Molecular Sites and Mechanisms of Action of Alcohol on the NMDA Receptor GluN2B Subunit

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MOLECULAR SITES AND MECHANISMS OF ACTION OF ALCOHOL ON THE NMDA RECEPOR GLUN2B SUBUNIT

by

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ABSTRACT
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Yulin Zhao, B.S.

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Alcohol abuse and alcoholism are behavioral disorders involving altered synaptic transmission in the brain. The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate-gated ion channel, has been shown to be one of the most important target sites of alcohol in the central nervous system. The NMDA receptor is formed between two obligatory GluN1 subunits and two GluN2 subunits (2A-2D). Previous studies have been mainly focused on the GluN2A subunit-containing NMDA receptor to study the molecular sites and mechanisms of alcohol action on the NMDA receptors. However, a major role for the GluN2B subunits in the action of alcohol has been pointed out recently.

To test the hypothesis that alcohol interacts with specific residues in the membrane-associated (M) domains of the GluN2B subunit to modulate ion channel gating and alcohol sensitivity, we performed site-directed mutagenesis at positions in the GluN2B subunit, transfected mutant receptors into human embryonic kidney (HEK 293) cells, and did whole-cell patch-clamp recording on these cells. One position in the GluN2B subunit showed significant influence on ethanol sensitivity and ion channel functions. Moreover, the manner in which mutations at positions in the GluN2B subunit alter ethanol sensitivity differ from that of the cognate positions in the GluN2A subunit.

The predicted structure of the NMDA receptor indicates a close apposition of the alcohol-sensitive positions in the M3 and M4 domains between the GluN1 and GluN2 subunits. By using both two-way ANOVA and mutant cycle analysis, significant interactions affecting ethanol inhibition and glutamate potency were observed at three pairs of positions in GluN1/GluN2B: Met818/Phe637, Phe639/Leu825, and Gly638/Met824; the last pair also interact with each other to regulate ion channel desensitization.

To study whether characteristics of NMDA receptor mutations observed in a non-neuronal cell line are also observed in central nervous system (CNS) neurons, we transfected our constructed GluN1/GluN2A NMDA receptors into rat cortical neurons from postnatal day 2 or 3 by using electroporation. Because there is only few endogenous GluN2A subunits in rat cortical neurons early in development, the currents recorded from these transfected neurons are attributable to the introduced NMDA receptors. Electrophysiological recording in these transfected neurons so far yielded currents that closely resemble those observed in HEK293 cells.

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For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

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ABBREVIATIONS

NMDA receptor, *N*-methyl-D-aspartate receptor

Nicotinic ACh-like receptors, nicotinic acetylcholine–like receptors

ATP, Adenosine Tri-Phosphate

LGICs, ligand-gated ion channels

AMPA receptor, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

ATD, amino-terminal domain

LBD, ligand-binding domain

M domains, membrane-associated domains

CTD, carboxy-terminal domain

HEK-293, human embryonic kidney cells (lineage 293)

tsA201, transformed HEK-293 cells

LTP, long-term potentiation

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

EC_{50}, half maximal effective concentration

IC_{50}, half-maximal inhibitory concentration

I_{ss}:I_p, steady-state current to peak current ratio

S.E.M., standard error of mean

I. INTRODUCTION

General Introduction

The purpose of this thesis is to understand the molecular sites and mechanisms through which alcohol modulates the function of GluN2B subunit-containing *N*-methyl-D-aspartate (NMDA) receptor. This dissertation provides new information about 1) the location of ethanol-sensitive positions in the GluN2B subunit of the NMDA receptor; 2) whether these positions can also regulate ion channel gating; 3) how positions among different subunits interact to form sites of alcohol action; and 4) whether the effects of NMDA receptor mutations on alcohol modulation and ion channel gating observed in a non-neuronal cell line can also be observed in mammalian CNS neurons.

The NMDA receptor, a type of ionotropic glutamate receptor, is highly expressed in the central nervous system (CNS). As one type of important neurotransmitter-activated ion channel in the brain, NMDA receptors play critical roles in multiple aspects of brain function, such as motor function, cognition, sensory processing, as well as plasticity involved in learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Dingledine *et al*. 1999; Paoletti and Neyton, 2007).

Alcohol addiction, characterized by compulsive and uncontrolled consumption of alcoholic beverages despite the negative consequences, is a costly and detrimental chronic brain disorder. It is thought to be associated with disorganized motor function, cognition, and learning and memory processes (Weiss and Porrino, 2002; Koob, 2003), all of which involve NMDA receptors. Following the initial finding of Lovinger and colleagues (1989) that alcohol inhibits NMDA receptors, a large number of studies have

established that the NMDA receptor is a major target of alcohol action in the brain and plays a role in several ethanol-associated phenomena such as tolerance, dependence, withdrawal and relapse (Khanna et al., 1993; Krystal *et al*., 2003b; Vengeliene *et al*., 2005; Chandrasekar, 2013). However, the molecular sites and mechanisms of alcohol action on the NMDA receptor are still incompletely understood.

The NMDA receptor is a heterotetramer formed between two obligatory GluN1 subunits and two GluN2 subunits, while four different isoforms of the GluN2 subunits are expressed in the brain (GluN2A-GluN2D) (Kew et al., 1998; Dingledine et al., 1999). Although the GluN2A subunit-containing NMDA receptor predominates in the mammalian brain, recent research has pointed to the importance of GluN2B subunit-containing NMDA receptors in brain function and alcohol action (Chazot, 2004; Nagy, 2004; Boyce-Rustay and Holmes, 2005; Gogas, 2006; Kash *et al*., 2008). Considering the importance of the GluN2B subunit in the actions of alcohol, the lack of information about the molecular sites and mechanisms of alcohol action on GluN2B subunit containing-NMDA receptors, represents a critical gap in the understanding of alcohol action in the brain.

Findings presented in this dissertation provide key information for the interaction between the GluN2B subunit and the ethanol molecule by identifying candidate sites of ethanol action on the GluN2B subunit protein. Furthermore, studies in this dissertation may aid in developing a better structural model of the M3 and M4 domains of the GluN2B subunit and in understanding the relation between the structure and the function of these domains as well as the effects of alcohol on these domains. Finally, studies in which wild-type and mutant NMDA receptors were transfected into rat cortical neurons

during the early development period have demonstrated that effects of NMDA receptor mutations on alcohol sensitivity and ion channel gating observed in a non-neuronal cell line are not cell-specific phenomena.

Alcohol addiction

Alcohol addiction is defined as a chronic and often progressive disorder, which is characterized by preoccupation with alcohol, problems controlling excessive consumption, compulsive use of alcohol, and the presence of either physical dependence on alcohol or withdrawal symptoms when rapidly decreasing or stopping drinking (American Psychiatric Association, 1994). Among addictions, alcohol abuse and dependence are both profound in their impact on society and global in scope. Unhealthy alcohol consumption can affect various organ systems, such as liver, heart and stomach, and the brain is the major target of acute alcohol actions (Grant *et al*., 2005; Hasin *et al*., 2007). Chronic ethanol exposure also has complex and long-lasting effects on the brain at the morphological, functional and behavioral levels. Studies focused on structural changes in alcohol addiction have shown that administration of ethanol to rat pups can lead to oxidative stress in the developing cerebellum and neurotoxicity-induced loss of Purkinje cells in this brain region (Ramezani *et al*., 2012). And effects of ethanol are not only seen during development. Chronic alcohol assumption also results in neuropathologically-detectable gray and white matter injury in mature brain, which is accompanied by brain shrinkage (Kril *et al*., 1997; Harper *et al*., 2003; Bartsch *et al*., 2007). Metabolic impairments were found in the mediofrontal and dorsolateral prefrontal cortex following chronic alcohol consumption, which suggests an effect of chronic

alcoholism on brain metabolic processes (Dao-Castellana *et al*., 1998). Alcohol addiction is thought to be associated with alterations in certain brain functions, such as decision-making, and impairment is thought to be associated with brain shrinkage (Le Berre *et al*., 2012). Moreover, individuals abusing alcohol often show blackout behavior and disrupted performance on a variety of short-term memory tasks, which indicates impaired learning and memory following binge drinking of alcohol, and acute ethanol can impair semantic and figural memory (Miller *et al*., 1978; Lister *et al*., 1991; Acheson *et al*., 1998).

Unlike other drugs of abuse, alcohol targets are widespread in the brain, and actions involve a number of brain neurochemical systems, with each system supporting part of the mechanism of alcohol reward and alcohol-seeking behavior, which presents unique challenges to study the basis of alcohol addiction. Alcohol has been demonstrated to be associated with dopamine function in the mesolimbic "reward" system (Weiss and Porrino, 2002). It has been reported that ethanol can increase the firing of dopamine neurons in the ventral tegmental area (VTA) (Gessa *et al*., 1985; Brodie *et al*., 1990), and ethanol elevates dopamine concentration in the nucleus accumbens core (NAc) (Di Chiara and Imperato, 1988). A number of brain regions play a role in the development of alcohol dependence, including the central nucleus of the amygdala, the bed nucleus of the stria terminalis (BNST), and a transition zone in the medial part of the nucleus accumbens (NA). Within these nuclei, alcohol plays a key role in dysregulation of the brain stress systems. For example, brain corticotrophin-releasing factor (CRF) activity was significantly increased during alcohol dependence reinforcement (Koob, 2003). In addition, a large body of research indicates that the function of neurotransmitter-gated ion

channels are abnormally altered during alcohol exposure. Lovinger *et al*. (1989) first showed that NMDA-activated current was inhibited by ethanol in hippocampal neurons. And this key publication also showed that alcohol-associated NMDA receptor inhibition may contribute to the neural impairments induced by alcohol intoxication. In subsequent years, several other types of ionotropic receptors were reported to be disrupted by alcohol, including γ-aminobutyric acid $(GABA)_A$ receptor and glycine receptor, that alcohol can enhance their function (Mihic *et al*., 1997). In addition to its effects on these three receptors, the function of neuronal nicotinic acetylcholine (nAch) –like receptor and 5-hydroxytryptamine 3 ($5\text{-}HT_3$) receptor also can be potentiated by alcohol exposure (Lovinger, 1997). And among these ligand-gated ion channels (LGICs), the NMDA receptor and $GABA_A$ receptor has been intensely studied, and viewed as major targets of alcohol action in the brain. However, actions of alcohol on $GABA_A$ receptors strongly depend on variables, such as the subunit composition, that most subunit compositions only respond to alcohol action at high concentrations (>60 mM) (Tsai and Coyle, 1998; Vengeliene *et al*., 2008; Chandrasekar, 2013).

NMDA receptor structure

The NMDA receptor, a member of the ionotropic glutamate receptor family in the brain, is formed as heterotetrameric assemblies of integral membrane protein subunits. Early studies about the stoichiometry of NMDA receptors proposed a pentameric structure based on the size of chemically cross-linked NMDA receptor protein as well as analysis of sensitivity to channel blockers (Brose *et al*., 1993; Ferrer-Monteil and Montal, 1996), although other studies from the same time period concluded that the NMDA

receptor is a tetrameric protein consisting of two GluN1 and two GluN2, or two GluN3 subunits in the early postnatal phase (Behe *et al*., 1995; Pachernegg *et al*., 2012), and that homomeric subunits could arrange as tetramers (Mano and Teichberg, 1998). Eventually, sufficient evidence was presented to support a heterotetrameric arrangement for the NMDA receptor, which could be activated by concurrent binding of glycine or D-serine to the GluN1 subunit and glutamate to the GluN2 subunit. A GluN1 subunit can combine with two of the same types of GluN2 subunit, and also can combine with two different GluN2 subunits to form triheteromeric receptors. A number of studies have demonstrated the presence of GluN1/GluN2A/GluN2B, GluN1/GluN2A/GluN2C, GluN1/GluN2B/GluN2D, and GluN1/GluN2A/GluN2D NMDA receptors in different brain regions as well as different neuronal subpopulations (Chazot *et al*., 1994; Chazot and Stephenson, 1997; Luo *et al*., 1997; Dunah *et al*., 1998; Brickley *et al*., 2003; Jones and Gibb, 2005; Karakas and Furukawa, 2014).

Expression of functional NMDA receptors requires the co-expression of two obligatory GluN1 subunits and at least one type of GluN2A-D subunit or GluN3A-B subunit. There are eight different GluN1 subunits generated from alternative splicing variants of the same gene, and six separate genes encode four different GluN2 subunits (A, B, C, and D) as well as two different GluN3 subunits (A, B; Dingledine *et al*., 1999; Kaniakova *et al*., 2012; Chandrasekar, 2013; Paoletti and Neyton, 2007). Recent studies using x-ray crystallography have reported the structure of the GluN1/GluN2B NMDA receptor, which has an overall twofold symmetry, a dimer-of-dimers arrangement in which the GluN1 and GluN2B subunits occupy the A-C and B-D positions, respectively, defined previously by the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

(AMPA) receptor (Lee *et al*., 2014; Karakas and Furukawa, 2014; Sobolevsky et al., 2009; Figure 1.1).

