as well as phosphorylation sites for protein kinase A (PKA), and protein kinase C (PKC)[170, 171].

Although the functional role of each structural element in the entire subunit has been well characterized, the nature of the conformational changes and molecular determinants underlying NMDARs are still largely unknown. Numerous evidence shows that the LBD is structurally and functionally linked to the MD. The dynamics between these two structural elements can affect each other reciprocally. From the early work on GluN1 (F639W), it is known that this position not only alters alcohol action of the NMDARs, but affects channel properties, such as glycine potency. The increased affinity for glycine but not glutamate[137] is consistent with the evidence that GluN1 subunit contains glycine binding site, whereas GluN2 subunits provide glutamate binding sites[1]. Our previous work on GluN2A (M823W) showed that mutation at the methionine position only altered the steady state glutamate $EC_{50}$ values, instead of glycine $EC_{50}$ values[110]. Taken together, sites in the MD also can influence ion channel gating and may be involved in transducing agonist binding into ion channel gating[110, 172, 173]. Mutations at MD not only affect agonist potencies, but receptor kinetics, such as mean open time (MOT), deactivation, and desensitization. GluN2A (M823W) mutant receptors have dramatically altered apparent desensitization, and increased mean open time[110]. GluN2A (F637W) and (F636W) mutant receptors also show alterations in glutamate potency, desensitization and MOT.

## Results

### *Effects of mutations at GluN2C (Met-821) on glutamate potency and desensitization.*

According to previous work on the cognate site GluN2A (Met-823), mutations at this position not only affected alcohol action of the receptors, but physiological characteristics, such as glutamate potency and desensitization. To test whether the GluN2C (Met-821) residue can also affect glutamate sensitivity and desensitization, we performed concentration-response experiments for glutamate in the functional mutants using a rapid solution exchange apparatus in lifted cells. For the functional mutants, significant differences were obtained in the $EC_{50}$ values for glutamate-activated peak ($P$<0.001; ANOVA) and steady-state ($P$<0.001; ANOVA) current and for the steady-state to peak current ratio ($I_{ss}$:$I_p$; $P$<0.0001; ANOVA). The $EC_{50}$ values for glutamate-activated peak current were altered among the majority of functional mutants, but the $EC_{50}$ values for steady-state current were changed only in the M821I, M821L, and M821S mutants. Surprisingly, the tryptophan mutant only had a difference in the peak $EC_{50}$ value, but not steady-state $EC_{50}$ value. The slope factors of the glutamate concentration-response curves for both peak and steady-state did not differ significantly from the wild type values in any of the mutants. Apparent desensitization was affected by most of the

mutations, even when steady-state $EC_{50}$ values were unchanged. It also should be noted that there was no observable desensitization in the wild type GluN2C subunit(Figure 7).

In the previous studies, we reported that a linear correlation was obtained between either peak $EC_{50}$ values *versus* steady-state $EC_{50}$ values or peak/steady-state $EC_{50}$ values *versus* the steady-state to peak current ratio ($I_{ss}$:$I_p$)[110, 113]. However, we did not obtain any kind of correlation (Figure 8).

***Effects of GluN2C (Met-821) Mutants on Deactivation.***

One of the prominent features of glutamate receptors in gating kinetics are time course of deactivation, which mediate a majority of excitatory synaptic transmission. One unique physiological property of GluN2-containing NMDAR is unusually prolonged deactivation time course following the removal of glutamate. To measure the maximal response, the co-agonist glycine was present in all solutions (50μM), which saturated all the glycine binding sites, and current responses were evoked with 300μM glutamate. GluN2C-containing NMDAR deactivated slowly with a single exponential time course with time constants ranging from 1098 to 2183 ms (Figure 9). We subsequently analyzed time constants of deactivation for other mutants at the Met-821 position. There was a significant difference among the mutants compared to wild type

($P$<0.0001; ANOVA). M821L, M821T, M821W mutants have the most significant changes for the deactivation. It should be noted that the steady-state $EC_{50}$ value of tryptophan mutant does not have difference compared to wild type receptor, while deactivation time course of this mutant becomes faster than wild type.

## Discussion

The results of the present study demonstrate that alcohol sensitive position Met-821 in the M4 domain of the GluN2C subunit is involved in regulating ion channel gating and receptor kinetics. The tryptophan mutant can alter glutamate-activated peak current, but not steady-state current $EC_{50}$ values. The steady-state to peak current ratio ($I_{ss}$:$I_p$) also changed because of the attribution of the significantly increased peak $EC_{50}$ values. We performed linear regression analysis between either peak $EC_{50}$ values *versus* steady-state $EC_{50}$ values or peak/steady-state $EC_{50}$ values *versus* the steady-state to peak current ratio, and found that there was no correlation between any of these pharmacological parameters. In the studies of cognate site GluN2A (Met-823), we reported that steady-state $EC_{50}$ values were highly correlated with steady-state to peak current ratio, which was interpreted as agonist trapping on desensitized receptors causing increased affinity[110]. The present studies of GluN2C (Met-823) showed that a majority of mutations at these position significantly increase

the peak $EC_{50}$ values without affecting the steady-state $EC_{50}$ values. In contrast, mutations at GluN2A (Met-823) only affected steady-state $EC_{50}$ values instead of peak $EC_{50}$ values[110]. Taken together, the underlying mechanism at Met-821 appears to differ from the trapping mechanism observed at GluN2A (Met-823).

GluN2C-containing NMDAR deactivated slowly with a single exponential time course with time constants ranging from 1098 to 2183 ms, which is approximately 40-fold longer compared to receptors containing GluN2A[66, 174] . The deactivation tau of the M821W mutant 1291±160 ms, is significantly different from wild-type and other mutants at this position. Several lines of investigation have identified there are several factors that can regulate deactivation time constant of NMDARs, such as the rates of ligand association and dissociation, which are the primary determinants of the deactivation time course[10]. We have showed that dual tryptophan mutant combinations GluN1 (L819W)/GluN2A (F637W) can significantly interact with each other to alter deactivation[114]. The observations in this study provide additional evidence that mutations at MD also can affect time course of deactivation.

# Chapter 5. A Methionine (Met-821) Position in the M4 Domain of GluN2C Subunit Can Interact with Positions in the M3 Domains of GluN1 Subunit.

## Introduction

Ethanol is one of the common drugs of abuse and acts at high concentrations (millimolar) on multiple targets in the central nervous system to regulate neuronal activities[2, 3]. Among those are mainly channels gated by the neurotransmitter glutamate, the major excitatory neurotransmitter in the mammalian brain. The N-methyl-D-aspartate (NMDA) receptors, subtypes belonging to the glutamate receptor family, play the most crucial role for the inhibitory effect of ethanol[84, 85]. The first evidence that ethanol inhibited NMDAR evoked currents in hippocampal neurons was shown by Lovinger *et al*. (1989)[91]. Subsequent studies using radiolabeled neurotransmitter release, calcium uptake, and ratiometric calcium indicators confirmed that NMDARs are inhibited by alcohol across a wide range of brain regions[175]. Although there is numerous studies leading to the conclusion that ethanol inhibits NMDAR function, the mechanism of alcohol action remains unclear. The inhibitory effect of alcohol action on NMDA-evoked currents is in a voltage-independent manner and is not involved in altering single channel conductance, suggesting that alcohol molecule does not act as an ion channel pore blocker[92, 176]. There is also evidence showing that ethanol inhibition is not relate to competitive

inhibition at the glycine and glutamate binding sites[86, 101, 103, 107, 123], or allosteric modulation[103, 121]. In addition, although the phosphorylation sites in the CTD, in some instances, modulate ethanol sensitivity of the NMDARs[95, 175, 177], receptors lacking the CTD still show sensitivity to ethanol[99].

Ronald et al. first found a phenylalanine residue (Phe-639) in the M3 domain of the GluN1 subunit influences alcohol sensitivity[137]. The previous studies from our laboratory also identified a number of alcohol sensitive sites, including two phenylalanine residues (Phe-636) and (Phe-637) in the M3 domain of the GluN2A subunit[113, 115], a methionine residue (Met-823) and an alanine residue (Ala-825) in the M4 domain of the GluN2A subunit[111, 114]. Although these residues can alter ethanol sensitivity of NMDARs, mutations at these sites did not abolish ethanol inhibition. Studies in $GABA_A$ and glycine receptors showed residues in transmembrane domains two and three forming sites of alcohol and anesthetic action[109, 178]. Based on these results, we tested the role of dual tryptophan mutants GluN2A (F637W M23W) in influencing ethanol sensitivity and receptor function. We observed that dual mutations at Phe-637 and Met823 in GluN2A subunit can interactively influence ethanol sensitivity and receptor kinetics, which suggests that these two positions are functionally linked because modulation of ethanol by dual mutants is not additive[116]. After this study was performed, the high-resolution structure of an ionotropic glutamate receptor was reported [1]. Our group subsequently showed that sites

of alcohol action are formed by groups of 4-6 residues clustered in small regions at the M3-M4 intersubunit interfaces between GluN1 and GluN2A subunit[114]. By using two-way ANOVA and mutant cycle analysis of log-transformed ethanol IC50 values, significant interactions affecting ethanol inhibition were observed at four pairs of positions in GluN1/GluN2A: Gly-638/Met-823, Phe-639/Leu-824, Met-818/Phe-636, and Leu-819/Phe-637[114] (Figure 1). Unlike the functional interaction between Phe637 and Met823 in GluN2A subunit, these side chains appear to physically interact with one another, which is consistent with the proposed model that places these pairs of positions in close proximity [114].

Unlike GluN2A-containing NMDAR, which predominates in mammalian brain and has been shown to be involved in multiple brain functions, GluN2C-containing NMDAR are mainly expressed in cerebellar granule neurons[20, 25-27], thalamus[25], olfactory bulb[25], oligodendrocytes[117], and hippocampal interneurons[20]. The GluN2C subunit has unique electrophysiological and pharmacologic properties that differ from those of the GluN2A subunit. For example, the GluN2C-contianing NMDA receptor has a lower open probability, being open for only ~1% of the time during agonist activation[64], a lower single-channel conductance, shorter open time[20, 118], higher affinity for the agonist and coagonist glutamate and glycine[57, 119], and much less sensitivity to ethanol compared to GluN2A-containing NMDAR[121-123]. To understand the role of GluN2C subunit in regulating ethanol sensitivity and channel gating

function, the cognate position Met-821 in the M4 domain was substituted by tryptophan and showed different alcohol sensitivity, glutamate potency and desensitization. Dual tryptophan mutants GluN1 (G638W)/ GluN2C (M821W) and GluN1 (F639W)/ GluN2C (M821W) showed abnormal channel gating activity in the presence of glutamate, which may suggest apparent interactions between these two pairs of positions at the intersubunit interface in the M3 and M4 domains of the GluN1 and GluN2C subunits. In this study we introduced cysteine mutations in the GluN1 at G638, F639 and in the GluN2C at M821. Significant changes in ethanol sensitivity, agonist affinity, and deactivation have been observed and mutant cycle analysis also showed an interaction between the dual cysteine mutants. Based on these findings, we propose that two pairs of positions can interact and play a crucial role for ethanol sensitivity modulation and channel gating activity.

## Results

### *Effects of Dual Tryptophan Mutants on Glutamate Activation.*

Our previous studies have identified significant interactions altering ethanol sensitivity between the GluN1 (G638) and GluN2A (M823) positions[114]. We predicted that the cognate sites in GluN2C-containing NMDAR M3-M4 intersubunit interfaces would exhibit a similar interaction consistent with a site

of alcohol action. Using tryptophan substitution, we first tested whether single tryptophan mutants at GluN1 (G638), GluN1 (F639), GluN2C (M821) could alter ethanol sensitivity. Tryptophan is the largest amino acid and has consistently produced the greatest effect on ion channel behavior in previous studies from this laboratory[110, 111, 113-115]. All of the single tryptophan mutants exhibited increased alcohol sensitivity, in which the ethanol $IC_{50}$ value was significantly decreased ($p < 0.0001$; ANOVA) (Figure 10). We next coexpressed either GluN1 (G638W) or GluN1 (F639W) with GluN2C (M821W), which is predicted to be in close proximity. However, neither G638W/M821W nor F639W/M821W exhibited observable glutamate-activated currents.

***Coexpressed Cysteine Mutants in the M3 and M4 Domains of GluN1 and GluN2C Subunits Interact to Regulate Ethanol Inhibition.***

The GluN1 (G638W)/GluN2C (M821W) GluN1 (F639W)/GluN2C (M821W) dual tryptophan mutants were not sensitive to glutamate, but both mutants appeared to respond to ethanol, in that very small apparent spontaneous currents in these mutants were inhibited by 100mM ethanol. These may be due to the side chain of tryptophan at one position, which may interact with the side chain of the other one to disrupt the ion channel gating activity. To test whether the sites in the M3 and M4 domains intersubunit interfaces can interact, we made cysteine substitution at G638, F639 and M821 positions. All of the single cysteine

mutants did not alter ethanol sensitivity, but ethanol $IC_{50}$ value in the mutant combination G638C/M821C differed significantly from the wild type ($p < 0.0001$; ANOVA) (Figure 11). Interestingly, we observed the opposite results in the mutations F639C/C744A and F639C/C744A/M821C, in which the redox site Cys744 is substituted by alanine, decreased ethanol sensitivity. We next used both two-way analysis of variance (ANOVA) on log-transformed $IC_{50}$ values and mutant cycle analysis to calculate whether there were significant free energy changes. We found an interaction between the mutant combinations of G638C/M821C and G638C/C744A/M821C, as both types of analysis were statistically significant. In contrast, we did not detect any interaction in F639C/M821C, but in F639C/C744A/M821C (Figure 12).

***Coexpressed Cysteine Mutants in the M3 and M4 Domains of GluN1 and GluN2C Subunits Can Regulate Glutamate Potency.***

To determine whether dual cysteine mutants also influence glutamate potency, we compared $EC_{50}$ values for glutamate activation of steady-state currents. Both single cysteine mutants GluN1 (F639C) and GluN2C (M821C) showed different glutamate $EC_{50}$ values and highly significant differences were obtained in dual cysteine mutants F639C/C744A/M821C ($p < 0.0001$; ANOVA) (Figure 13). Interestingly, although G638C/M821C exhibited significantly altered $IC_{50}$ value, it was not involved in changing steady-state glutamate potency;

F639C/M821C did not change ethanol sensitivity, but varied the agonist affinity. After removing the redox site Cys744, we observed both dual cysteine mutants G638C/C744A/M821C and F639C/C744A/M821C significantly altered glutamate $EC_{50}$ values.

***Coexpressed Cysteine Mutants in the M3 and M4 Domains of GluN1 and GluN2C Subunits Can Regulate NMDA Receptor Kinetics.***

Our previous results have shown that dual tryptophan mutants GluN1 (G638W)/GluN2A (M823W) can interact to alter NMDA receptor kinetics[114]. If the side chain of cysteine at G638 or F639 in the GluN1 M3 domains interact with the side chain of cysteine at M821 in the GluN2C M4 domains and are involved in regulating ion channel function, dual cysteine mutants should also affect relevant ion channel behaviors. We measured time of decay among the mutants: the cysteine mutants containing redox site Cys744 all showed markedly decreased time constant of deactivation compared to wild type, while the mutants with C744A all increased the time of decay. We observed extremely prolonged deactivation in the mutant GluN1 (F639CC744A)/GluN2C (Figure 14). Then, we analyzed interactions among the dual cysteine mutants respect to deactivation. In the group containing redox site C744, although time constants of deactivation differed among the various mutants, we did not observed significant interactions with respect to deactivation between

G638C/M821C and F639C/M821C; while in the group with C744A, significant interaction was obtained in the mutant combination F639C/C744A/M821C (Figure 15).

***Cross-linking Between Cysteine Substitutions in the M3 and M4 Domains of GluN1 and GluN2C Subunits.***

To confirm whether G638/F639 and M821 are sufficiently spatially proximal to interact, we examined the effects of applying the reducing reagent DTT among the mutants. Previous work from other laboratories has reported that a pair of cysteine residues, Cys744 and Cys798 in GluN1, is responsible for the potentiation of GluN1/GluN2A by DTT, and that redox modulation can be completely abolished in the mutant GluN1 (C744A, C798A)/GluN2C[179-181]. To eliminate the effect of endogenous redox sites, we substituted cysteine at C744 with alanine. However, we did not observe DTT-potentiated currents in either GluN1/GluN2C or GluN1(C744A)/GluN2C. We then examined the effects of DTT on all the single and dual cysteine mutations. As expected, significant potentiation was only obtained among dual cysteine mutants. There was no effect of DTT treatment on current amplitude in any single cysteine mutant. In contrast, when either the GluN1 (G638C) or GluN1 (F639C) mutants (with or without the C744A mutation) were expressed with GluN2C (M821C), DTT treatment significantly increased current amplitudes (Figure 16). We also

compared time constants of deactivation after DTT exposure among the mutants, and observed significant increases in time constants of decay in dual cysteine mutants containing the Cys744 residue, but not in those containing C744A (Figure 17).

## Discussion

These experiments were aimed at determining whether residues in GluN1 M3 and GluN2C M4 at the intersubunit interfaces interact to alter ethanol sensitivity and NMDA receptor ion channel behavior. We have observed that single tryptophan substitutions at GluN1 (G638), GluN1 (F639), or GluN2C (M821) can alter ethanol $IC_{50}$ values, but neither GluN1 (G638W)/GluN2C (M821W) nor GluN1 (F639W)/GluN2C (M821W) receptors exhibit glutamate-activated current. However, both dual tryptophan mutants exhibit small-amplitude spontaneous currents that respond to ethanol. Apparent ethanol inhibition has been observed between these two mutants. These results are mostly in agreement with the results from our previous findings[114] and suggest apparent interaction between G638W/M821W or F639W/M821W may be too strong to disrupt NMDA receptor function. Based on the x-ray crystallographic structure of the GluA2 subunit[1] and the structural model of the NMDAR M domains[114], we predict these two pairs of residues are in close proximity to produce effects on ethanol sensitivity and NMDA receptor function.

Our previous work demonstrated that the side chains of one amino acid can interact with one another to alter ethanol sensitivity[114]. In this study, we made cysteine mutations at the above positions to determine the effects of amino acid side chain interaction. Using both two-way ANOVA and mutant cycle analysis, significant interaction was observed between GluN1 (G638C)/GluN2C (M821C), which is consistent with our previous results[114]. Redox site modulation experiments also demonstrated that a disulfide-bond is spontaneously formed between these two positions that alters current amplitude, and that the disulfide bond can be reduced by DTT to enhance glutamate-activated currents. Although this study does not directly answer the question about which coexpressed tryptophan mutations at GluN1 (G638)/GluN2C (M821) interacts to alter ethanol sensitivity and NMDA receptor function, we have demonstrated that the side chain of one cysteine can interact with one another in mediating the action of alcohol. These results are consistent with our previous findings in GluN1 (G638W)/GluN2A (M823W)[114].

NMDARs are regulated by an extracellular redox state and both GluN1 and GluN2 subunits are involved in redox modulation. Two disulfide bonds have been found within GluN1 subunit, including Cys744 and Cys798 in the LBD and SVC79ED and RGC308VG in the ATD[179, 181]; Cys87 and Cys320 are proposed to form a disulfide bond within the ATD of GluN2A subunit[150]. The

disulfide bonds formed within the ATD are responsible for the modification of the $Zn^{2+}$ binding site[179], so in the present study, we only considered the endogenous redox site Cys744 and Cys798 and mutated the cysteine at C744 residue into alanine. In the study of ethanol inhibition, we found that only the dual cysteine mutant G638C/M821C altered ethanol sensitivity, as this mutant had decreased ethanol $IC_{50}$ values, whereas in the group with C744A, GluN1 (F639C/C744A) exhibited extremely low ethanol sensitivity and the dual cysteine mutation F639C/C744A/M821C also significantly decreased ethanol sensitivity. When we removed GluN1 (F639C/C744A)/GluN2C from the analysis group, a significant difference was obtained in the dual cysteine mutant GluN1 (G638C/C744A)/GluN2C (M821C). Although the involvement of Cys744 altered ethanol sensitivity among the mutants, the trend of change is similar in the group with or without C774A. Because the Cys744 is in the LBD, the change in this position may alter agonist affinity. In the glutamate concentration-response experiments, GluN1 (Cys-744)/GluN2C significantly decreased steady-state glutamate $EC_{50}$ values compared to wild type GluN2C-containing NMDAR. The cysteine mutants containing C744A all showed decreased glutamate $EC_{50}$ values except the dual cysteine mutant G638C/C744A/M821C. Time of decay also showed that mutants containing C744A increased Tau values, which is consistent with the increased agonist affinity. Taken together, the cysteine mutants containing C744A alter ethanol sensitivity in an additive manner. The alcohol molecule acts on the membrane-associated domain

instead of the LBD as an antagonist[86, 99, 101-103, 111], so it is likely that the mutation at Cys744 causes a long distance effect on the conformational change of the MD to affect ethanol sensitivity. We have demonstrated that the change in the methionine position in the GluN2A subunit can cause agonist trapping in the LBD to alter ethanol sensitivity[110, 111], the change in the LBD may subsequently affect the alcohol action in the MD. In this study we used both mutant cycle analysis and two-way analysis of variance to test for interactions among dual cysteine mutants. Ethanol $IC_{50}$ values may represent fewer kinetic rates compared with steady-state glutamate $EC_{50}$ values. It has been demonstrated that the main action of ethanol on NMDAR kinetics is to decrease mean open time[92], whereas glutamate EC50 depends on both agonist binding and ion channel gating[114, 182]. Alcohol action in the mutants containing C744A may involve multiple kinetic rates, so we only analyzed the interaction among the dual cysteine mutants containing Cys744. A significant interaction with respect to ethanol sensitivity was obtained in the mutant combination G638C/M821C, but not in the mutant F639C/M821C.

The redox site modulation by reducing reagent DTT showed potentiated currents in all the dual cysteine mutants. The time constant of decay after DTT treatment was significantly increased in dual cysteine mutants containing Cys744. Breaking disulfide bonds between two pairs of cysteine residues in the GluN1 M3 and GluN2C M4 domain intersubunit interface can regulate receptor

kinetics. We showed that mutations at GluN2A (Met-823) can affect agonist affinity by trapping the agonist in the closed, desensitized state[110]. Although apparent desensitization was not changed by these mutations, these mutations are in domains involved in regulating gating and we obtained a significant interaction between G638C/M821C with respect to ethanol sensitivity. However, we did not detect a significant interaction with respect to deactivation in either G638C/M821C or F639C/M821C. The interaction we observed between the GluN1 M3 and GluN2C M4 domains cysteine residues may mainly alter the local conformational change, which is only reflected in a change in ethanol sensitivity.

In summary, taking the results of this study together with those of previous studies, we predict an interaction between G638C/M821C involved in regulating ethanol action on NMDAR(Figure 18). It will be of interest in future studies to test how adjacent amino acid side chains contribute to alter alcohol action and ion channel gating within the M3-M4 interfaces.

## Chapter 6. General Discussion

We found that the GluN2C (Met-821) is an alcohol sensitive residue, which is also responsible for regulating ion channel gating. It is possible that observed changes in ethanol sensitivity among the mutants at the methionine position result from changes in agonist potency or ion channel gating kinetics. Plotting ethanol $IC_{50}$ values against both peak and steady-state glutamate $EC_{50}$ values revealed there is no correlation. A significant linear relation was obtained when ethanol $IC_{50}$ was plotted *versus* maximal $I_{ss}$:$I_p$ values ($\boldsymbol{R^2}$ = 0.501; $\boldsymbol{P}$ < 0.05) (Figure 19).

In the study of GluN2A (Met-823), we found that ethanol IC50 was negatively correlated with the maximal $I_{ss}$:$I_p$ values, such that the greater the desensitization in a given mutant, the lower its ethanol sensitivity[111]. It is possible that GluN2A (Met-823) influences ethanol sensitivity indirectly via changes in desensitization. In contrast, we observed a positive linear relation between ethanol sensitivity and apparent desensitization among the various mutants at GluN2C (Met-821). Ethanol has been shown to influence desensitization in several ligand-gated ion channels, including ***α***-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)[183], 5-hydroxytryptamine (5-HT), and GABA receptors[184]. The opposite effects of mutations at GluN2C (Met-821), and its cognate position GluN2A (Met-823) on

ethanol sensitivity and desensitization may suggest that ethanol inhibition of NMDARs does not involve changes in desensitization.

Although mutants at GluN2C (Met-821) can alter glutamate $EC_{50}$ values, we did not detect any correlation between ethanol sensitivity and glutamate potency. Most mutants showed significantly increased peak glutamate $EC_{50}$ values, but not steady-state glutamate $EC_{50}$ values. It is possible that the alcohol molecule physically interacts with the M821 position and leads to the limited conformational changes only in the MD, which will not affect the conformation of the LBD. Unlike the trapping theory we observed in GluN2A (Met-823), the changes in the GluN2C (Met-821) position only cause short distance changes, which only alter the local environment.