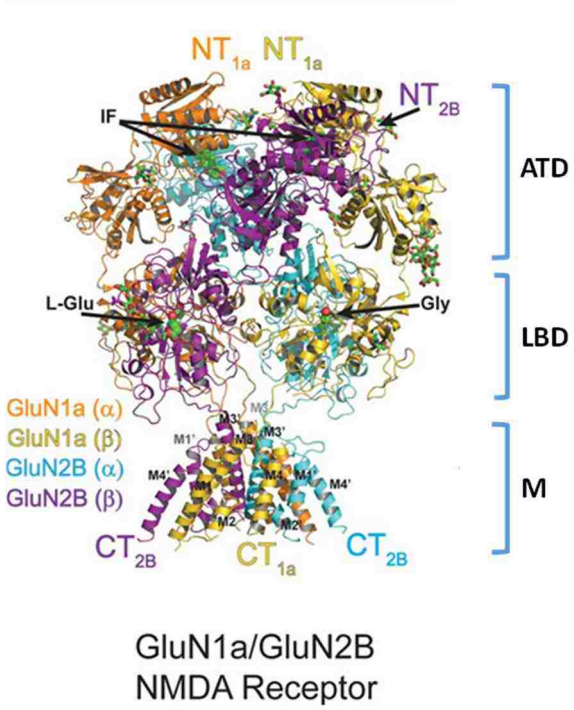

Figure 1.1. Overall structure of the heterotetrameric GluN1/GluN2B NMDA receptor

The GluN2B-containing NMDA receptor structure with the amino (AT) and carboxy (CT) domains located on the top and bottom, respectively (Karakas and Furukawa, 2014). Two GluN1 subunits are colored in orange and yellow, and two GluN2B subunits are colored in cyan and purple, which clearly indicate that the NMDA receptor is composed of four subunits in a dimer of dimers manner [Protein Data Bank (PDB) ID: 3KG2]. Ifenprodil (IF), is located at the GluN1-GluN2B ATD heterodimer interfaces, and the agonists, glycine (Gly) and L-glutamate (L-Glu), bind at the LBD clamshells, as indicated by arrows. Note that the large intracellular C-terminal domains are not shown, as their structures could not be resolved.

The structure of a single NMDAR subunit can be described by a large amino-terminal domain (ATD), an extracellular ligand-binding domain (LBD) (S1S2), four membrane-associated domains (M1-M4), of which M2 is a re-entrant membrane loop, and an intracellular carboxy-terminal domain (CTD) (Figure 1.2). The ATD is highly homologous with the bacterial protein leucine/isoleucine/valine-binding protein (LIVBP), one of the bacterial periplasmic binding proteins (PBPs) (Masuko *et al*., 1999; Paoletti *et al*., 2000). Numerous studies have used truncation mutants that lack the entire ATD to study the role of this region. However, these truncated subunits seem to assemble into functional NMDA receptors with altered open probability, deactivation, and desensitization compared with wild-type receptor (Fayyazuddin *et al*., 2000; Matsuda *et al*., 2005; Rachline *et al*., 2005; Gielen *et al*., 2009; Yuan *et al*., 2009). In turn, the nonessential nature of this region seems to be consistent with its regulatory role in the receptor. For example, the ATD of the GluN2 subunit contains the binding sites of many NMDA receptor modulators, such as zinc, ifenprodil, protons, and polyamines (Choi and Lipton, 1999; Fayyazuddin *et al*., 2000; Gallagher *et al*., 1996; Gallagher *et al*., 1997).

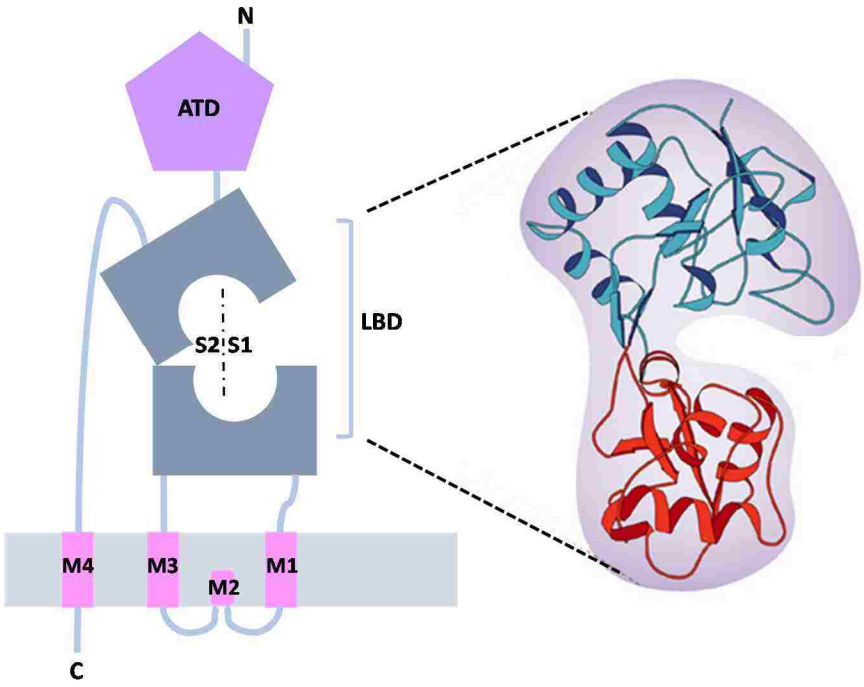

Figure 1.2. Single subunit of NMDA receptor

The amino-terminal domain (ATD) is followed by the S1 half-domain (Pre-M1), two transmembrane domains (M1, M3) with a re-entrant P loop (M2), the S2 half-domain (linker between M3 and M4), and the last transmembrane domain (M4). The carboxyl terminus is located in the cytoplasm, where it can interact with proteins of the postsynaptic density. The ligand-binding domain (LBD) is formed by S1 and S2 half-domains (Armstrong and Gouaux, 2000).

The ligand binding domain (LBD) is formed by two polypeptide segments, named S1 and S2, and this domain adopts a clamshell-like structure that undergoes a conformational change to enclose the ligand upon binding. The S1 segment, located on the amino-terminal side of M1, forms half of the clamshell, and the segment S2 between the M3 and M4 domains forms the other half of the clamshell (Armstrong and Gouaux,

2000; Furukawa and Gouaux, 2003; Sobolevsky *et al*., 2009). This region shows sequence homology with the bacterial glutamine-binding protein QBP (Stern-Bach *et al*., 1994; Hsiao *et al*., 1996) and contains the agonist binding pocket within the cleft between S1 and S2 segment. The initial step for activation of the NMDA receptor requires the simultaneous binding of either glycine or D-serine in the GluN1 subunit and glutamate in the GluN2 subunit. Agonists of the NMDA receptor uniformly contain sequences that correspond to the α-amino and α-carboxyl groups, which form atomic interactions with the regions of the binding pocket (Armstrong *et al*., 1998; Armstrong and Gouaux, 2000; Gill *et al*., 2008; Furukawa *et al*., 2005).

The re-entrant membrane loop (M2) is homologous to the P-loop sequences of other channels, which, together with the upper region of the M3 domain, contributes to formation of the pore facing the cytoplasm, whereas the M3 forms the outer boundary of the cavity facing the extracellular side. The M1 helix is located on the exterior of M2 and M3, and the M4 domain from one subunit is associated with the ion channel core of an adjacent subunit, which indicates not only that the M2 loop and M3 domain are essential for ion channel gating, but also that the M1 and M4 helices are important for regulating ion channel gating (Schneggenburger and Ascher, 1997; Villarroel *et al*., 1998; Ren *et al*., 2003a). The ion channel pore of the NMDA receptor has a small segment of amino acid sequence that is similar to those found in the inverted ion channel domain of K^+ channels (Wood *et al*., 1995; Wo and Oswald, 1995; Kuner *et al*., 2003). The P-loop sequences are responsible for the selection of ion permeability as well as the affinity for the pore blocker Mg^{2+} (Verdoorn *et al*., 1991; Burnashev *et al*., 1992). The M3 domain of

GluN1 and GluN2 subunits have also been demonstrated to contribute to the regulation of the surface delivery of NMDA receptors (Kaniakova *et al*., 2012).

The CTD of the NMDA receptor shows no sequence homology to any known proteins; however, it encodes short docking motifs for intracellular binding proteins. This region is thought to be responsible for membrane targeting, stabilization, post-translational modifications, targeting for degradation, and regulating channel function. Deletion of this domain in some subunits (e.g., GluN1, GluN2A) does not completely abolish channel functions but does alter them (Kohr and Seeburg, 1996; Vissel et al., 2001; Peoples and Stewart, 2000). For example, the C-terminal domain of the GluN1 subunit contains a number of regulatory protein binding sites, including sites for calmodulin, scaffolding proteins, and phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC) (Wang *et al*., 2006; Blanke et al., 2009) (Figure 1.3).

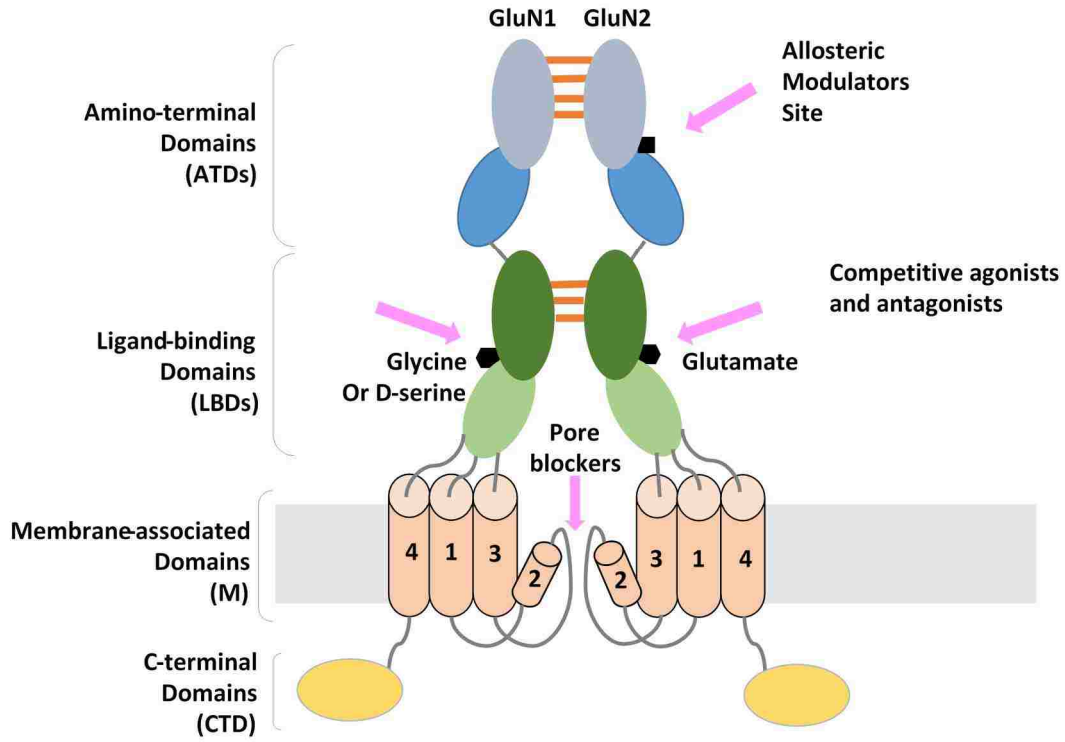

Figure 1.3. The NMDA receptor is believed to assemble as a tetramer

Two GluN1 and two GluN2 subunits are arranged in a "dimer of dimers" quaternary architecture. For clarity, only one of the two heterodimers is shown. In the extracellular region, the subunits dimerize at the level of the ATDs. Glutamate binds at the GluN2 LBD binding site, whereas glycine (or D-serine) binds at the GluN1 LBD binding site. Sites in the GluN2 ATD are known to bind allosteric modulators such as zinc (GluN2A and GluN2B ATDs) or ifenprodil-like compounds (GluN2B ATDs). Ion channel domain contains binding sites for pore blockers such as Mg^{2+}, MK-801 or ketamine.