Although highly homologous sequences are found among all the GluN2 subunits, several different amino acids are found in the M4 domain between GluN2A and GluN2C subunit (Figure 20). There is only one different residue in the M3 domain between GluN2A and GluN2C subunit, and it is located at the C-terminal end of the M3 domain. It is unlikely there is either a physical or functional interaction between that position and the sites in the M4 domain because of the very long distance. We then compared the amino acids near the methionine residue between GluN2A and GluN2C subunit. We found that the Ala-825 and Ala-826, which are downstream of the GluN2A (Met-823), are Leu-823 and Val-824 in the GluN2C subunit. Both leucine and valine are

hydrophobic amino acids and the side chains are larger than alanine. These two amino acids may form a small hydrophobic environment near the methionine position, which can significantly alter the alcohol molecule interacts with the GluN2C (Met-821) position. We also found another five different amino acids located near the C-terminal end of the M4 domain. However, the difference in the physical and chemical properties among these amino acids is not significant. So, it is unlikely those amino acids can cause long-term effects on the alcohol modulation of the GluN2C (Met-821) position.

In the present studies, we showed that the Met-821 position involved in regulating ethanol sensitivity and ion channel gating. We also showed Gly-638 and Met-821 positions in the M3-M4 intersubunit interfaces between GluN1 and GluN2C subunits interactively regulate ethanol sensitivity. The results we observed from GluN2C-containing NMDAR are different from the previous discoveries in the NMDAR containing GluN2A subunit. The difference may mainly lies in the sequence difference between GluN2A and GluN2C M4 domains and small hydrophobic environment formed near the methionine position.

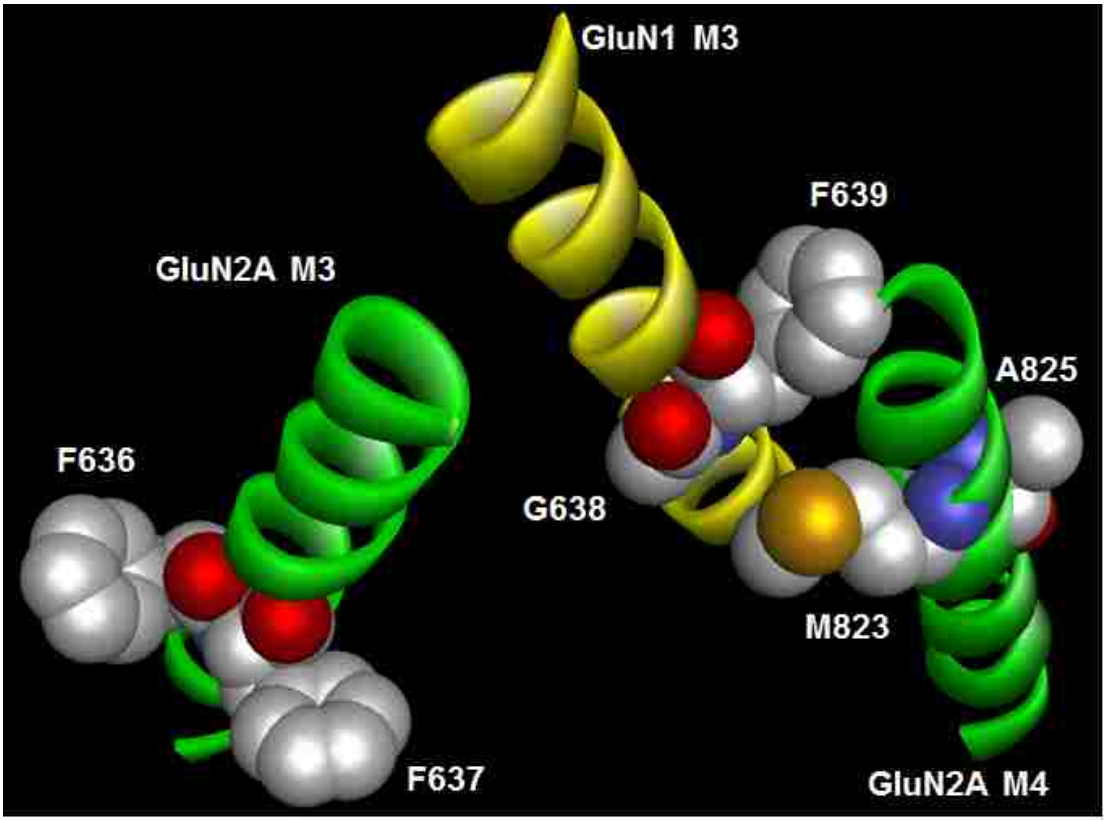


**Figure 1. The structural model of M domains of the NMDARs**. Shown is a model of the NMDA receptor M domains from [1]. M domains of the GluN1 subunit are shown in ***yellow***, and those of the GluN2A subunit are shown in ***green***. Five alcohol sensitive positions are illustrated by CPK models.

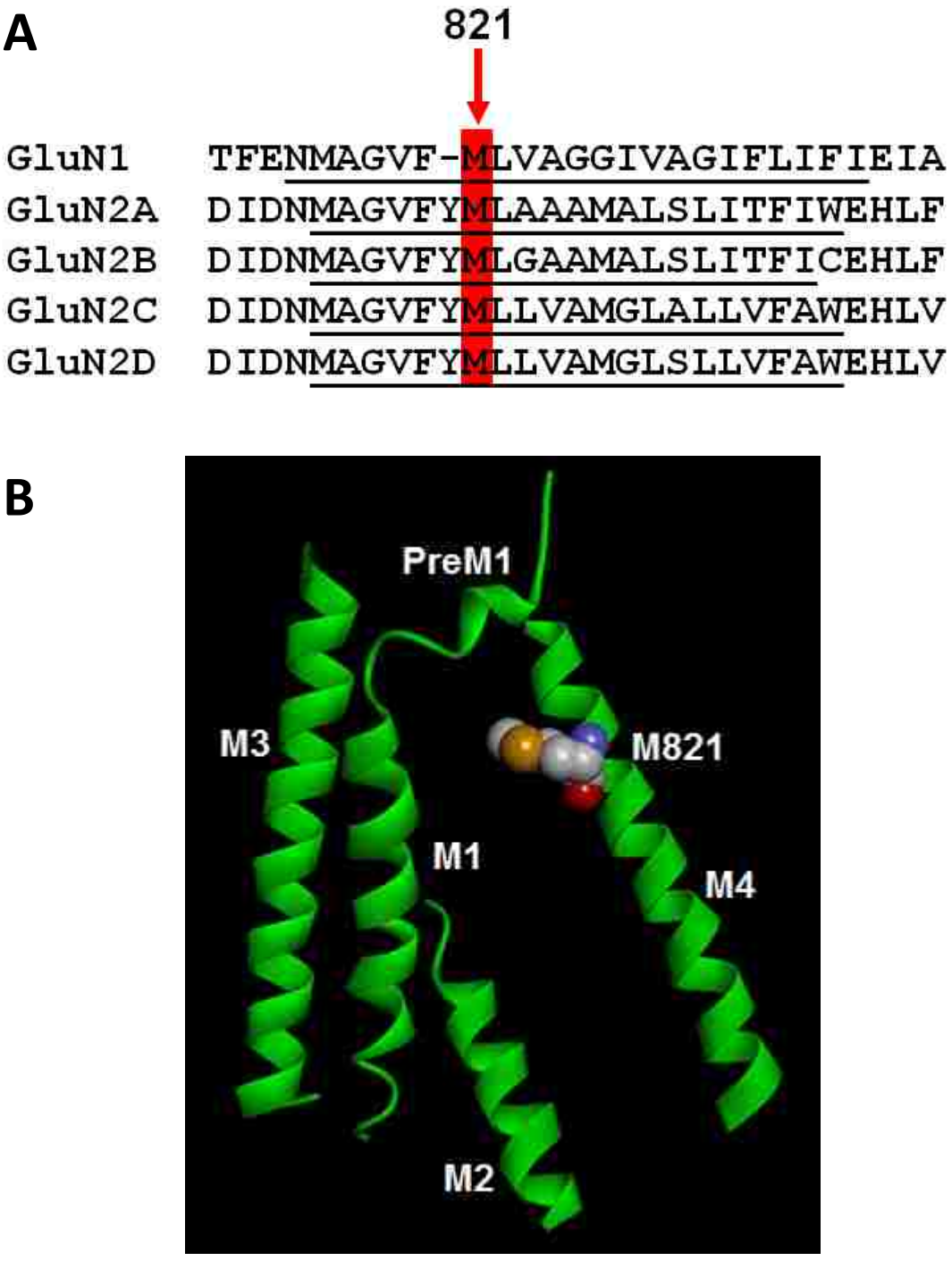


**Figure 2. A methionine residue in the M4 domain is highly conserved in all GluN subunit**.A, shown is the sequences of the M4 domains of the GluN subunits. The *arrow* shows the position of the conserved methionine. B, model of the NMDA receptor M domains from [1]. Met-821 position is illustrated by CPK model.

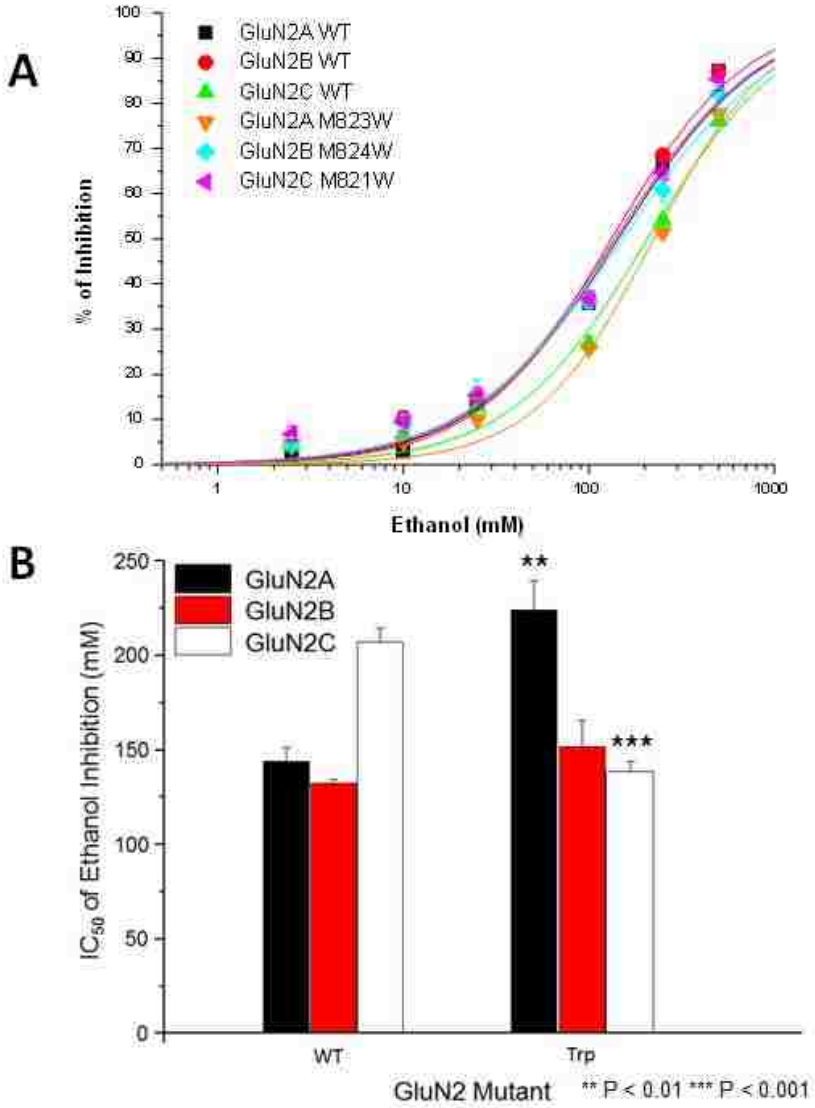


**Figure 3. Effects of ethanol on tryptophan mutations at the methionine residue in the M4 domain of GluN2 subunits**. A, concentration-response curves for ethanol inhibition of glutamate-activated current in cells expressing tryptophan mutations in GluN2 subunits. Data are the means ± S.E. of n = 6–9 cells; error bars not visible were smaller than the size of the symbols. Curves shown are the best fits to the equation given under "Methods" B, graphs plot $IC_{50}$ values for ethanol in tryptophan mutations in GluN2 subunits. Asterisks indicate $IC_{50}$ values that differed significantly from that for wild type (***, $p < 0.001$; ANOVA followed by Tukey's test).

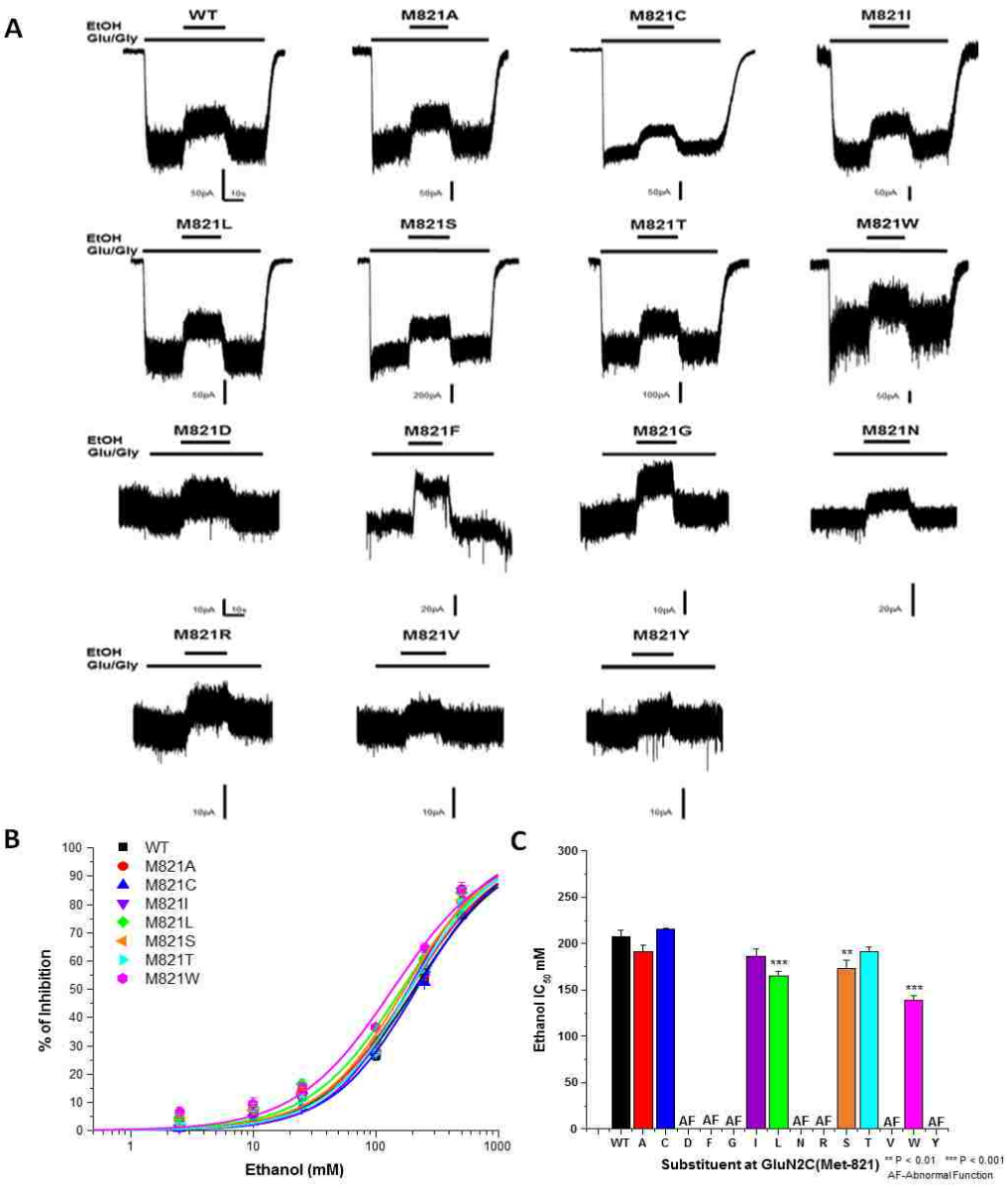


**Figure 4. Single mutations at Met-821 residue in the M4 domain of GluN2C subunit can alter ethanol inhibition**. A, records are currents activated by 300 μM glutamate and 50 μM glycine in the absence and presence of 100mM ethanol in cells expressing various mutant GluN2C subunit. B, shown are concentration-response curves for ethanol inhibition of glutamate-activated current in cells expressing various single site substitution mutations in GluN2C. Data are the means ± S.E. of n = 6–9 cells; error bars not visible were smaller than the size of the symbols. Curves shown are the best fits to the equation given under "Methods" C, graphs plot $IC_{50}$ values for ethanol in various single site substitution mutations in GluN2C. Asterisks indicate $IC_{50}$ values that differed significantly from that for wild type GluN1/GluN2C subunits (**, $p < 0.01$ ***, $p < 0.001$; ANOVA followed by Dunnett's test).

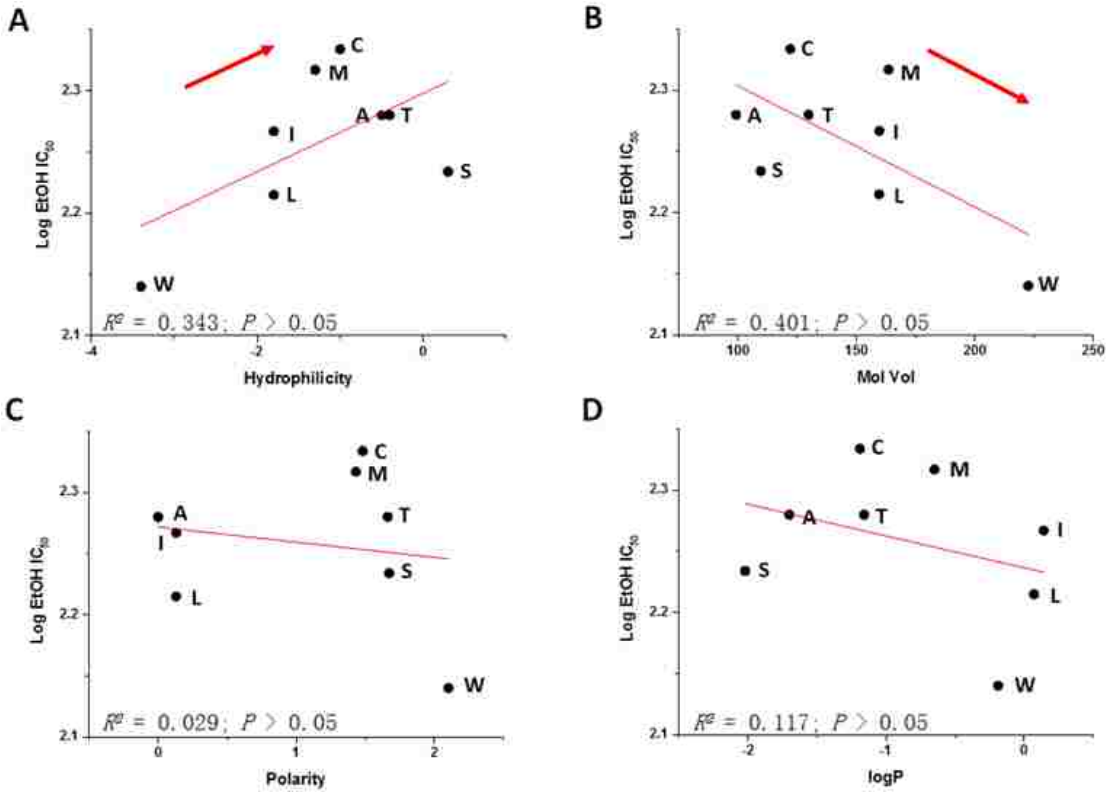


**Figure 5. Relation of amino acid physicochemical parameters of the substituent at Met-821 to ethanol sensitivity**.Graphs plot Log $IC_{50}$ for ethanol versus hydrophilicity (A), molecular volume in $Å^3$ (B), polarity (C), and LogP (D). The lines shown are the least-squares fits to the data. No significant linear relations were obtained between log $IC_{50}$ and physicochemical parameters of the substituents.

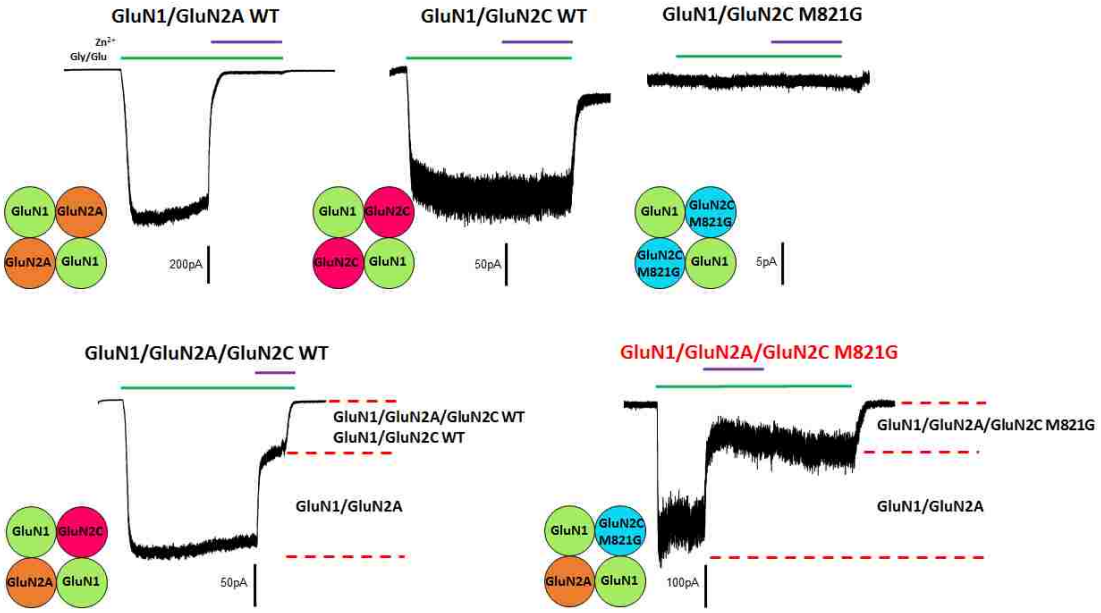


**Figure 6. Triheteromeric GluN1/GluN2A/GluN2C M821G NMDAR Can Restore Ion Channel Gating Function.** Records are currents activated by 300 μM glutamate and 50 μM glycine in the absence and presence of 200nM $Zn^{2+}$ in cells expressing diheteromeric or triheteromeric NMDARs.