NMDA receptor kinetic model

The general concept of activation of the NMDA receptor is that glutamate binds in the cleft of the S1-S2 segments of a single NMDA receptor GluN2 subunit (glycine or D-

serine bind similarly to the GluN1 subunit), which form a clam-shell-like motif. The conserved structural elements for this agonist-binding site among different types of ionotropic glutamate receptors are an arginine side chain on helix D, which forms the major binding site for the ligand α-carboxyl group, and a glutamate side chain, which binds the agonist α-amino group (Mayer, 2006; Watkins et al., 1990). As discussed previously, the NMDA receptor is formed by heterodimers, and these dimers play a key role in coupling the agonists binding to the activation of ion channel gating. As illustrated in **Figure 1.4**, in domain 1 (D1), which is part of the ligand binding domain, dimers are

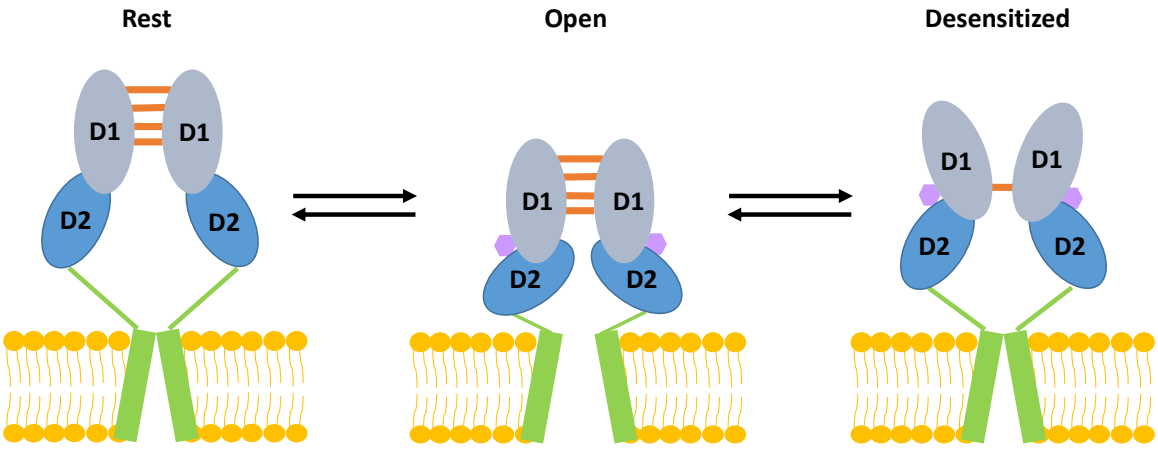

Figure 1.4. NMDA receptor kinetic model of structural changes accompanying ion channel opening and desensitization

Diagrammatic representations of the resting, activated and desensitized states to illustrate how domain closure is linked to breaking of contacts between two D1s.

connected together by hydrophobic contacts made on the surface of two helices, as well as hydrogen bonds and salt bridges. However, the domain 2 (D2) is free to move from the

resting state to the agonist-bound state. Immediately after the binding of agonists, D2 moves toward D1 and produces a scissors-like outward motion of the linkers between the membrane-associated domains and ligand-binding domains. In turn, contraction of the ligand-binding domains in a dimer assembly upon agonists binding could result in the movement of the membrane-associated domains, causing gating of the ion channel. The channel opens because the dimer only forms through the D1 surface, but leaves D2 free to move. In other ionotropic glutamate receptor types, such as AMPA and kainate receptors, each subunit contains an agonist binding domain, which is linked with the membrane-associated domains of the same subunit, so it is reasonable to imagine that agonist-binding in an individual subunit could result in partial opening of the ion channel. This is not the case for NMDA receptors, however, in which binding of either glutamate or glycine alone does not trigger ion channel gating (Armstrong and Gouaux, 2000; Banke *et al*., 2003; Schorge *et al*., 2005).

This model also suggests that the desensitization state occurs when the hydrophobic contacts between ligand-binding domains are broken during the conformational rearrangement of the dimer. In this state, even though the agonists remain bound to their binding sites, the uncoupled agonist-binding domains in a dimer could rotate around the central axis, relieving strain on the linkers between the ligand-binding domain and membrane-associated domains, allowing the ion channel to close (Sun *et al*., 2002). As described previously, there are at least two mechanisms by which NMDA receptor-mediated responses decay over time, in addition to true desensitization: the first is manifested as a decrease in glycine affinity, and the second is calcium-dependent

inactivation, which depends upon calcium interaction with the GluN1 C-terminal domain (Mcbain and Mayer, 1994; Clark *et al*., 1990).

NMDA receptor function in the brain

During brain development, the subunit composition of NMDA receptors changes in response to neuronal activity from predominantly GluN2B subunit-containing to primarily GluN2A subunit-containing NMDA receptors (Bellone and Nicoll, 2007). However, this replacement is not absolute, GluN2B subunits are still expressed in many regions in the adult brain and play a major role in multiple brain functions. The molecular and chemical mechanisms for the change in expression have not yet to be fully understood. It is possible that neuronal activity during this period drives the trafficking and insertion of the GluN2A subunit as well as the endocytosis of the GluN2B subunit (Barria and Malinow, 2002; Groc *et al*., 2006; Paoletti *et al*., 2013). Because this switch in subunit composition occurs during a critical time window when synaptogenesis, circuit refinement, and acquisition of learning abilities occur (Gambrill and Barria, 2011; Sanchez *et al*., 2012; Dumas, 2005), the change in subunit composition may explain the present of these processes during brain development.

On the synaptic level, NMDA receptor subunit composition plays an important role in the process of synaptogenesis, synapse maturation, and synapse stabilization. One previous study concluded that GluN2 subunits are key elements of normal development of synapses, in which the GluN2A subunit acts to stabilize synapse formation, whereas the GluN2B subunit is required for spine retraction and new spine formation (Aizenman and Cline, 2007; Wu *et al*., 1996; Isaac *et al*., 1997; Gambrill and Barria, 2011). A large

number of studies have further explored the role of NMDA receptor-mediated synaptic activity in the functional development of neurons and neuronal circuits. Although NMDA receptors are not essential for brain development, because transgenic mice lacking individual NMDA receptor subunits appears normal, at least at birth, their activities are vital for regulating the integration of newborn neurons into mature brain circuits (Tahiro *et al*., 2006; Forrest *et al*., 1994; Kutsuwada *et al*., 1996). NMDA receptors are known to have a role in axon arbor refinement since experiments on retinotectal topographic map indicated an evolutionarily-conserved role of the NMDA receptor in activity-dependent sensory map formation (Cline *et al*., 1987; Cline *et al*., 1989; Simon *et al*., 1992). In addition to exon arbor elaboration, blockade of NMDA receptor activity with APV also showed a reduced dendritic growth rate (Rajan and Cline, 1998). Moreover, during the early phase of the subunit type switch, GluN2A subunit-containing NMDA receptors progressively predominate in the synapses, which leads to a balance of plasticity and stability, and more balanced synapse function is optimal for information processing and formation of various associative learning abilities (Quinlan *et al*., 1999; Quinlan *et al*., 2004; Dumas, 2005).

The fundamental physiological function of the NMDA receptor in the brain is to regulate calcium flux in response to synaptic activity as well as to play an important role in excitatory synaptic neurotransmission. NMDA receptors regulate functional and structural plasticity by activating multiple calcium-dependent pathways upon the removal of magnesium block by coincident strong depolarization. Although NMDA receptors activate more slowly than other ionotropic glutamate receptors, they have higher affinity for glutamate and much longer synaptic potentials compared with AMPA receptors,

which are responsible for the initial membrane depolarization that overcomes NMDA receptor magnesium block (Mayer *et al*., 1984; Huang and Bergles, 2004). Coactivation of NMDA receptors in multiple synapses enhances excitatory postsynaptic potentials (EPSPs) to facilitate temporal summation and allow neurons to detect and react to the synchrony of inputs (Zito and Scheuss, 2009).

Some of the features of NMDA receptors, such as their prolonged EPSP duration, magnesium block, and calcium permeability, allow them to play important roles in certain forms of both positive and negative synaptic plasticity. High frequency synaptic input-induced excitatory postsynaptic potentials (EPSPs) are the basis of the paradigm of long-term potentiation (LTP), which involves phosphorylation and enhanced trafficking of AMPA receptors to the postsynaptic membrane (Bliss and Lomo, 1973; Johnston, 1992). The role of NMDA receptors in some forms of LTP in the brain is well demonstrated in previous studies, especially in the hippocampal CA1 region (Izquierdo, 1994; Rockstroh *et al*., 1996). Long-term depression (LTD), which can be induced by prolonged low frequency (0.5 to 3 Hz) stimulation, is the negative counterpart of LTP. NMDA receptor-dependent LTD decreases synaptic potentials by dephosphorylation-induced reduction of open probability and downregulation of AMPA receptors in the hippocampal CA1 area (Dudek and Bear, 1992; Bear and Fitzjohn, 1999; Isaac, 2001) (Figure 1.5).

Both NMDA receptor-mediated LTP and LTD are proposed to be cellular mechanisms by which the brain can change in response to external stimuli, and LTP in particular plays a crucial role in various types of learning and memory (Riedel *et al*., 2003). During learning and memory processes, activation of NMDA receptors has been

shown to be required for initiation of LTP in multiple brain regions, including the hippocampus, amygdala, and medial septum. Studies in experimental animals suggest that the glutamate receptor system and LTP are closely linked to the induction of new memory formation, and blockade of NMDA receptors before learning can impair memory formation (Izquierdo and Medina, 1993; Constantine-Paton, 1994). By using

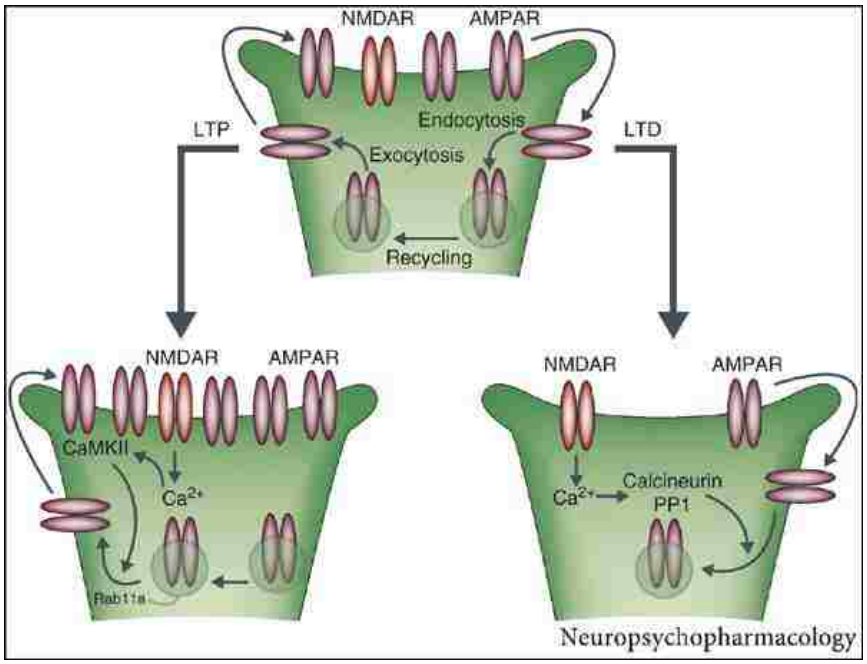

Figure 1.5. Model of LTP and LTD

During the basal state, AMPA receptors cycle between the postsynaptic membrane and intracellular compartments in an equilibrium (depicted on top). Following induction of LTP, there is enhanced AMPA receptor exocytosis and insertion into the membrane of post-synaptic neuron, and this process is driven by calcium-induced CAMKII activity and fusion of recycling endosomes (depicted on bottom left). However, following induction of LTD, AMPA receptor endocytosis process is enhanced, which is also calcium-dependent (depicted on bottom right) (Citri and Malenka, 2007).

NMDA receptor antagonists and blockers, such as MK-801, learning impairments following NMDA receptor inhibition have been reported in nonhuman primates, and prevention of the induction of LTP by NMDA receptor antagonists was shown to contribute to these learning impairments (Harder *et al*., 1998; Morris et al., 1986). fMRI studies in humans also reported that an NMDA receptor antagonist evoked a range of schizophrenia-like symptoms (Honey *et al*., 2005; Krystal *et al*., 1994). Administration of a pure NMDA receptor antagonist has been shown to disrupt memory formation in humans (Rowland *et al*., 2005). Not surprisingly, because NMDAR-associated brain functions can be disrupted by alcohol, it is well established that NMDAR is a major target of alcohol actions in the brain (Woodward *et al*., 1999; Krystal *et al*., 2003a; Peoples *et al*., 2003; Gass and Olive, 2008; Vengeliene *et al*., 2008).

Although appropriate NMDA receptor activation is essential for neuronal survival and physiological functions, abnormal activation contributes to multiple pathological processes in the brain. Overactivation of NMDA receptors has been demonstrated to be involved in cell death triggered by seizure and ischemic stroke. Under normal conditions, calcium entry through the NMDA receptor ion channel can mediate synaptic plasticity in the brain. However, excessive NMDA receptor activation could induce unusually high intracellular calcium, which triggers a series of cytoplasmic and nuclear processes that are involved in neuronal cell death. For instance, upon entry of excessive calcium into the cell, enzymes are activated to degrade either essential proteins or certain DNA, which is considered to be the mechanism of neuronal cell death in seizure and ischemic stroke (Dirnagl *et al*., 1999;). Moreover, enhanced oxidative stress, together with calcium overload, can induce cell death through apoptosis in neurodegenerative diseases.

Increased cytoplasmic calcium can enhance the mitochondrial electron transport function, increasing reactive oxygen species in these neurons and leading to oxidative stress in the brain. It has been demonstrated that dysfunction of the NMDA receptors, located both synaptically and extrasynaptically, are associated with certain neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, as well as Huntington's disease. Overactivation of NMDA receptors can lead to increased calcium influx, which cause excessive oxidative phosphorylation and increased production of reactive oxygen species. This eventually leads to oxidative stress-caused neuronal death, which plays an important role in the mechanism of neurodegenerative diseases (Michaelis, 1998; Greene and Greenamyre, 1996; Zhou *et al*., 2013).

NMDA receptor and alcohol

Since alcohol can directly inhibit NMDA receptor in the brain, it is important to understand how the NMDA receptor structure and function are modified in response to alcohol exposure at different levels (Woodward *et al*., 1999; Krystal *et al*., 2003a; Peoples *et al*., 2003; Krystal *et al*., 2003b). At the behavioral level, the NMDA receptor has been linked to many alcohol exposure-associated behaviors. Systemic administration of NMDA receptor inhibitors significantly inhibited rapid tolerance in the intoxicated paradigm in mice, which supports a potential role of the NMDA receptor in the attenuation of alcohol intoxication (Khanna *et al*., 1997). Furthermore, repeated administration of NMDA receptor antagonists decreased ethanol consumption in a concentration-dependent manner (Vengeliene *et al*., 2005). Like other types of substance addiction, withdrawal is also a hallmark for physical dependence to ethanol, and the

NMDA receptor plays an important role during this process. For instance, excessive NMDA receptor activity due to withdrawal from chronic ethanol has been proposed to be the main cause of neuronal cell death in ethanol withdrawal period (De Witte *et al*., 2003; Fadda and Rossetti, 1998).

At the level of synaptic physiology, it is well established that calcium influx through NMDA receptors regulates synaptic plasticity, especially LTP, via calcium-regulated signaling processes (Malenka and Nicoll, 1999; Bliss and Collingridge, 1993). A previous study reported that LTP in the dentate gyrus of the hippocampus was inhibited by intraperitoneal injection of non-intoxicating concentrations of ethanol (Givens and McMahon, 1995). In other brain regions, ethanol inhibition of LTP was also observed, including in dorsomedial striatum (Yin *et al*., 2007) and dorsolateral bed nucleus of the stria terminalis (Weitlauf et al., 2004).

At the receptor level, Lovinger and colleagues initially reported that acutely applied ethanol could inhibit NMDA-activated ion currents in cultured mouse hippocampal neurons (Lovinger *et al*., 1989). Inhibitory actions of ethanol on NMDA receptor-mediated excitatory postsynaptic potentials (EPSPs) in slices from different brain regions were subsequently demonstrated, such as hippocampus, cortex, basolateral amygdala, nucleus accumbens as well as dorsal striatum (Lovinger *et al*., 1990; Calton *et al*., 1999; Nie *et al*., 1994; Yin *et al*., 2007; Wirkner *et al*., 2000). In cultured cortical neurons, single channel recording showed that ethanol can decrease the open channel probability and mean open time of native NMDA receptor (Wright *et al*., 1996). Moreover, when recombinant NMDA receptors were transfected into cell lines, such as human embryonic kidney (HEK) 293 cells, reduction in NMDA receptor-mediated

excitatory currents have also been reported (Peoples and Stewart, 2000). These changes in ion channel kinetics are believed to underlie behavioral changes found in alcohol addiction patients (Koob *et al*., 2003). Although the rapid reduction of channel activity in response to ethanol implies that there is a direct interaction between ethanol and NMDA receptor, the precise mechanism by which ethanol can inhibit NMDA receptor ion channel kinetics is still incompletely understood. And it seems one of the useful ways to study ethanol action on NMDA receptor precisely is to transfect different combinations of NMDA receptor subunits into nonneuronal mammalian cell culture model, such as HEK 293 cells, which do not contain any endogenous NMDA receptor subunits.

In addition to acute inhibitory effect of ethanol on NMDA receptor, chronic treatment of ethanol has more complex and long-lasting effects on the expression and function of the NMDA receptors (Lovinger, 1997). Neuroadaptive changes in NMDA receptor functions during long-term alcohol treatment may play an important role in the development of alcohol tolerance and dependence (Fadda and Rossetti, 1998). Previous studies showed that chronic ethanol exposure in adult and fetal cortical neuron cells upregulated NMDA receptor subunit genes expression, which eventually contribute to upregulation of NMDA receptor subunit levels (Anji and Kumari, 2006). In order to compensate prolonged inhibitory effect caused by chronic ethanol exposure, NMDA receptor mediated functions were also upregulated. During prolonged ethanol exposure, both synaptic NMDA receptor clustering and synaptic NMDA currents were significant increased (Carpenter-Hyland *et al*., 2004).

Molecular sites of alcohol action on NMDA receptor

Compared to other antagonists of the NMDA receptor, alcohol has different features when acting on the NMDA receptors. Alcohol acts as a noncompetitive antagonist at a site different from other NMDA receptor modulators (Peoples *et al*., 1997). However, unlike other NMDA receptor modulators, alcohol does not have a known site of action, so it is of interest to determine the molecular sites of alcohol action on the NMDA receptor (Peoples and Stewart, 2000; Honse *et al*., 2004; Ren *et al*., 2012). This information can also be beneficial for the development of pharmacological therapies for alcohol addiction. A number of studies have demonstrated that alcohol and NMDA receptor antagonists can produce similar subjective effects in animals and humans (Grant *et al*., 1991; Krystal *et al*., 1998; Hodge *et al*., 1998).

Early studies tested whether ethanol could act on the agonist or co-agonist site or other modulatory sites. However, the effect of ethanol on NMDA receptors in cultured mouse hippocampal neurons showed that ethanol decreased the E_{max} of the NMDA concentration-response curve without affecting the EC_{50} value. This indicates that the mechanism of ethanol inhibition is non-competitive with respect to agonists of NMDA receptor (Gothert and Fink, 1989; Gonzales and Woodward, 1990; Rabe and Tabakoff, 1990; Peoples *et al*., 1997; Peoples and Stewart, 2000). Studies on the role of the co-agonist (glycine) site in mediating the effect of ethanol are more controversial. Some studies reported a possible interaction of alcohol with the glycine co-agonist site based on apparent reversal by glycine of ethanol inhibition (Hoffman *et al*., 1989; Rabe and Tabakoff, 1990; Dildy-Mayfield and Leslie, 1991; Buller *et al*., 1995), while in other studies using full glycine concentration-response curves, the action of ethanol was shown

to be noncompetitive with respect to glycine (Gonzales and Woodward, 1990; Peoples and Weight, 1992; Woodward, 1994; Chu *et al*., 1995; Mirshahi and Woodward, 1995; Peoples *et al*., 1997). It seems the most probable that the glycine-binding site may regulate ethanol sensitivity, however, it is not the site of alcohol action by itself.

A number of studies also tried to identify the relationship between other regulatory sites in the NMDA receptor and the inhibitory effect of ethanol. For example, studies assessing whether the effect of ethanol on NMDA receptors in cultured cortical neurons was dependent upon the concentration of magnesium (Mg^{2+}), demonstrated that ethanol inhibition of NMDA-activated currents was not affected by changes in Mg^{2+} concentration (Peoples *et al*., 1997). Furthermore, the absence of any effect of other regulatory agents on the inhibition by ethanol of the NMDA receptor eliminated the possibility that ethanol can interact with regulatory site of the NMDA receptor (Snell *et al*., 1994; Peoples *et al*., 1997).

A number of studies also addressed whether ethanol could bind within the channel pore region. The inability to demonstrate the binding sites of ethanol within the channel pore of NMDA receptor was reported by both whole-cell and single-channel analysis results from cultured neurons (Wright *et al*., 1996; Popp *et al*., 1999). Taken together, these results can be interpreted to indicate that ethanol does not interact with a known site in the NMDA receptor, and that alcohol may affect NMDA receptor function via an unidentified site, effects on membrane lipids, or other proteins that interact with and modulate its activity, such as PKC. To test these possibilities, a previous study used C-terminal truncation mutant NMDA receptor subunits to investigate a possible role for intracellular proteins that can modulate the activity of NMDA receptor. The observations

of this study showed that C-terminal truncation did not eliminate ethanol sensitivity of the NMDA receptor (Peoples and Stewart, 2000). This would argue against the possibility that alcohol inhibition of NMDA receptor is mediated primarily through the interaction of alcohol with either intracellular regulatory protein binding sites or the second messenger system, and showed in addition that the site of alcohol action of the NMDA receptor was not located in the C-terminal domain (Figure 1.6).

Studies performed to locate the region of the NMDA receptor containing the site of alcohol action eliminated the agonist and coagonist sites (*see above*), the channel pore (Wright *et al*., 1996), and the C-terminal domains (Peoples and Stewart, 2000), making the M domains a likely candidate region for a site of alcohol action. The first report of an alcohol-sensitive amino acid in the NMDA receptor M domains was from the laboratory of Woodward and colleagues (Ronald *et al*., 2001). This study demonstrated that a phenylalanine residue located in the third membrane-associated domain of GluN1 subunit, Phe639, can influence ethanol sensitivity, but the variable ethanol sensitivity among different mutants in a subsequent study (Smothers and Woodward, 2006) implied that this is not the only position for ethanol action. A study from our laboratory first identified a position of alcohol action in the fourth membrane-associated domain of the GluN2A subunit of NMDAR, Met823, which is exposed to extracellular face of membrane (Ren *et al*., 2003b). We also reported an important role for this residue in the regulation of ion channel gating, such as in the steady-state NMDA EC_{50}, apparent desensitization, mean open time as well as peak current density (Ren *et al*., 2003a). Ethanol sensitivity among various mutants at Met823 was correlated with the steady-state to peak current ratio (apparent desensitization) based on these two studies. A linear

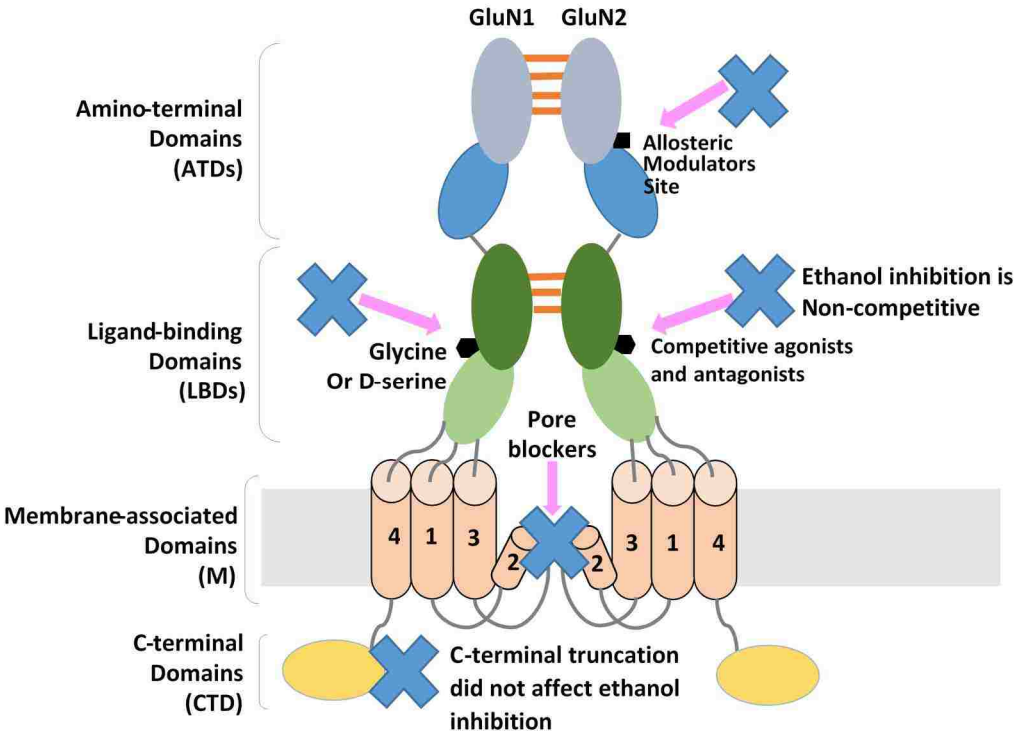

Figure 1.6. Molecular sites of alcohol action on NMDA receptor

One heterodimer of NMDA receptor is shown here. Glutamate binds at the GluN2 LBD binding site, whereas glycine (or D-serine) binds at the GluN1 LBD binding site. Sites in the GluN2 ATD are known to bind allosteric modulators such as zinc (GluN2A and GluN2B ATDs) or ifenprodil-like compounds (GluN2B ATDs). Ion channel domain contains binding sites for pore blockers such as Mg^{2+}, MK-801 or ketamine. Blue crosses indicate non ethanol-sensitive sites reported by precious studies.

relation between apparent desensitization and the substituent amino acid hydropathy was found in these studies, as was a linear relation between ethanol sensitivity and hydrophilicity of the substituent at this position, which indicates that ethanol interact with this site in a manner that involves hydrophobic binding. Besides these observations, ethanol sensitivity was correlated with molecular volume of the substituent at this

position, which is consistent with results of ethanol action on other types of receptors, that is, that ethanol binds to a site in a manner that is dependent upon volume occupation of this site (Ren *et al*., 2003a&b; Mihic *et al*., 1997; Wick *et al*., 1998; Mascia *et al*., 2000).