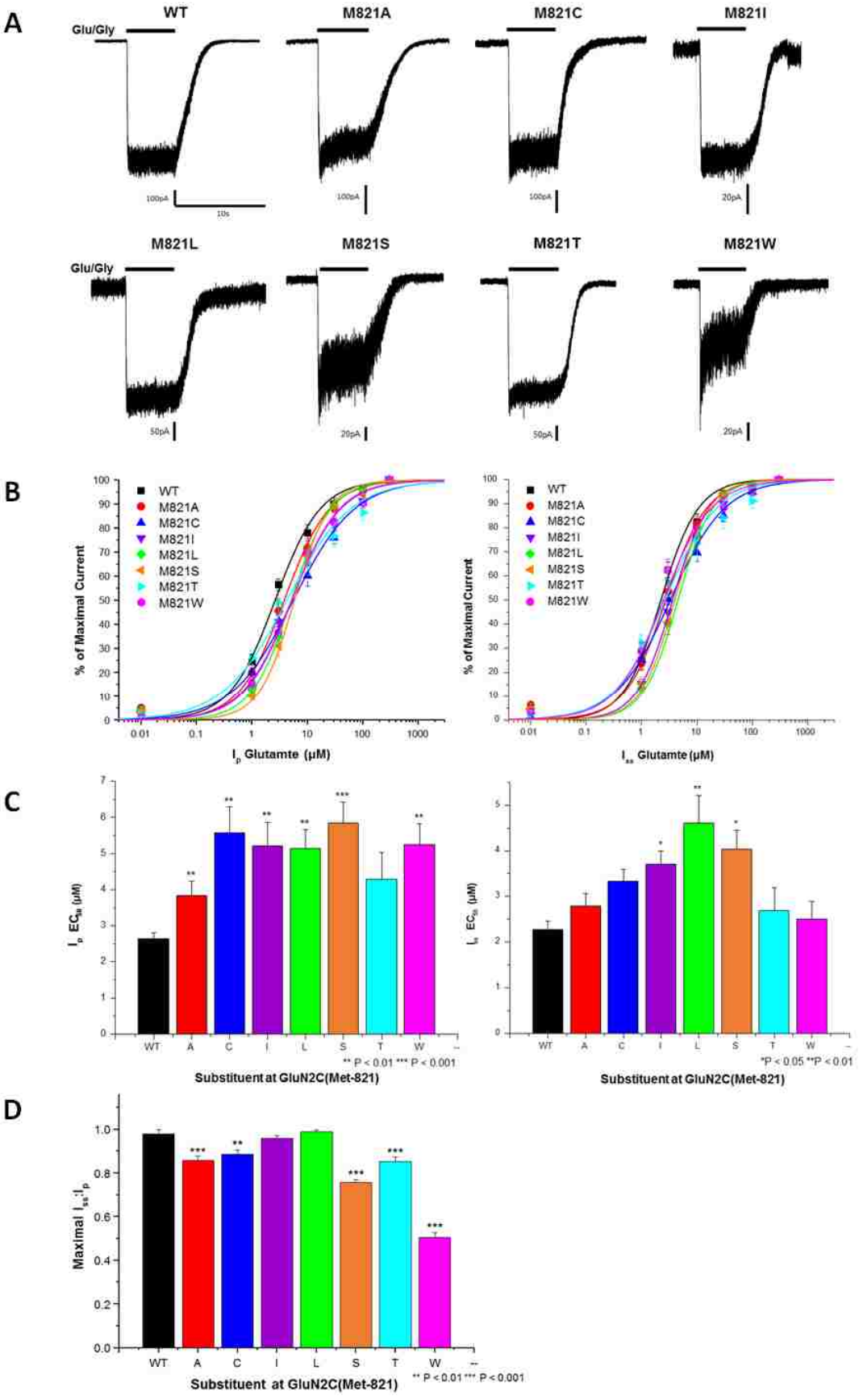
A
WT
M821A
M821C
M821I
Glu/Gly
100pA
10s
100pA
100pA
20pA
M821L
M821S
M821T
M821W
Glu/Gly
50pA
20pA
50pA
20pA
B
WT
M821A
M821C
M821I
M821L
M821S
M821T
M821W
% of Maximal Current
I_p Glutamte (μM)
I_ss Glutamte (μM)
C
I_p EC50 (μM)
I_ss EC50 (μM)
WT A C I L S T W
** P < 0.01 *** P < 0.001
*P < 0.05 **P < 0.01
Substituent at GluN2C(Met-821)
D
Maximal I_ss:I_p
WT A C I L S T W
** P < 0.01 *** P < 0.001
Substituent at GluN2C(Met-821)

**Figure 7. Single mutations at Met-821 residue in the M4 domain of GluN2C subunit can alter the $EC_{50}$ for peak and steady-state glutamate-activated current and apparent desensitization**. A, records are currents activated by 300 μM glutamate in the presence of 50 μM glycine in cells expressing various mutant GluN2C subunit. B, concentration-response curves for glutamate-activated current in cells expressing various single site substitution mutations in GluN2C. Data are the means ± S.E. of n = 6–9 cells; error bars not visible were smaller than the size of the symbols. Curves shown are the best fits to the equation given under “Methods” C, graphs plot glutamate $EC_{50}$ values in various single site substitution mutations in GluN2C. D, bar graph shows the average values of maximal steady-state to peak current ratio ($I_{ss}$:$I_p$) in lifted cells coexpressing GluN1 and WT GluN2C subunits or GluN2C subunits containing various mutations at M821. Asterisks indicate $EC_{50}$ values that differed significantly from that for wild type GluN1/GluN2C subunits (*, $p < 0.05$ **, $p < 0.01$ ***, $p < 0.001$; ANOVA followed by Dunnett's test).

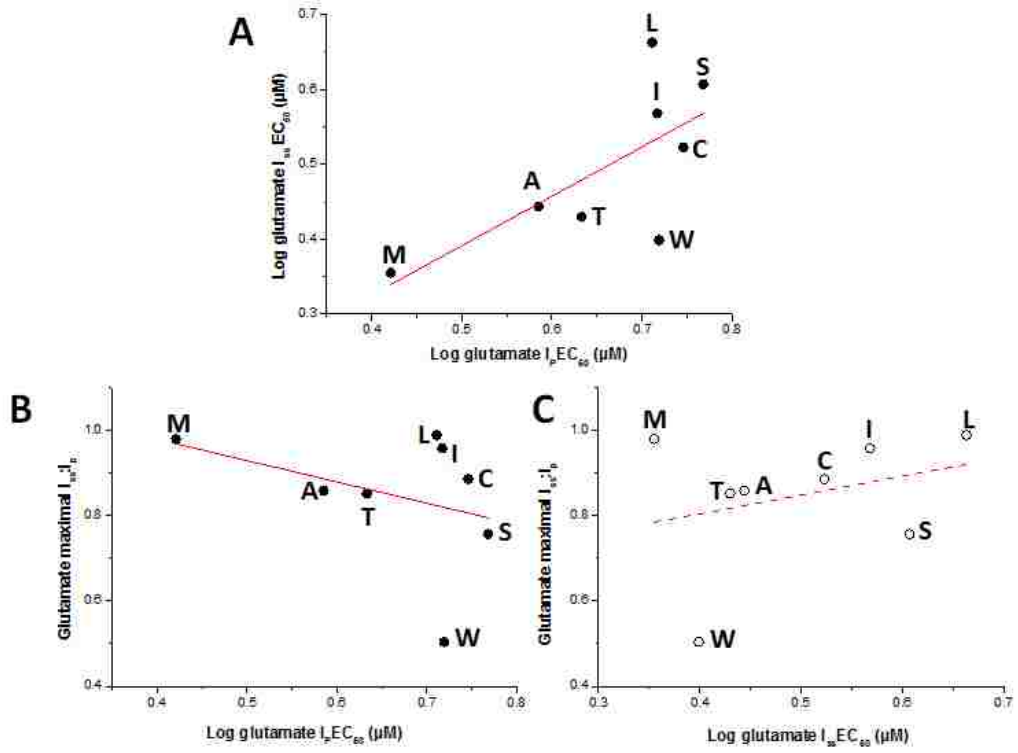


**Figure 8. Relationship between glutamate $EC_{50}$ and apparent desensitization**. A, the graph plots values of glutamate log $EC_{50}$ for steady-state current versus values of glutamate log $EC_{50}$ for peak current in the series of mutants. Glutamate $EC_{50}$ values for peak and steady-state current were not statistically significantly correlated ($R^2$ = 0.482; $P$ > 0.05). The line shown is the least-squares fit to the data. B-C, the graph plots the maximal $I_{ss}$:$I_p$ versus peak (●) and steady-state (○) log $EC_{50}$ values for glutamate-activated current in various GluN2C(Met-821) mutant subunits. Maximal $I_{ss}$:$I_p$ for glutamate was not correlated with steady-state glutamate log $EC_{50}$ ($R^2$ = 0.092; $P$ > 0.05) and peak glutamate log $EC_{50}$ ($R^2$ = 0.129; $P$ > 0.05). The lines shown are the least squares fits to the data: peak (solid line) and steady-state (dash line).

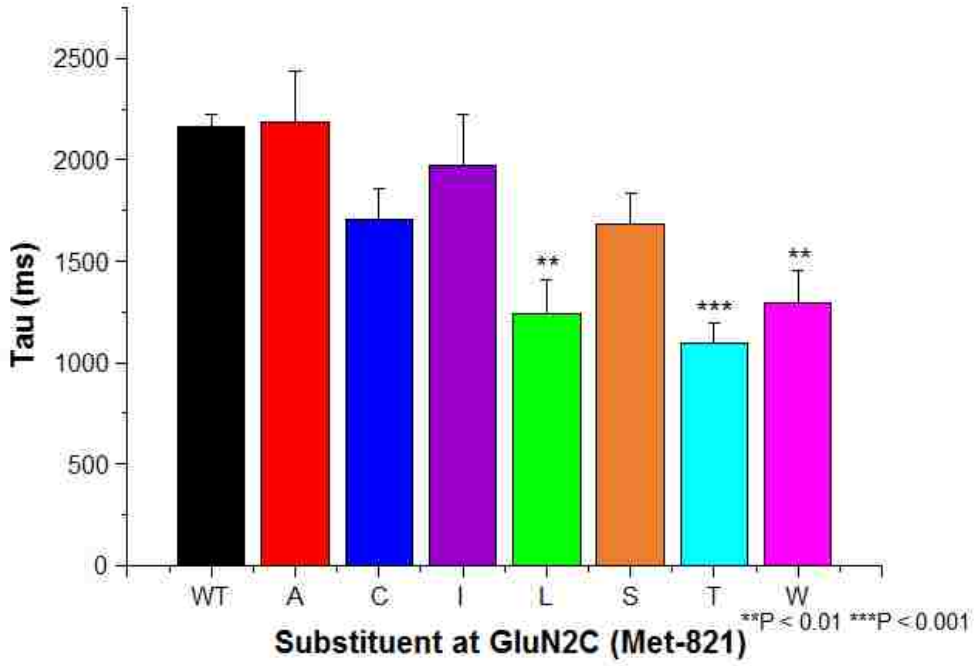


**Figure 9. The deactivation time constant (τ) of substituent at GluN2C (Met-821).** Bar graph shows the average values of deactivation time course in the presence of 300 300 μM glutamate and 50 μM glycine. Asterisks indicate Tau that differed significantly from that for wild type GluN1/GluN2C subunits (**, $p < 0.01$ ***, $p < 0.001$; ANOVA followed by Dunnett's test).

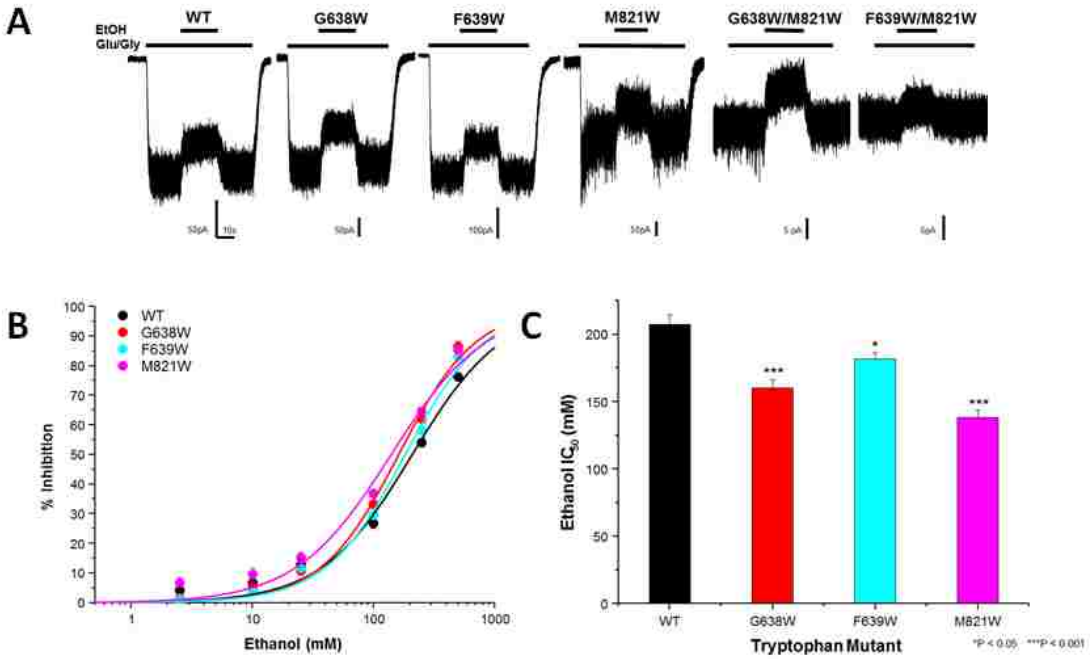


**Figure 10. Single tryptophan mutations in the M3 and M4 domains of GluN1 and GluN2C subunits can alter ethanol inhibition**. A, records are currents activated by 300 μM glutamate and 50 μM glycine in the absence and presence of 100mM ethanol in cells expressing various mutants. B, concentration-response curves for ethanol inhibition of glutamate-activated current in cells expressing various single tryptophan substitution mutations. Data are the means ± S.E. of n = 6–9 cells; error bars not visible were smaller than the size of the symbols. Curves shown are the best fits to the equation given under "Methods" C, graphs plot $IC_{50}$ values for ethanol in various single tryptophan substitution mutations. Asterisks indicate $IC_{50}$ values that differed significantly from that for wild type GluN1/GluN2C subunits (*, $p < 0.05$ ***, $p < 0.001$; ANOVA followed by Dunnett's test).