Ethanol produces its effects at concentrations in the millimolar range, and interacts with multiple sites in other ethanol-sensitive neurotransmitter receptors (Franks and Lieb, 1994). It should thus be noted that GluN2A(Met823) could not completely account for the action of ethanol on the NMDA receptor. Studies from this laboratory successively identified a small number of ethanol-sensitive residues (Ala825 and Phe637) in both the M4 and M3 domains of the GluN2A subunit. After functional screening of the M4 domains of the GluN2A subunit, the former position was found to regulate ethanol IC_{50} as well as NMDA receptor functions (Honse *et al*., 2004). The latter position, Phe637, is the cognate position of GluN1(Phe639). Various mutations at this position showed altered glutamate EC_{50}, maximal steady-state current (I_{ss}) to peak current (I_p) ratios, and mean open time of glutamate-activated currents, and ethanol sensitivity was significantly correlated with both glutamate peak current and steady-state EC_{50}. However, apparent desensitization was not correlated with any of these characteristics. Moreover, unlike GluN2A(Met823), ethanol sensitivity of the amino acid substituent at this position was not correlated with any of the physicochemical parameters (Ren *et al*., 2007). Recently, another ethanol-sensitive position in GluN2A subunit has been identified in our laboratory, Phe636, and a series of substitutions of amino acids at this position exhibited significantly altered glutamate potency and apparent desensitization (Ren *et al*., 2013). The common elements among those three identified positions in the GluN2A subunit

appear to be that they are hydrophobic, do not line the ion channel lumen, have limited accessibility from the cytoplasmic side of the membrane, and are located in regions that influence ion channel gating.

GluN2B subunit and alcohol action in the brain

Overall sensitivity of an individual NMDA receptor to ethanol depends on specific combinations of GluN1 and GluN2 subunits. Both GluN2A and GluN2B subunit-containing NMDA receptors are more sensitive to the inhibitory actions of ethanol compared to GluN2C and GluN2D-containing NMDA receptor (Chu *et al*., 1995; Mirshahi and Woodward, 1995; Masood *et al*., 1994; Blevins *et al*., 1997). However, comparison of the degree of ethanol sensitivity between GluN2A and GluN2B subunit-containing NMDA receptor still remains inconclusive. Although previous studies about alcohol actions on the NMDA receptor have been primarily focused on the GluN2A-containing NMDA receptor, accumulating evidence has shown the importance of the GluN2B subunit in the action of alcohol.

In 1997, one group observed that GluN2B phosphorylation level was increased following ethanol administration (Miyakawa *et al*., 1997). Moreover, chronic ethanol treatment upregulated the GluN2B subunit in mouse hippocampus (Kash *et al*., 2009), and ethanol inhibition of NMDAR-mediated synaptic transmission was dependent on the GluN2B subunit expression in this brain region (Kash *et al*., 2008). Another study reported that GluN2B subunit knockout eliminated the enhanced long-term potentiation (LTP) by chronic application of ethanol in the bed nucleus of the stria terminalis (BNST)

in mice (Wills *et al*., 2011). Behavioral studies have also shown that a GluN2B-selective antagonist reduced rat operant self-administration of ethanol (Wang *et al*., 2007).

However, there is still little information on the molecular mechanism of alcohol modulation of GluN2B-containing NMDAR. It is also unclear whether ethanol-sensitive positions in the GluN2B subunit are important for ion channel gating. Based on the fact that the third and fourth transmembrane domains of GluN2A and GluN2B are highly homologous, *studies in this thesis initially tested positions in the GluN2B subunit that correspond to ethanol-sensitive sites in the GluN2A subunit. However, not all of these positions showed significant ethanol sensitivity changes upon amino acids substitutions. In order to look for ethanol sensitive positions, the search was expanded to include other adjacent positions. Whether ethanol-sensitive positions are also important for ion channel gating was determined by using glutamate concentration-response experiments.*

Interaction between ethanol-sensitive positions

Because ethanol can interact with multiple positions in NMDA receptor as mentioned previously, it is possible that those positions would interact functionally to regulate ethanol sensitivity. Our laboratory has initially obtained evidence by using dual mutations at Phe637 and Met823 in the GluN2A subunit, which can both influence receptor function and alcohol action. Results from this study demonstrated that modulation of ethanol by dual mutagenesis was not additive, indicating that these two positions are functionally linked (Ren *et al*., 2008). This result was consistent with the model proposed by our collaborator Dr. Donard Dwyer, in which these two positions are located in close proximity. These positions, however, do not appear to form a unitary site

of alcohol action since they are separated by an estimated distance of 13–14 Å, which means they are not close enough to form a common binding site for one ethanol molecule. The NMDA receptor model in this study, however, was based on the protein aquaporin, and was developed prior to the structural determination of any of the ionotropic glutamate receptors.

In the model of the NMDA receptor derived from the x-ray crystallographic structure of the related GluA2 glutamate receptor, the outward face of the M3 domains of one subunit orient toward, and seems to form interactions with, the M4 domain of the other type of subunit (Sobolevsky *et al*., 2009). This implies the presence of sites of ethanol action in NMDA receptor, because the AMPA receptor and NMDA receptor have highly homologous structures. Ronald *et al.* initially reported that mutations at Phe639 in the M3 domain of the GluN1 subunit could alter ethanol sensitivity of NMDAR (Ronald *et al*., 2001). Our laboratory has also identified two ethanol-sensitive positions in the M3 domain, Phe636 and Phe637, and two in the M4 domains of GluN2A subunits, Met823 and Ala825 (Ren *et al*., 2013; Ren *et al*., 2007; Ren *et al*., 2003a; Ren *et al*., 2003b; Honse *et al*., 2004). Based on those previously identified ethanol-sensitive positions as well as the structural model, there are four putative pairs of positions in GluN1/GluN2A: Gly638/Met823, Phe639/Leu824, Met818/Phe636, and Leu819/Phe639, that can interact functionally to regulate ethanol sensitivity. By using both two-way analysis and mutant cycle analysis of log-transformed ethanol IC_{50} values from mutagenesis at both positions, our laboratory reported significant interactions with respect to ethanol sensitivity. Unlike the interaction between Phe637 and Met823 in the same GluN2A subunit, these sites can

alter ethanol action in a manner that suggests that the side chains of these pairs of residues physically interact with one another (Ren *et al*., 2012).

Considering the high homology of the M3 and M4 sequences in the GluN2A and GluN2B subunits, it is likely that similar sites of ethanol action exist between the GluN1 and GluN2B subunits. *In this thesis, studies directed toward the discovery of ethanol-sensitive sites at the M3-M4 intersubunit interfaces of the GluN2B-containing NMDA receptor were conducted based on the study of ethanol-sensitive positions in the GluN2B subunit.*

II. MATERIALS AND METHODS

Materials

All drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), except for BAPTA, tetracesium salt (Life Technologies, Carlsbad, CA). Chemicals used to make recording solutions were the highest purity available. Ethanol (95%; prepared from grain) was obtained from Pharmco (Brookfield, CA).

NMDA receptor subunit plasmids and site-directed mutagenesis

Plasmids containing the sequences encoding each NMDA receptor subunit (GluN1, GluN2) were used to heterologously express receptors in model cells.

Site-directed mutagenesis in plasmids containing GluN1 or GluN2 subunit cDNA was performed using a PCR-based system, the QuikChange II kit (Stratagene, La Jolla, CA). Mutagenic oligonucleotide primers were designed following criteria: 1) Both forward and reverse mutagenic primers contained the desired mutation and annealed to the same sequence on opposite strands of the plasmid, 2) primer length was between 25 and 45 bases, 3) primer melting temperature (Tm) was $\geq 78\,^{o}C$, 4) Primers had a minimum GC content of $\geq 40\%$, 5) primers terminated in one or more C or G bases. In each mutagenesis reaction, two complimentary oligonucleotides were added into a tube containing reaction buffer, appropriate dsDNA template, ddH_2O and dNTP. *pfuUltra* HF DNA polymerase was added into the mixture to amplify the entire plasmid. Following PCR amplification, we added *Dpn* I restriction enzyme to PCR product, to eliminate the original template by digesting methylated DNA.

After *Dpn* I digestion, 1 µl of the treated DNA from each reaction was added to separate aliquots of the XL1-Blue supercompetent cells. Following a 45 s heat pulse and

1 hour preincubation at 37 ºC, an appropriate volume of cells were plated onto LB-ampicillin agar plate. The ampicillin was used for selecting cells containing our vector, which contains an ampicillin resistance gene. After > 16 hours incubation at 37ºC, colonies were picked and used to inoculate 5 ml LB cultures containing ampicillin (50 μg/ml). Plasmid DNA was then extracted by using the QIAprep miniprep kit (Qiagen, Valencia, CA). This step yielded around 100 μg pure plasmid, and all mutants were verified by double-strand DNA sequencing.

Cell culture and transfection

A transformed human embryonic kidney cell line, tsA-201, was used for expression of wild-type and mutant NMDA receptors. Cells were cultured in flasks containing Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA) with 10% heat-inactivated sterile fetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37 ºC. For use in experiments, cells were seeded on 35mm dishes coated with poly-D-lysine and 0.001% fibronectin.

We used two ways to transfect heterologous NMDA receptors into tsA-201 cells, calcium phosphate (Invitrogen, Carlsbad, CA) and lipofectamine 3000 (Invitrogen). The calcium phosphate transfection method introduces DNA into tsA-201 cells by forming a calcium phosphate-DNA precipitate, facilitating the binding of the DNA to the cell surface. DNA eventually enters the cell by endocytosis. Lipofectamine consists of cationic lipids, which interact with the phosphate backbone of the nucleic acid. This interaction is via the cationic head groups of the lipids and does not result in the formation of micelles or liposomes surrounding the nucleic acid. The cationic lipids also

mediate the interaction of the nucleic acid with the negatively charged cell membrane. The resulting DNA complexes enter the cell through endocytosis.

tsA-201 cells at 30 – 50% confluence were transfected with cDNA for the GluN1 and GluN2 wild-type or mutant subunits and green fluorescent protein (GFP) in a 2:2:1 ratio, respectively, using either transfection method above. GFP served as a marker for cells that were transfected. NMDA receptor antagonists, 200 μM *dl*-2-amino-5-phosphonovaleric acid (APV) and 100 μM ketamine, were added to the culture medium immediately after transfection to protect cells from receptor-mediated excitotoxicity. Cells were recorded from 18-48 hours post-transfection depending on the transfection efficiency. Antagonists were removed before recording by extensive washing.

Electrophysiological recording

Patch-clamp recordings were performed using an Axopatch 1D or 200B (Molecular Devices, Sunnyvale, CA, USA) amplifier. Whole-cell configuration (Figure. 2.1) was achieved by pressing a fire-polished glass pipette against the surface of a cell and applying light suction, followed by rupture of the membrane using negative pressure. Gigaohm seals were formed using patch-pipettes with tip resistances of 2-4 MΩ, and series resistances of 4-6 MΩ were compensated by 80%. To increase the speed of solution exchange around the cell, cells were lifted off the surface of the dish after obtaining a gigaohm seal. Cells and patches were voltage-clamped at -50 mV. Data were acquired at 2-10 kHz (whole-cell recording) or 50 kHz (single-channel recording) on a computer using a DigiData interface and pClamp software (Axon Instruments). During the recording, cells were superfused at 1 – 2 ml/min in an extracellular medium containing (in mM): NaCl, 150; KCl, 5; $CaCl_2$, 0.2; HEPES, 10; glucose, 10; pH was

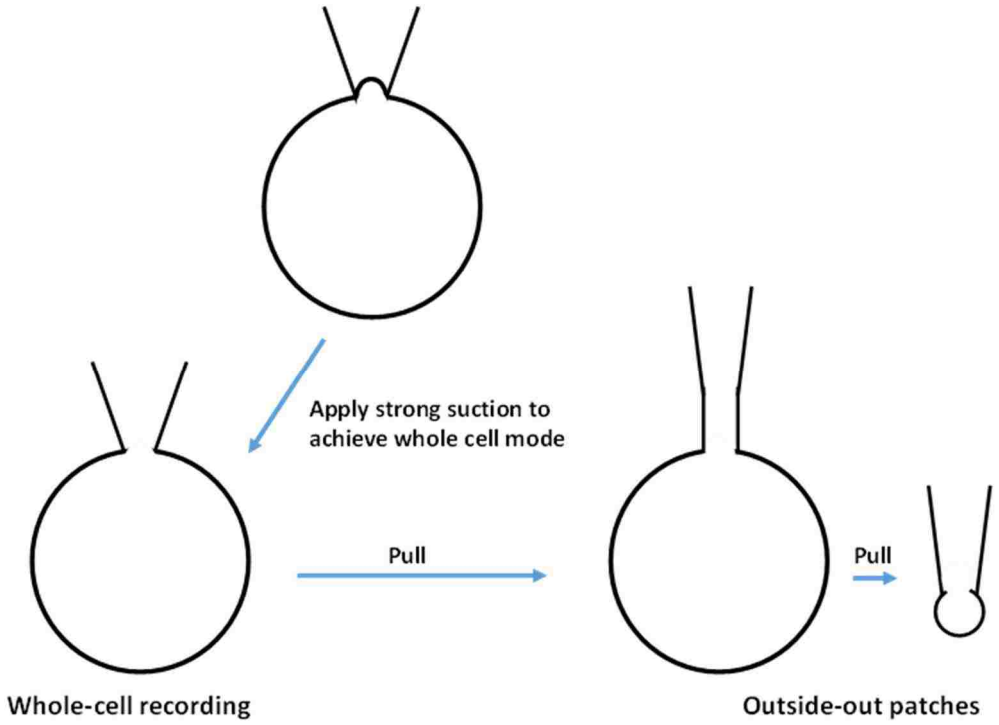

Figure 2.1. Whole-cell and outside-out patch-clamp configurations

In whole-cell recording mode (*left*), a gigaohm seal is established followed by application of strong suction to rupture the membrane patch under the pipette tip. Pulling the patch-pipette away from the cell after obtaining whole-cell mode can result in the formation of an outside-out patch (*right*).

adjusted to 7.4 using NaOH and osmolality to 340 mOsmol/kg using sucrose. Low Ca^{2+} was used to minimize NMDA receptor inactivation, which is dependent on extracellular calcium levels (Zilberter *et al*., 1991), and Mg^{2+} was excluded from the solution to eliminate voltage-dependent NMDA receptor block. The intracellular (patch pipette) recording solution contained (in mM): CsCl, 140; Mg_4ATP, 2; BAPTA, 10; pH 7.2 and 310 mOsmol/kg. Solutions containing agonists and drugs were prepared fresh daily. EDTA was added into recording solution to eliminate the fast component of apparent desensitization due to high-affinity Zn^{2+} inhibition (Ren *et al*., 2003a; Erreger and

Traynelis, 2005). In order to accomplish rapid-solution exchange, solutions were applied to cells using a stepper motor-driven solution exchange system (Warner Instruments, Hamden, CT, USA) and three-barrel square glass tubing of internal diameter 600 µm (Figure 2.2). The three barrels were connected to reservoirs filled with different solutions

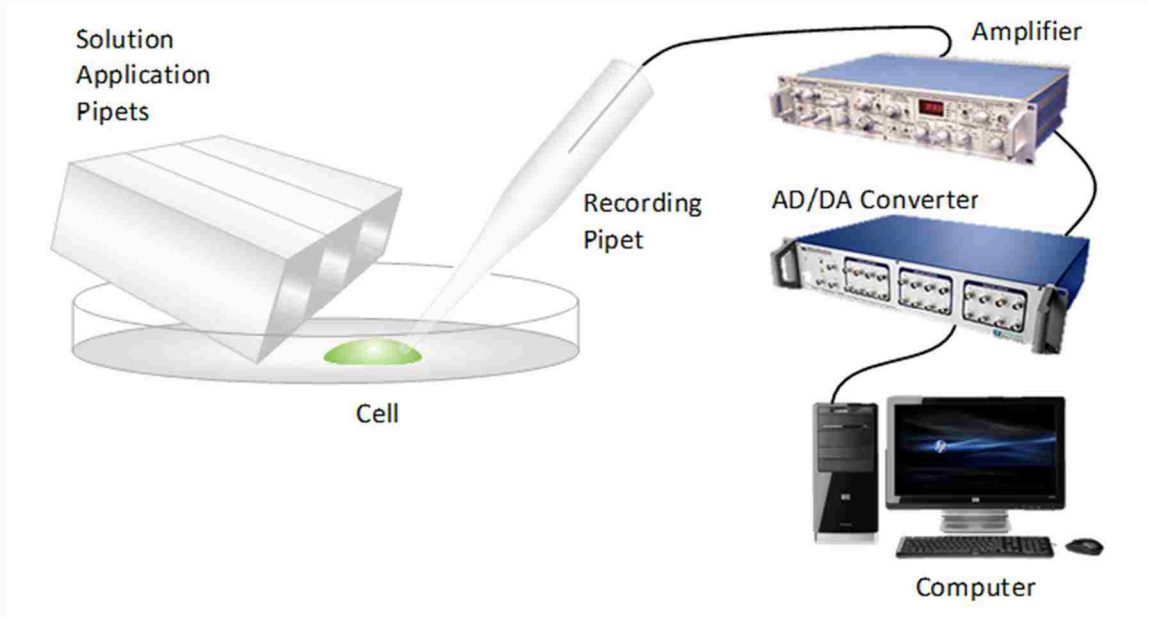

Figure 2.2. Whole-cell recording

After establishment of a whole-cell or outside-out recording mode, the three-barrel array is placed close to the cell or membrane patch. The barrels can be rapidly shifted laterally using a stepper motor under computer control to expose the patch to recording solutions flowing out of different barrels, while the electrode remains stationary. Ionic current is measured by a high-gain amplifier, digitized, and recorded on a computer.

and the movement of the barrel array was controlled by a computer. The 10-90% rise time for whole-cell solution exchange using this system has been previously shown to be ~1.5 ms (Ren *et al*., 2003a).

Concentration-response experiments

For ethanol inhibition curves, a serious of ethanol concentrations ranging from 2.5 - 500 mM were prepared in external solution containing glutamate and glycine. In most cases, solutions contained 50 μM glycine, which is a saturating concentration for NMDA receptors, and 10 μM glutamate. In cases, in which mutant receptors were less sensitive to glutamate compared to wild-type receptors, glutamate concentrations were increased. The three-barrel array was configured so that the first barrel contained regular external solution, the second contained agonist solution, and the third contained agonists and various concentrations of ethanol. The third barrel was connected to a seven-tube manifold made of fused silica tubing to allow application of multiple alcohol concentrations. In an experiment, the barrels are positioned so that the external solution flowing from the first barrel bathes the cell. The array is shifted laterally one position to expose the patch to the cell to the solution containing glycine and glutamate, shifted to the third position to apply ethanol solution to the cell. Concentrations of ethanol were applied in random order to minimize any time-dependent effects (e.g., rundown). The entire series of ethanol concentrations were tested in a single cell. In most of cases, 4-7 cells were obtained for each mutant combination tested, and these cells were from different recording days. Therefore, each concentration-response curve relies on data from several cells collected on at least two days. Ethanol (100 mM) inhibition current is shown in **Figure 2.3 (left)**.

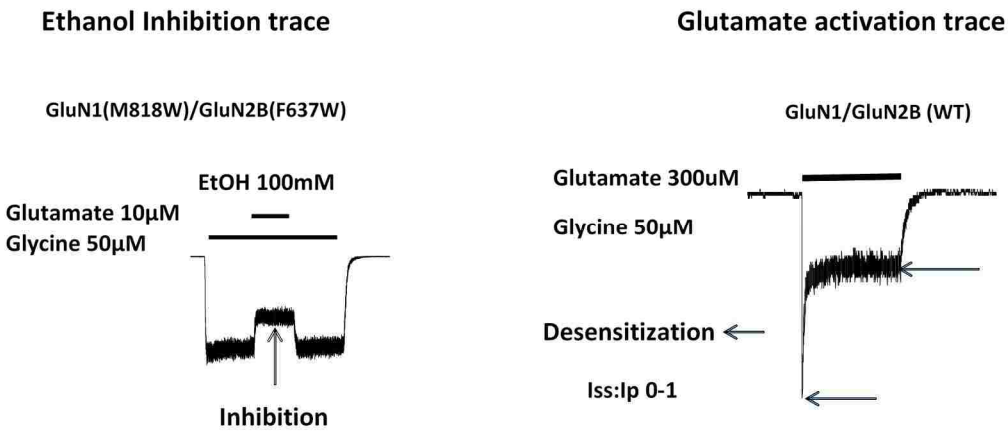

Figure 2.3. Traces for typical ethanol inhibition and glutamate activation

The trace on the left shows typical inhibition of GluN2B-containing NMDA receptor-mediated current by 100 mM ethanol. Current was activated by application of 10 μM glutamate as well as 50 μM glycine. The trace on the right is a typical current activated by maximal concentration of glutamate (300 μM), showing desensitization. Apparent desensitization was quantified using the maximal steady-state to peak current ratio.

In glutamate concentration-response experiments, current responses evoked by a series of glutamate concentrations were compared to the current response evoked by a saturating concentration of glutamate (300 μM). A supramaximal concentration of glycine (50 μM) was used to saturate the glycine binding site. Only two out of three barrels were used in this experiment: the first was filled with external solution and the second with different concentrations of glutamate solution. In order to increase the speed of the solution exchange in these experiments, cells were lifted off the surface of the dish after obtaining a gigaohm seal. The 10% - 90% rise time for solution exchange in lifted cells for this system is ~ 1.5 ms. Concentrations of glutamate were applied in random order, and the maximal response to glutamate in each cell was determined by applying a

saturating concentration of glutamate at the beginning and end of each experiment. The entire range of glutamate concentrations was applied to the same cell to allow a full concentration-response curve to be generated for each cell. Concentration-response curves for each mutant were generated by averaging the curve fits (see below) from 5-8 cells. An example of glutamate-activated maximal current in a typical cell expressing NMDA receptors is shown in **Figure 2.3 (right)**. This current trace shows activation, desensitization, as well as deactivation of current.

Data analysis of ethanol inhibition and macroscopic kinetics ($EC_{50,}$ desensitization and deactivation)

In concentration-response experiments, IC_{50} or EC_{50} and n (slope factor) were calculated using the equation: $y = E_{max} / 1 + (IC_{50}$ or $EC_{50} / x)^n$, where y is the measured current amplitude, x is concentration, and E_{max} is the maximal current amplitude. This identified the concentration that gives a half-maximal inhibition / response, termed IC_{50} / $EC_{50.}$ We measured two types of EC_{50} values from glutamate concentration-response experiments, peak current EC_{50} as well as steady-state current $EC_{50.}$ Statistical differences among concentration-response curve 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 Dunnett test to compare value from each mutant to the wild-type value. Linear relations of mean values of log IC_{50} or EC_{50} were made using linear regression analysis by using the program StatView (SAS Institute, Inc., Cary, NC). In some cases, during ligand application a distinct desensitization phase of the current response was observed. Values for maximal steady-state to peak current ratio (I_{ss}:I_p) were obtained from application of a supramaximal agonist concentration. Linear

relations of mean values of maximal steady-state to peak current ratio (I_{ss}:I_p) for the various mutants were also made using linear regression analysis. For deactivation rate analysis, time constants (τ) of deactivation were determined from fits of the current decay following the removal of glutamate (in the continued presence of glycine) to an exponential function using Clampfit (Axon Instruments). In most cells, deactivation was best fitted using a bi-exponential function; in these cases, the weighted time constant is reported. For cells in which deactivation was adequately fitted by a single exponential function, this value is reported. All values are reported as the mean ± SE.

Calculation of physicochemical properties of amino acids

Molecular (Van der Waals) volumes of amino acids were calculated using Spartan Pro (Wavefunction, Inc., Irvine, CA) following structural optimization using the AM1 semi-empirical parameters. Values used for amino acid hydropathy, hydrophilicity, and polarity were reported previously (Zimmerman *et al*., 1968; Hopp *et al*., 1981).

Interaction analysis: Mutant cycle analysis and two-way ANOVA

Significant interactions with respect to ethanol sensitivity, glutamate potency, and apparent affinity among mutants at multiple positions were determined by two-way ANOVA of log-transformed IC_{50} values and by mutant cycle analysis. Mutant cycle analysis was performed essentially as described by Venkatachalan and Czajkowski, (2008). Tryptophan substitution mutations were introduced singly and in combination at two positions in GluN1 and GluN2B subunits proposed to interact, and ethanol IC_{50} and glutamate EC_{50} were determined in each mutant. The apparent interaction free energy $\Delta\Delta G_{INT}$ for mutations at two positions is the free energy difference between the parallel energies in the cycle (i.e., form the wild-type and either single mutant to the other single

mutant and the dual mutant). Apparent interaction free energies among mutated positions were calculated using natural logarithms (ln) of either ethanol IC_{50} or glutamate EC_{50} values obtained from wild-type and mutant subunit combinations, using the equation $\Delta\Delta G_{INT} = RT\ [\ln(WT) + \ln(mut1,mut2) - \ln(mut1) - \ln(mut2)]$. Statistically significant differences between mean values of $\Delta\Delta G_{INT} \pm$ S.E.M and zero energy were interpreted as indicating an interaction between two positions, since non-interacting positions should have an apparent interaction free energy of zero. The statistically significant differences were determined by using one sample *t* tests, with degrees of freedom $df = N_{WT} + N_{MUT1} + N_{MUT2} + N_{MUT1,MUT2} - 4$, with N_X equal to the number of cells used for each combination of wild-type and mutant subunits. S.E.M. was determined from propagated errors.