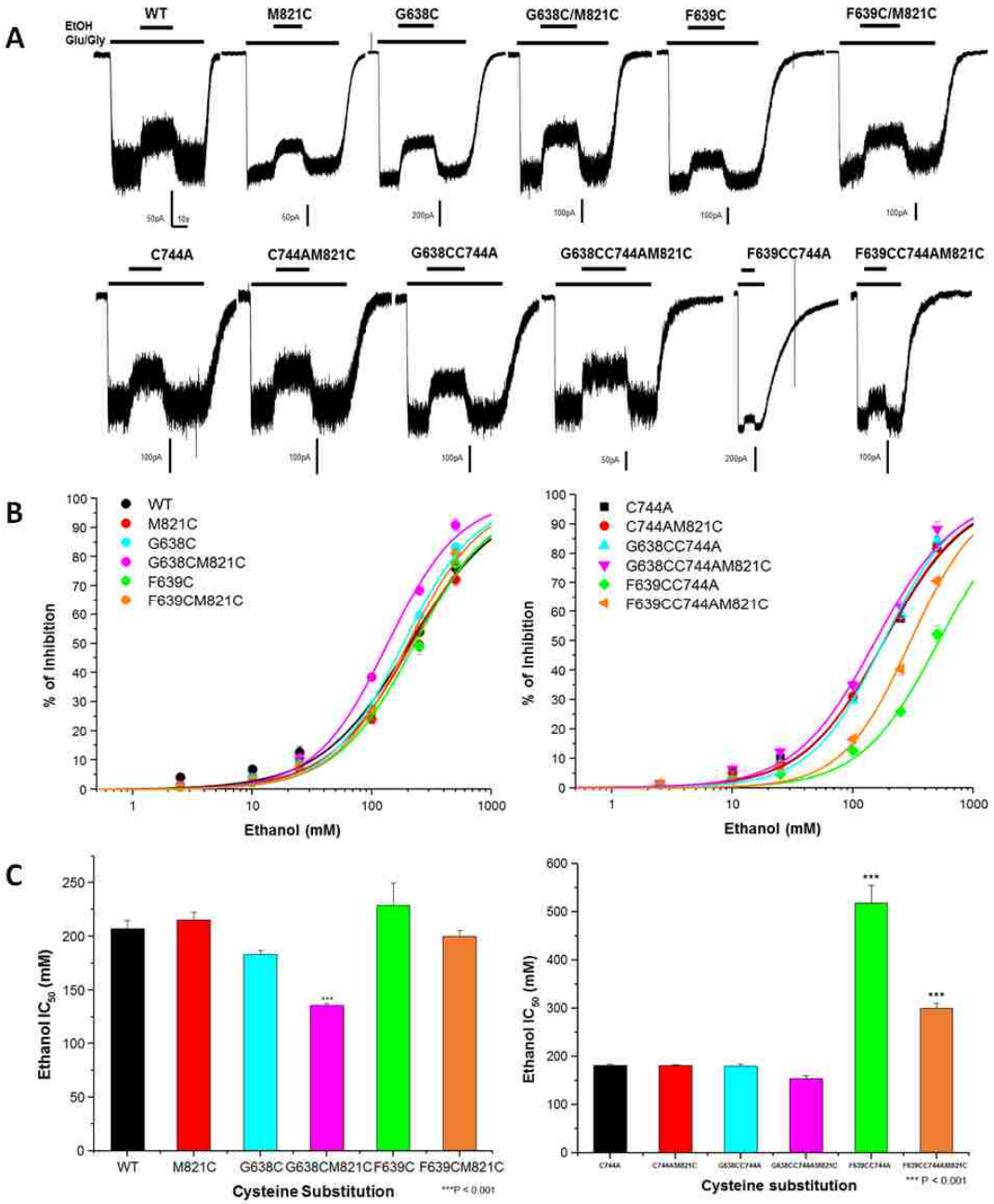


**Figure 11. Cysteine mutations in the M3 and M4 domains of GluN1 and GluN2C subunits can alter ethanol inhibition**. A, records are currents activated by 300 μM glutamate and 50 μM glycine in the absence and presence of 100mM ethanol in cells expressing various cysteine mutations. B, concentration-response curves for ethanol inhibition of glutamate-activated current in cells expressing various cysteine mutations. Data are the means ± S.E. of n = 6–9 cells; error bars not visible were smaller than the size of the symbols. Curves shown are the best fits to the equation given under "Methods" C, graphs plot $IC_{50}$ values for ethanol in various cysteine mutations. Asterisks indicate $IC_{50}$ values that differed significantly from that for wild type GluN1/GluN2C subunits (***, $p < 0.001$; ANOVA followed by Dunnett's test).

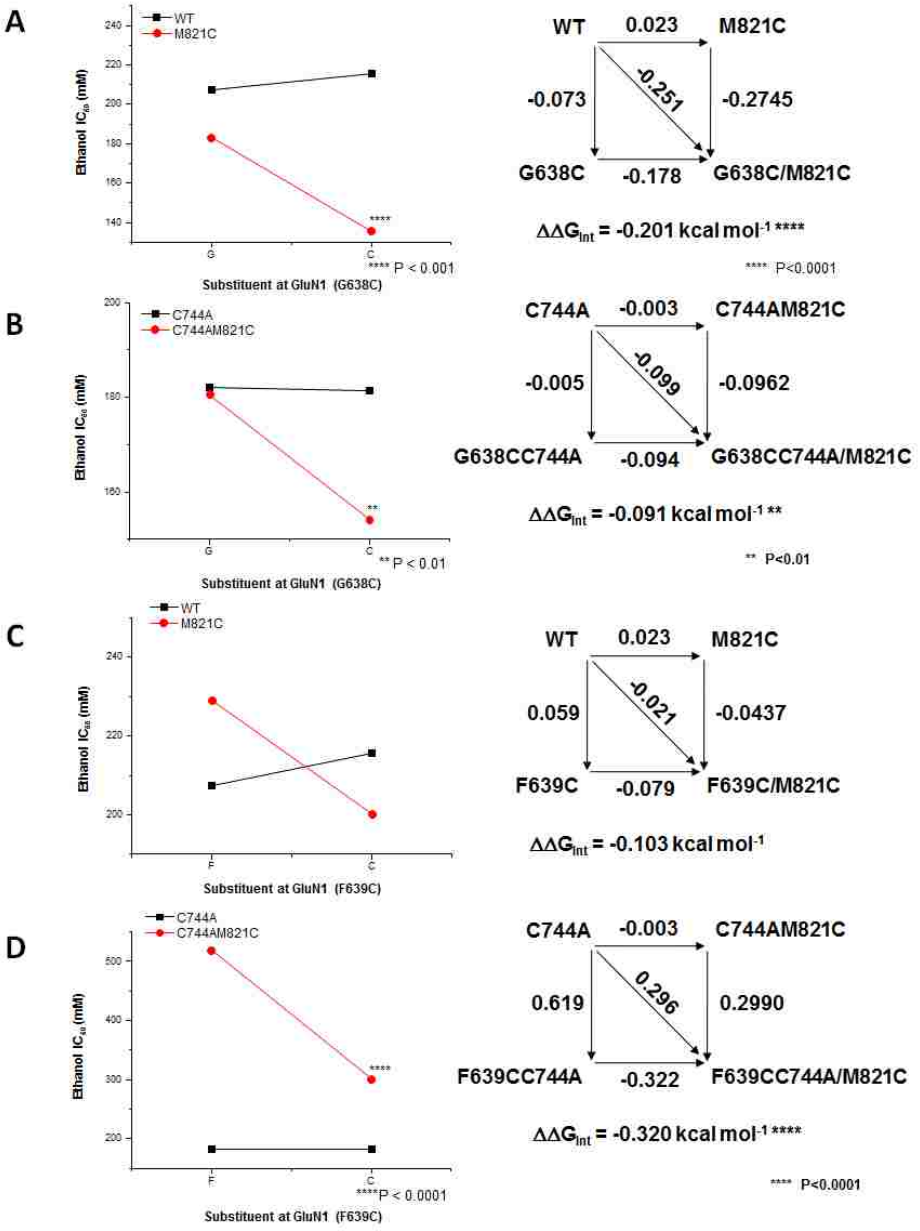


**Figure 12. Positions in the GluN1 subunit M3 domain interact with GluN2C (Met-821) in M4 domain to regulate NMDA receptor ethanol sensitivity.** A-D, graphs plot ethanol $IC_{50}$ values ***versus*** the substituent at position 638 or 639 in GluN1 for mutants at GluN2C position 821. ***Asterisks*** indicate significant interactions detected using log-transformed $IC_{50}$ values (**, $p < 0.01$; ****, $p < 0.0001$; two-way ANOVA) (left panel). Mutant cycle analysis of ethanol $IC_{50}$ values for the subunit combinations. Apparent free energy values associated with the various mutations ($\Delta G_x$) are given in kcal $mol^{-1}$. ***Asterisks*** indicate a statistically significant difference of the apparent interaction energy $\Delta\Delta G_{INT}$ from zero energy determined using a one-sample ***t*** test (**, $p < 0.01$; ****, $p < 0.0001$) (right panel).

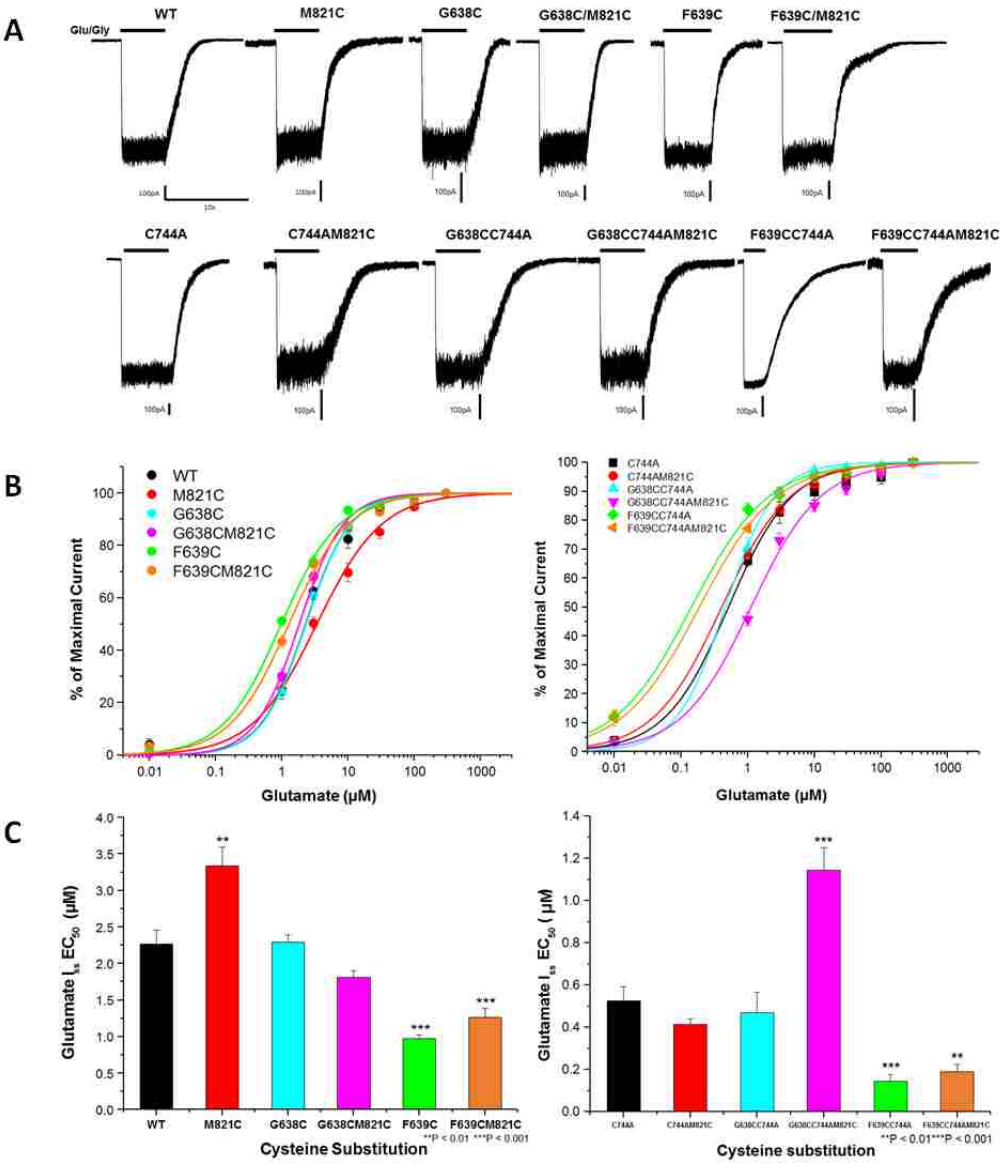


**Figure 13. Cysteine mutations in the M3 and M4 domains of GluN1 and GluN2C subunits can alter the $EC_{50}$ for steady-state glutamate-activated current**. A, records are currents activated by 300 μM glutamate in the presence of 50 μM glycine in cells expressing various cysteine mutations. B, concentration-response curves for glutamate-activated current in cells expressing various cysteine mutations. Data are the means ± S.E. of n = 6–9 cells; error bars not visible were smaller than the size of the symbols. Curves shown are the best fits to the equation given under "Methods" C, graphs plot glutamate $EC_{50}$ values in various cysteine mutations. Asterisks indicate $EC_{50}$ values that differed significantly from that for wild type GluN1/GluN2C subunits (**, $p < 0.01$ ***, $p < 0.001$; ANOVA followed by Dunnett's test).