Significant interaction with respect to maximal steady-state to peak current ratios in one mutant pair, GluN1(G638W) and GluN2B(M824W), was also determined by two-way ANOVA of maximal desensitization values and by mutant cycle analysis as described above for ethanol IC_{50} and glutamate EC_{50} values. All values are reported as means ± S.E.M.

It is important to note that significant coupling energy from these experiments may not just result from a direct interaction between side-chains of two residues, but could result from the unitary structural element composed of both residues, or from the secondary interactions between another residue and each of these two residues.

Designing the disulfide mutants

Based on the crystal structure of AMPA receptor and NMDA receptor (Sobolevsky *et al*., 2009; Lee *et al*., 2014), a series of amino acids in the M3 and M4

domain of GluN1 and GluN2B subunit were replaced with cysteine by using site-directed mutagenesis. Previous studies revealed that there are two redox-activated cysteine residues in the ligand-binding domain of the GluN1 subunit (Cys744, Cys798), which could form a flexible disulfide bond upon the application of redox reagents. Formation of a disulfide bond between them could affect channel kinetics. For example, after oxidizing agent application, glutamate-gated current was decreased; however, a reducing agent exerted the opposite effect on the current (Sullivan *et al*., 1994; Choi *et al*., 2001; Lipton *et al*., 2002). In order to eliminate kinetic changes caused by the redox site, I introduced an alanine substitution at GluN1(Cys744), and combined this mutation with cysteine substitutions at other residues in GluN1 subunit.

Cross-linking experiments

In order to study possible interactions between the M3 and M4 domains in adjacent subunits, we tested whether disulfide bonds could form between certain positions in these subunits. Redox reagents were used to either form or break disulfide bonds (Figure 2.4). Dithiothreitol (DTT; Sigma-Aldrich Co., St. Louis, MO, USA) was used as a reducing agent and freshly prepared daily at a concentration of 10 mM. Hydrogen peroxide and copper phenanthroline were used as oxidizing agents. Hydrogen peroxide, 0.1%, was prepared daily from a 30% stock solution (H_2O_2; Sigma-Aldrich Co., St. Louis, MO, USA). Copper phenanthroline was prepared at a 1 : 3 molar ratio of $CuCl_2$ in water and 1-10-phenanthroline dissolved in ethanol; the final concentration of ethanol was 0.0001% v/v; values reported in the experiment results indicate the $CuCl_2$ concentration. Solutions containing redox reagents were prepared in normal external solution. Cross-linking experiments for studying ion channel gating changes were

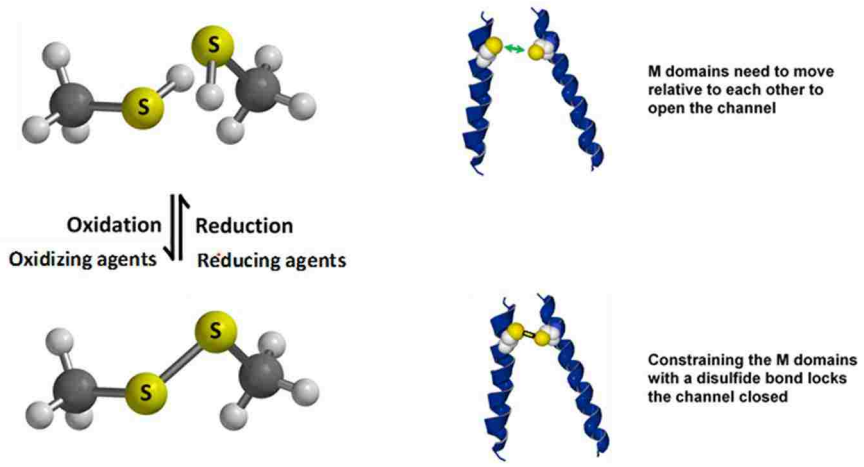

Figure 2.4. Crosslinking of introduced cysteines

Cysteines were introduced into positions suspected to interact. Two cysteines in close proximity could form a disulfide bond upon the application of oxidizing agents that could be broke by using reducing agents (*left*). The diagram of two adjacent M domains (*right*) illustrates one possibility, that is that formation of a disulfide bond between two M domains could "lock" the channel in a closed state.

performed as follows: solution containing maximal concentrations of agonists (300 μM glutamate and 50 μM glycine) was applied for 10 ms, followed by application of either oxidizing or reducing agent (1 - 2 min), and a final agonist application (10 ms). The procedures were repeated for 3-5 times to test effects of oxidizing/cross-linking and reducing compounds. Cross-linking experiments for studying sensitivity to ethanol were performed using 1 to 2 min application of redox reagents followed by application of 100 mM and 250 mM ethanol.

Previous results from this laboratory have suggested that DTT cannot gain access to the transmembrane domain region from outside of the cell. We therefore also applied DTT intracellularly by adding it to the solution in the recording pipette. In order to avoid

effects due to DTT reacting with the silver chloride – coated recording electrode, the tip of the patch-pipette was filled with a small volume of DTT-containing internal solution, and the remainder of the pipette, including the section containing the silver chloride wire, was backfilled with normal internal solution. DTT was prepared at a concentration of 10 mM in internal solution.

Molecular modeling

Molecular modeling was performed as described in previous studies from our laboratory and other laboratories (Dwyer, 2001; Dwyer, 2003; Ren *et al*., 2008). A model of the transmembrane region of the targeted subunit of NMDA receptor was built by using InsightII software from Biosym (now Accelrys; San Diego, CA). Available proteins from Protein Data Bank, which have similar arrangement with respect to our interested domain of NMDA receptor, served as the template for modeling. Since studies in our laboratory refined our models for GluN1/GluN2A NMDA receptor based on a recent study (Sobolevsky et al., 2009), the coordinates of the GluN2A subunit were used as the template to model of GluN2B subunits. Amino acid sequences were optimally aligned and models were built by assigning coordinates to structurally conserved regions. The initial model was then subjected to energy minimization with the AMBER force field (a total of 300 iterations of the steepest descent and conjugate gradient algorithms). The minimized structure served as a starting point for following refinement of the model.

Neuronal cell culture and transfection

Poly-D-lysine pretreated 35mm sterile dishes were coated overnight with 10 μg/ml laminin (Sigma), and rinsed three times with water before addition of 2 ml neuronal media. Neuronal culture medium was prepared by Dr. Murray Blackmore's

laboratory (Blackmore, *et al*., 2011). This medium contained Neurobasal medium (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), 5 µg/ml insulin (Sigma), 100 µM sodium pyruvate (Invitrogen), 4 µg/ml triiodothyronine (Sigma), 200 µM L-glutamine (Invitrogen), 50 µg/ml N-acetyl-L-cysteine (Sigma), B27 supplement (Invitrogen), 100 µg/ml transferrin (Sigma), 100 µg/ml bovine-serum albumin (Sigma), 63 ng/ml progesterone (Sigma), 16 µg/ml putrescine (Sigma) and 400ng/ml sodium selenite (Sigma).

Cortical neurons were also prepared by Dr. Blackmore's laboratory. The method used maximizes viability by first using sequential digestion with papain and trypsin, followed by trituration of cell clumps. During this process, the upper layer of the cell suspension is removed periodically to minimize mechanical stress on dissociated cells. Early postnatal rats (P2 or P3) were used to obtain neurons with minimal expression of native NMDA receptors. Rats were decapitated and the brains stored in ice-cold Hibernate E (minus $CaCl_2$) (Brainbits #HE-Ca 500). The meninges were removed, then the frontal cortex was isolated, minced with a razor blade, and incubated in 10 ml dissociation media (Hibernate E containing 20 U/ml papain and 150 µg/ml DNAse) at 37 ^{o}C in a shaking incubator for 30 min. If cell clumps were still visible, they were resuspended in Hibernate E and the suspension shaken at 37^{o}C for an additional 30 min. This process was repeated until no cell clumps were visible. Typically, a total of 12-14 ml of cell suspension would yield 8-10 million cells for transfection.

An electroporation method was used for cortical neuron transfection (Buchser *et al*., 2006). Cortical neurons were pelleted and resuspended in Internal Neuronal Buffer (INB), which contained (in mM): KCl, 135; $CaCl_2$, 0.2; $MgCl_2$, 2; HEPES, 10; EGTA, 5;

pH 7.3). Then 25 µl of cell suspension was placed in each well of a 96-well electroporation plate (Harvard Apparatus/BTX #45-0450) and mixed with 25 µl of INB containing 1 µg GFP reporter plasmid and 2 µg GluN1 subunit plasmid as well as 2 µg GluN2 subunit plasmid. The plate was placed in a plate handler (HT-200) attached to an ECM 830 square wave pulse generator (Harvard Apparatus/BTX), and each well received a single pulse of 350-475 V for 300 µs. Next, 100 µl of Hibernate E with 2% B27 supplement was added to each well to promote cell recovery, and cells were transferred to 35 mm dishes. Cortical neurons were used for recording 18-24 h post-electroporation.

Cortical neuron recording

Whole-cell patch-clamp recording was performed as previously described in *electrophysiological recording*. In order to eliminate currents caused by other receptor types, we added an antagonist of the glycine and acetylcholine receptor, strychnine, at a concentration of 1 µM into the external solution. When recorded neurons expressed GluN1/GluN2A NMDA receptor, either wild-type or mutant, a specific inhibitor of receptors composed of the GluN1 and GluN2B subunits, ifenprodil, was added into the recording solution in addition to strychnine. To distinguish introduced mutant subunits from native subunits, the GluN2A(M823W) mutant, which exhibits a high degree of desensitization, was transfected into neurons. Because the greatest desensitization was produced by application of maximal concentrations of glutamate and glycine, in this experiment, I also used maximal concentrations of both agonists (300 µM glutamate and 50 µM glycine). We then performed ethanol concentration-response experiments on neurons expressing various mutant NMDA receptors. In these neurons, inhibition by 100 mM and 250 mM ethanol were tested.

III. FUNCTIONAL SCREENING OF POSITIONS IN THE M3 AND M4 DOMAINS OF THE GLUN2B SUBUNIT AND COMPARISON OF THE ROLES OF THESE POSITIONS AMONG DIFFERENT GLUN2 SUBUNITS

Introduction

Alcohol binding sites on different receptors and ion channels have been characterized in a number of previous studies, however, the location and characteristics of these sites differ. For example, sites of alcohol action have been located in the cytoplasmic loop of a *Drosophila* potassium channel (Covarrubias *et al*., 1995), in the ion-channel lumen of muscle nicotinic acetylcholine receptors (Forman *et al*., 1995), and in the transmembrane domains of the $GABA_A$ and glycine receptors (Mihic *et al*., 1997; Ueno *et al*., 1999; Wick *et al*., 1998). Based on studies indicating that a phenylalanine residue in the M3 domain of the GluN1 subunit could regulate ethanol sensitivity of NMDA receptor (Ronald *et al*., 2001; Smothers and Woodward, 2006), previous studies in our laboratory have identified four ethanol-sensitive residues in the M3 and M4 domains of the GluN2A subunit of the NMDA receptor over the past several years by constructing various mutants at these positions. (Ren *et al*., 2003a; Ren *et al*., 2003b; Honse *et al*., 2004; Ren *et al*., 2007; Ren *et al*., 2013). These studies were based on the assumption that ethanol sensitive sites on the NMDA receptor are located in membrane-associated domains, which participate in regulating ion channel kinetics, and are accessible from the extracellular environment. Since truncation of the C-terminal of the GluN1 subunit of NMDA receptor did not alter ethanol sensitivity (Peoples and Stewart, 2000).

One position at the M4 domain of the GluN2A subunit, Met823, was first characterized by our laboratory as exerting important regulatory influence on the gating of the NMDA receptor ion channel. Moreover, ethanol sensitivity among various mutants at this position was correlated with apparent desensitization, such that the greater the desensitization is for a mutant, the lower its ethanol sensitivity is. This study also demonstrated that ethanol sensitivity of mutants was related to molecular volume and hydrophilicity of the substituents at this position (Ren *et al*., 2003a; Ren *et al*., 2003b). Besides this position, our laboratory used tryptophan-scanning mutagenesis in a region of the M4 domain which is not apparently accessible from the extracellular aqueous environment, and results showed that tryptophan substitution at Ala825 significantly altered both mean open time of the channel and concentration-response curve for ethanol inhibition (Honse *et al*., 2004). Thus, this residue, together with Met823 may form or contribute to an ethanol-binding pocket. Based on the importance of Phe639 on the ethanol action on the NMDA receptor, which is located in the M3 domain of the GluN1 subunit, characterized by Woodward's group (Ronald *et al*., 2001; Smothers and Woodward, 2006), as well as the highly homology of the M domains between the GluN1 and GluN2 subunits, our laboratory pointed out that the cognate position in the GluN2A subunit, Phe637, served an important regulatory role on both ion channel gating and ethanol sensitivity (Ren *et al*., 2007). Another position in the M3 domain of the GluN2A subunit, Phe636, was identified recently by our laboratory. Tryptophan mutagenesis at this position showed the lowest ethanol sensitivity among identified mutants in the GluN2A subunit. We also found that substitutions at this position could significantly

regulate apparent agonist affinity, apparent desensitization, and ion channel mean open time (Ren *et al*., 2013).