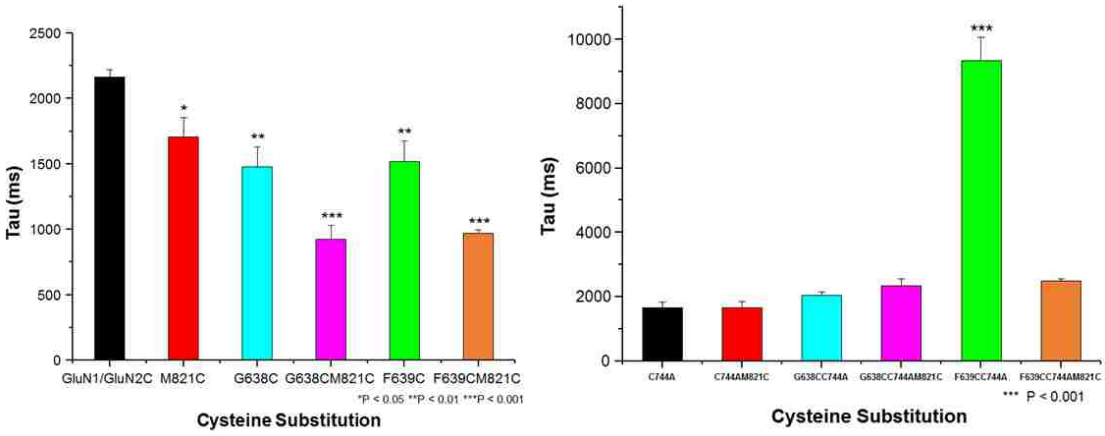


**Figure 14. The deactivation time constant (τ) of cysteine mutations in the M3 and M4 domains of GluN1 and GluN2C subunits.** Bar graph shows the average values of deactivation time course in the presence of 300 300 μM glutamate and 50 μM glycine. Asterisks indicate Tau values that differed significantly from that for wild type GluN1/GluN2C subunits (**, $p < 0.01$ ***, $p < 0.001$; ANOVA followed by Dunnett's test).

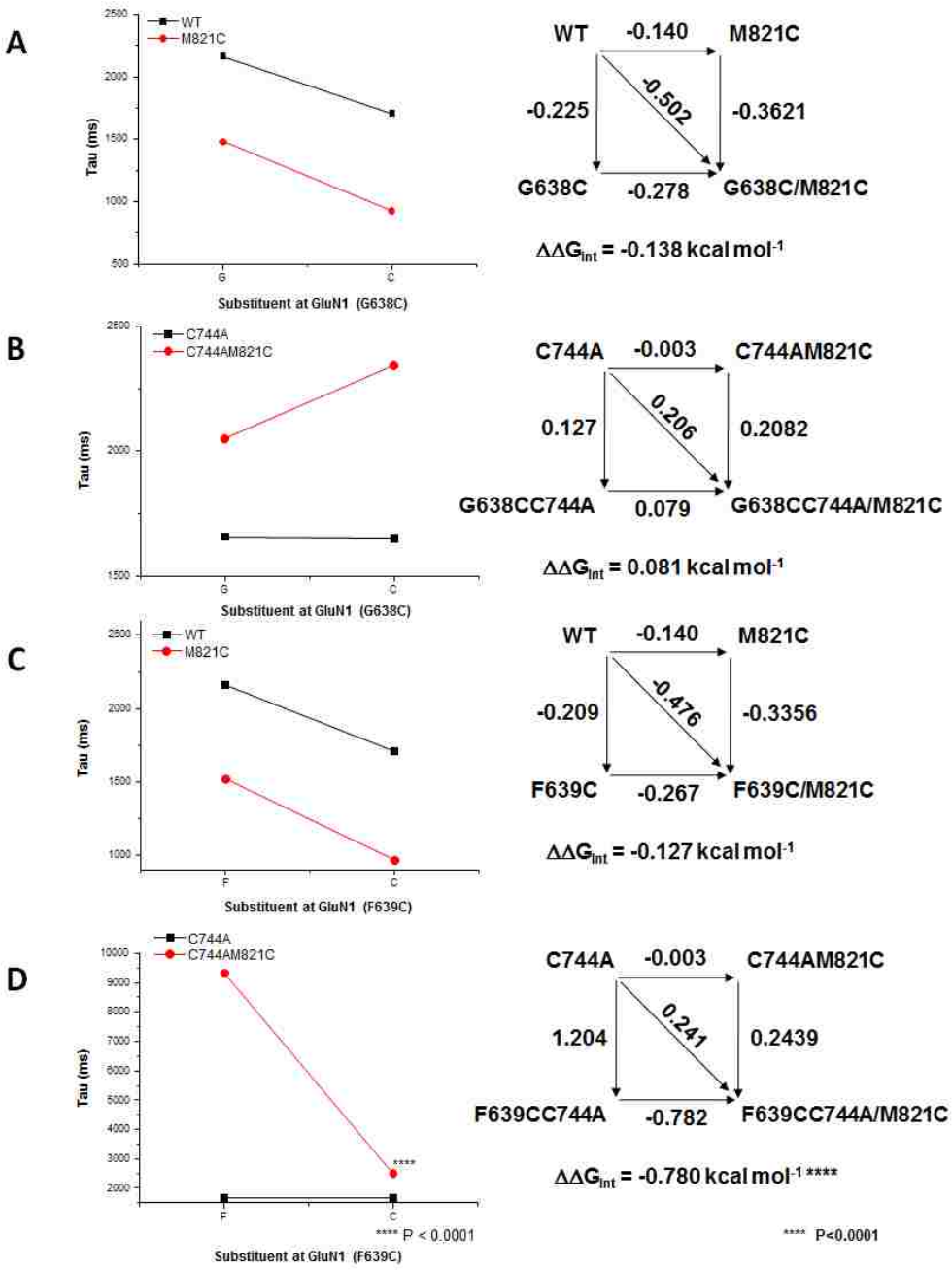


**Figure 15. Positions in the GluN1 subunit M3 domain interact with GluN2C (Met-821) in M4 domain to regulate NMDA receptor deactivation time course.** A-D, graphs plot deactivation time constants ***versus*** the substituent at position 638 or 639 in GluN1 for mutants at GluN2C position 821. ***Asterisks*** indicate significant interactions (****, ***p*** < 0.0001; two-way ANOVA) (left panel). Mutant cycle analysis of deactivation time constants for the subunit combinations. Apparent free energy values associated with the various mutations (Δ***Gx***) are given in kcal $mol^{-1}$. ***Asterisks*** indicate a statistically significant difference of the apparent interaction energy $\Delta\Delta G_{INT}$ from zero energy determined using a one-sample ***t*** test (****, ***p*** < 0.0001) (right panel).

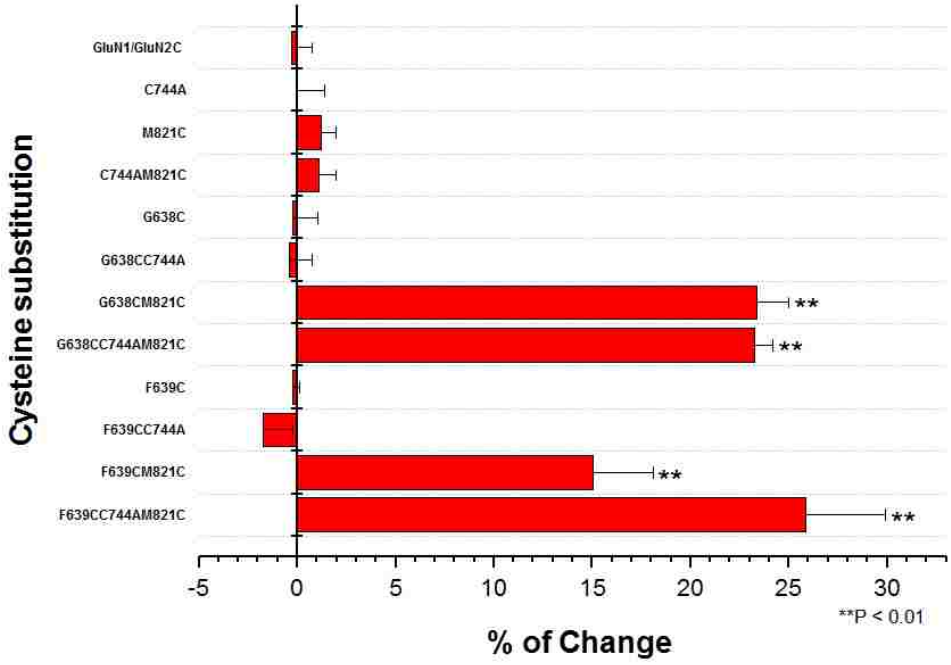


**Figure 16. Effects of DTT on cysteine mutations in the M3 and M4 domains of GluN1 and GluN2C subunits.** Bar graph summary of the percentage of change (mean± SEM) in 300 μM glutamate steady-state ($I_{ss}$) current amplitude after redox modification of wild type and cysteine mutations with reducing agent DTT. The percentage of change in $I_{ss}$ after DTT treatment is defined as $[((I_{after}/I_{inital}) - 1) \times 100]$. Negative values represent a decrease in $I_{ss}$ after DTT reaction, whereas positive values represent an increase in $I_{ss}$. Asterisks indicate the percentage of change that differed significantly from that for wild type GluN1/GluN2C subunits (**, $p < 0.01$; ANOVA followed by Dunnett's test).

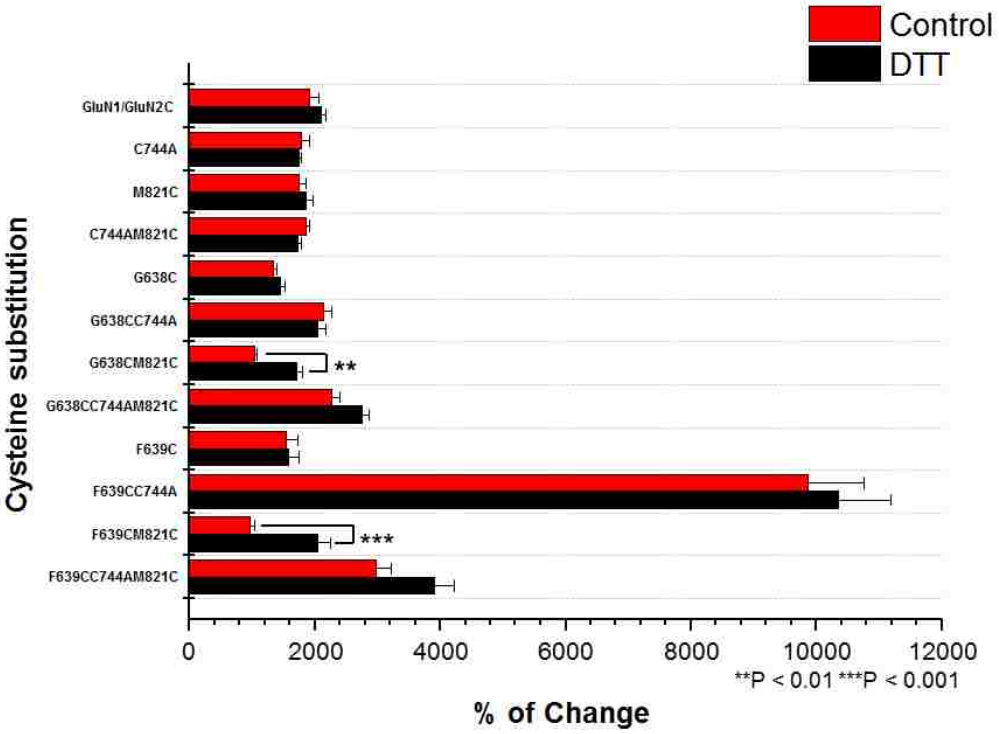


**Figure 17. Effects of DTT on the deactivation time constant (τ) of cysteine mutations in the M3 and M4 domains of GluN1 and GluN2C subunits.** Bar graph shows the average values of deactivation time course before and after DTT treatment in the presence of 300 μM glutamate and 50 μM glycine. Asterisks indicate Tau that differed significantly from that for wild type GluN1/GluN2C subunits (**, $p < 0.01$ ***, $p < 0.001$; ANOVA followed by Bonferroni's test).

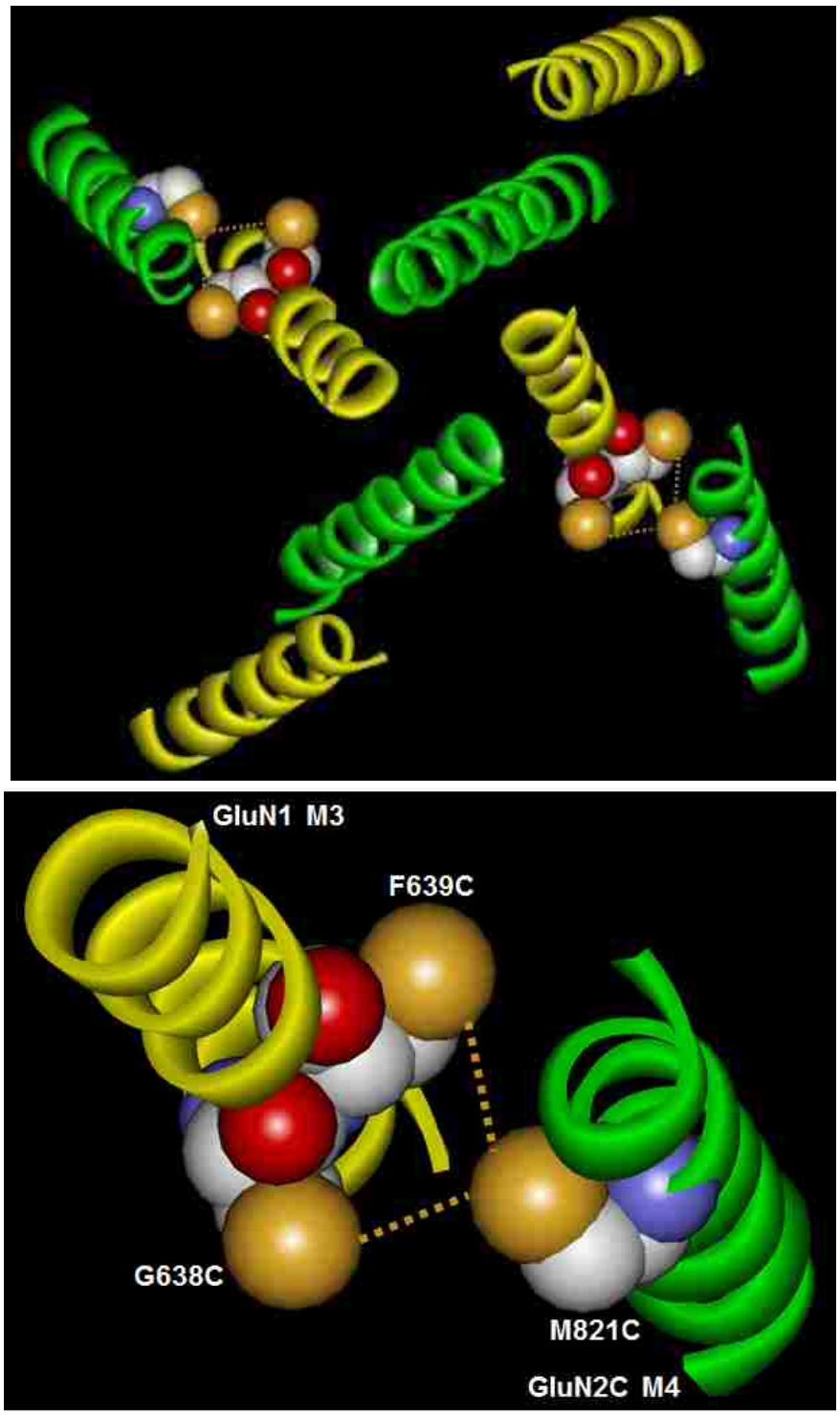


**Figure 18. Model of the MD of the NMDARs.** Model of the MDs of the GluN1/GluN2C subunit showing M3 and M4 helices (GluN1, yellow; GluN2C, green). Other MDs have been removed for clarity. Space-filling side groups are shown for the G638C and F639C positions in the M3 helices of GluN1, M821C position in the M4 helices of GluN2C. Disulfide bridges are represented by yellow dashed lines.

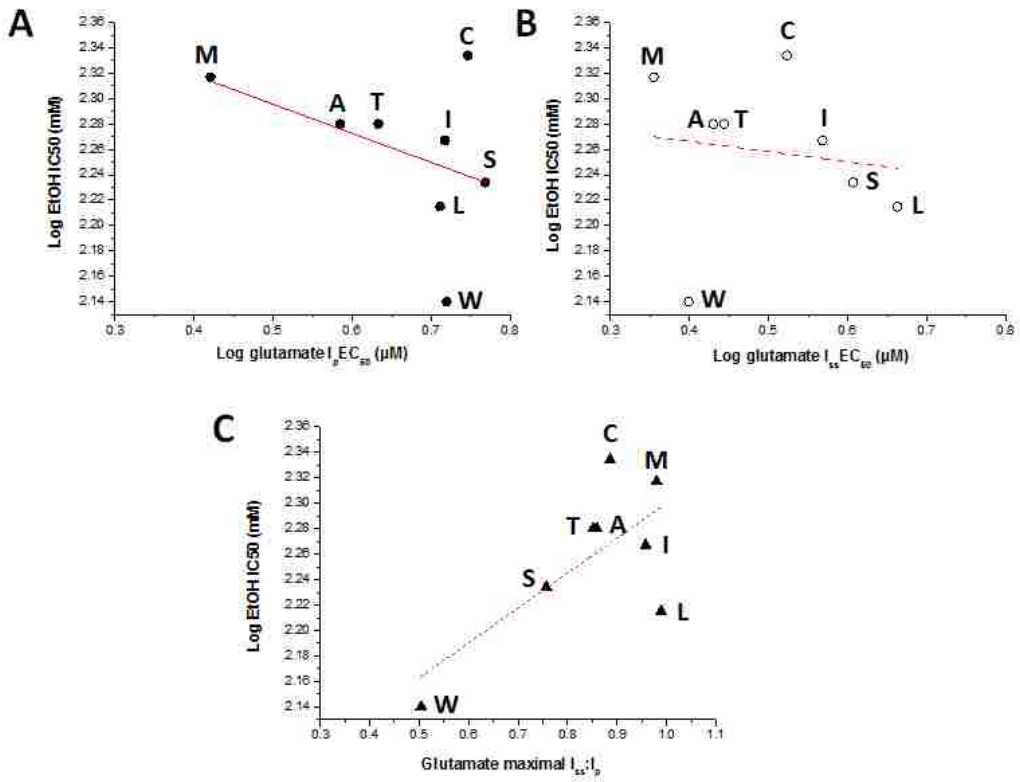


**Figure 19. Relation of agonist potency, ion channel gating to EtOH sensitivity in NMDAR containing GluN2C (Met-821) mutant subunits.** A-C, graph plot log $IC_{50}$ for EtOH *vs* log $EC_{50}$ for glutamate peak and steady-state current and maximal $I_{ss}$:$I_p$. Data points are labeled with the substituted amino acid for the various mutants at GluN2C (Met-821). The lines shown are the least squares fits to the data. No significant correlations were obtained between log EtOH $IC_{50}$ *vs* log $I_p$ $EC_{50}$ ($R^2$ = 0.182; $P$ > 0.05), log $I_{ss}$ $EC_{50}$ ($R^2$ = 0.019; $P$ > 0.05). A significant linear relation was obtained from log EtOH $IC_{50}$ *vs* maximal $I_{ss}$:$I_p$ ($R^2$ = 0.501; $P$ < 0.05).

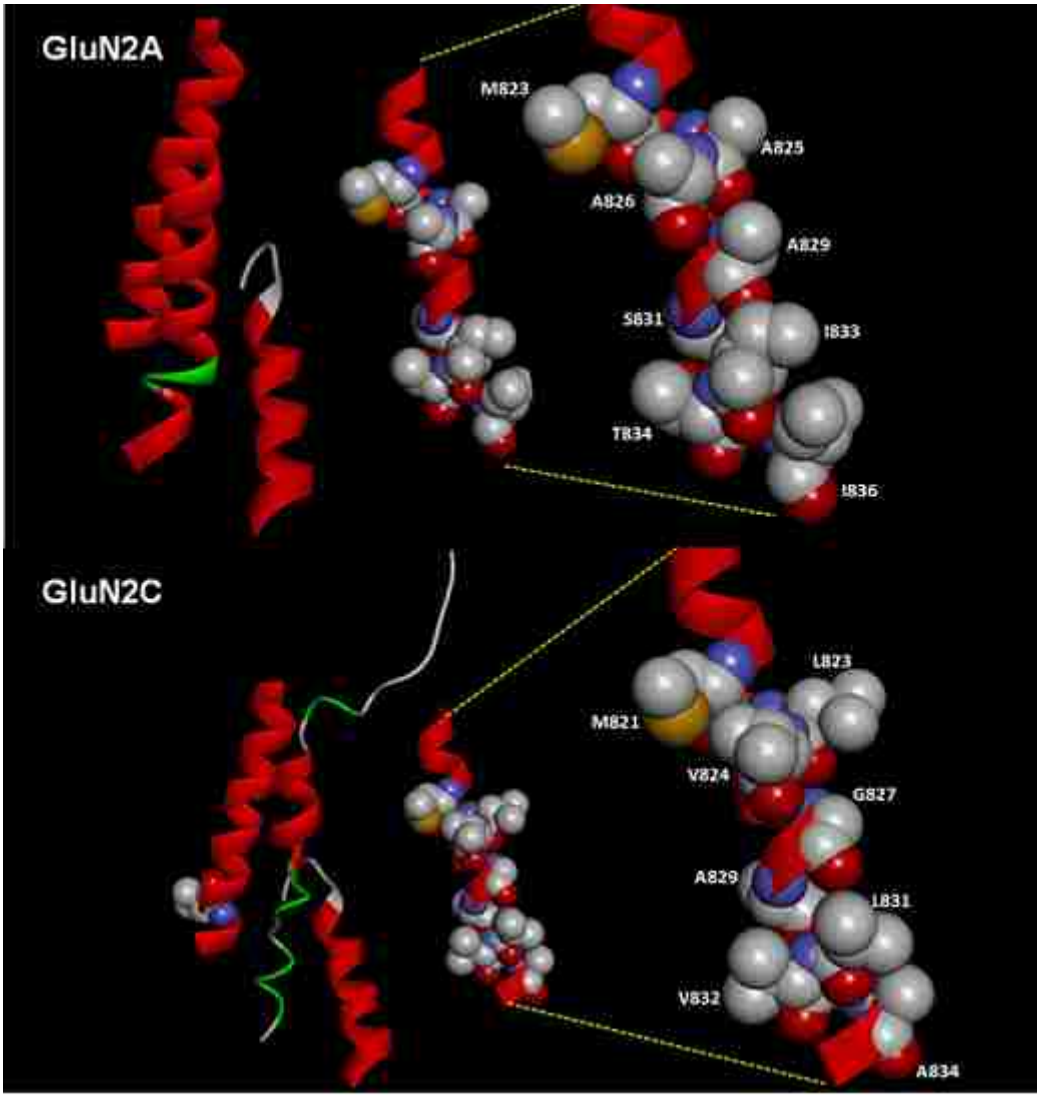


**Figure 20. Model of the MD of the NMDARs.** A, model of the MD of the GluN2A subunit. Space-filling side groups are shown for the different amino acid in the M3 and M4 domains compared to GluN2C subunit. GluN2A (Met-823) residue is labeled by yellow. B, model of the MD of the GluN2C subunit. Space-filling side groups are shown for the different positions in the M3 and M4 domains compared to GluN2A subunit. GluN2C (Met-821) is labeled by yellow.

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