Although these four positions do not exhibit a common motif, the common elements appear to be that they are all hydrophobic, do not line the ion channel lumen, have limited access from cytoplasmic side of membrane and are located in the regions that influence ion channel gating (figure 3.1). The GluN2A subunit has been studied in

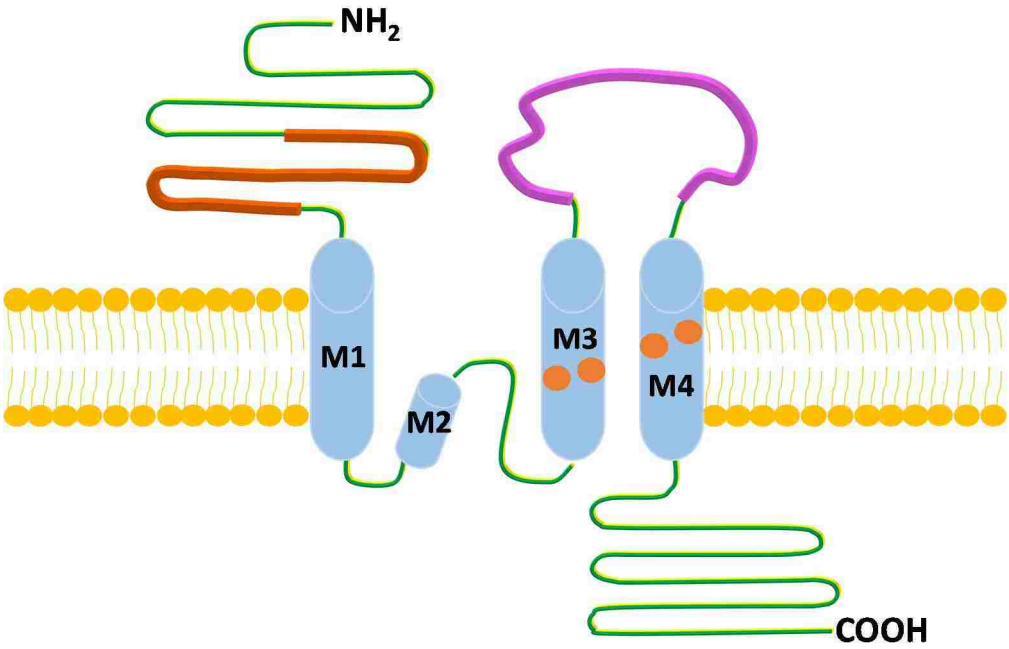

Figure 3.1. Common elements of ethanol-sensitive positions in GluN2A subunit

Previous studies in our laboratory have identified four ethanol-sensitive positions in GluN2A subunit. Two of them are in M3 domain, Phe636 and Phe637, and the other two are in M4 domain, Met823 and Leu825, which are shown in orange dots.

depth in the past, however, recent studies have pointed to a major role of the GluN2B subunit in normal brain functions as well as alcohol action in the brain. One study demonstrated the importance of the GluN2B subunit on NMDA receptor endocytosis and localization; furthermore, several pharmaceutical companies have developed GluN2B-selective antagonists as therapeutic drugs for several glutamate-mediated diseases (Gogas, 2006). Chronic pain could be enhanced by GluN2B subunit overexpression in case of any tissue or nerve injury (Zhuo, 2009). One recent study has characterized the GluN2B subunit as the key regulator for ethanol actions, and extrasynaptic GluN2B-containing NMDA receptors exert a unique role in facilitating long-term potentiation (LTP) in the bed nucleus of the stria terminalis (BNST) (Wills *et al*., 2012). Another study also suggested that co-exposure of long-term ethanol and corticosterone could sensitize the hippocampal CA1 region pyramidal neurons in a GluN2B subunit-dependent manner (Butler *et al*., 2013).

Because of the high sequence homology of the M3 and M4 domains between the GluN2A and GluN2B subunit, this thesis first tested residues in the GluN2B subunit that correspond to ethanol-sensitive positions in the GluN2A subunit. By using both alanine and tryptophan mutagenesis scanning at these positions, ethanol concentration-response experiments were initially performed to test ethanol sensitivity changes following site-directed mutagenesis compared with wild-type receptors. Because tryptophan is the largest and most hydrophobic amino acid, substitution of a tryptophan at these positions can significantly increase the molecular volume and hydrophobicity, whereas alanine is one of the smallest hydrophobic amino acids that (in contrast to glycine) is unlikely to destabilize α-helical regions. Thus, alanine substitution at an ethanol-sensitive position

may alter ethanol sensitivity, since ethanol sensitivity is shown to be related to the molecular volume of amino acid at the ethanol-sensitive position (Ren *et al*., 2003b; Smothers and Woodward, 2006; Ren *et al*., 2007; Salous *et al*., 2009). The manner in which mutations at positions in the GluN2B subunit alter ethanol sensitivity differs from that of the cognate positions in the GluN2A subunit. Glutamate potency and channel kinetics at all four positions after tryptophan and alanine substitution were also examined. Compared with related studies of the GluN2A subunit, only tryptophan mutagenesis at GluN2B(Phe637) showed altered EC_{50} values for glutamate-activated peak current and steady-state current, as well as increased deactivation tau. Both tryptophan and alanine substitutions at this position also showed decreased apparent desensitization compared with the wild-type receptor. Since both alanine and tryptophan mutagenesis at Phe637 exhibited altered ethanol sensitivity, this position deserves further analysis and is the subject of studies in the following chapter.

Results

Localization of candidate residues in the GluN2B subunit

The candidate positions in the M3 and M4 domains of the GluN2B subunit investigated in this thesis are shown in **figure 3.2**. This figure shows the highly homologous M3 and M4 domains among the GluN1, GluN2A and GluN2B subunits. Besides four residues in the GluN2B subunit, that correspond to residues in the GluN2A subunit, one adjacent residue in the M4 domain, Leu825, was also tested by replacing it with tryptophan.

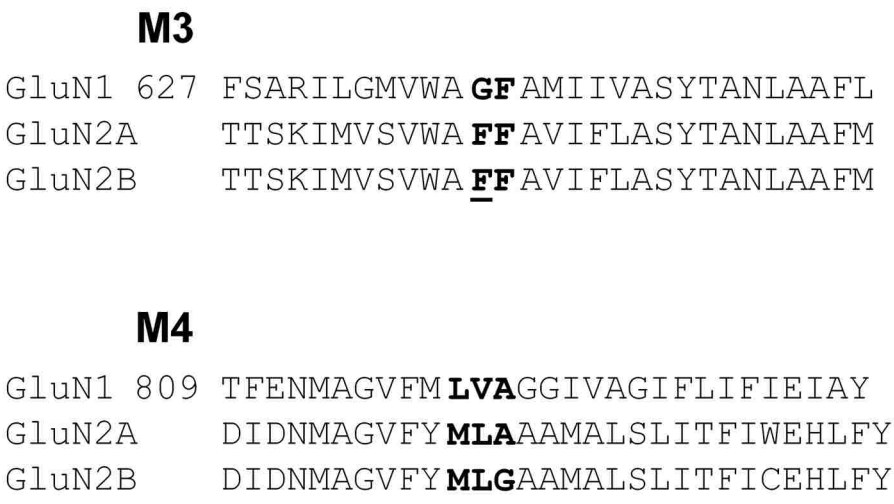

Figure 3.2. Partial sequences of the M3 and M4 domains in the GluN1, GluN2A and GluN2B subunit.

Ethanol sensitive positions in GluN2A and their cognate positions in GluN2B are indicated in bold. GluN2B(Phe637), which is the ethanol-sensitive position, is underlined.

Ethanol sensitivity of alanine and tryptophan substitution mutations at GluN2B M3 and M4 residues corresponding to GluN2A ethanol-sensitive positions

Since alanine is the smallest hydrophobic amino acid and tryptophan is the largest and most hydrophobic amino acid, it is possible that alanine and tryptophan substitution at these positions can produce significant change in ethanol sensitivity if these positions are ethanol-sensitive positions. Either alanine or tryptophan was initially introduced into positions in the GluN2B subunit, Phe637, Phe638, Met824, Leu825, and Gly826, corresponding to ethanol-sensitive positions in the GluN2A subunit by using site-directed mutagenesis. Mutants or wild-type NMDA receptors were then transfected into tsA201 cells one-day prior to the recording day. Tryptophan mutagenesis was also introduced to GluN2B(Leu825), an adjacent residue.

Traces shown in **figure 3.3** were recorded from tsA201 cells expressing either wild-type, alanine, or tryptophan substitution mutant receptors, which were activated by 10 μM glutamate and 50 μM glycine and inhibited by 100 mM ethanol. Mutants constructed here all yielded functional receptors. There is no noticeable difference in the degree of inhibition by 100 mM ethanol between alanine mutants and the wild-type NMDA receptors based on traces shown here. However, GluN2B(M824A) showed increased desensitization compared with wild-type current. GluN2B(F637A) showed almost no desensitization compared with the wild-type receptor; a detailed characterization about this position is given in the following chapter. It is obvious that receptors containing GluN2B(F637W) and GluN2B(G826W) mutant subunit showed less ethanol inhibition compared with wild-type receptors, and the former mutation also showed delayed deactivation. Moreover, the GluN2B(F637W) mutant also showed an obvious decrease in desensitization, which is consistent with results observed for alanine substitution at this position. Although the GluN2B(M824W) mutants did not show a visible change in ethanol sensitivity based on the currents, degree of desensitization was significantly increased compared with wild-type receptors, which reflects the change on ion channel kinetics. In order to test if ion channel functions are also changed in these mutants, glutamate concentration-response experiments were performed and will be characterized later.

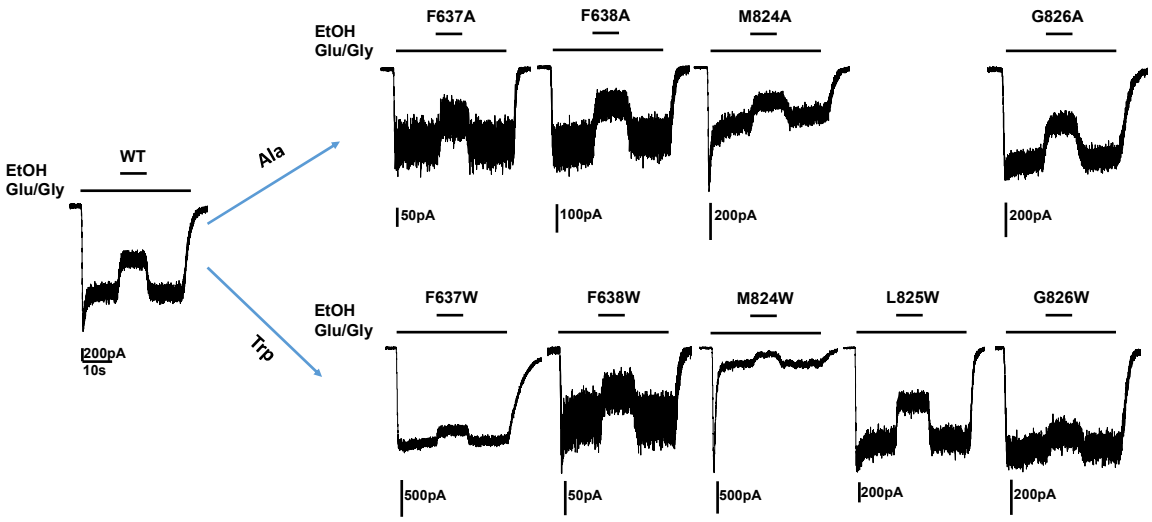

Figure 3.3. 100 mM ethanol inhibition traces of alanine and tryptophan substitutions in the GluN2B subunit

Traces show currents activated by 10 μM glutamate and 50 μM glycine and their inhibition by 100 mM ethanol in tsA-201 cells expressing either wild-type receptor or various alanine mutagenesis (upper) and tryptophan mutagenesis (lower) at positions in the GluN2B subunit. One-letter amino acid codes are used.

Ethanol concentration-response experiments were performed on cells expressing wild-type and mutant NMDA receptors. To test for ethanol sensitivity changes, a series of ethanol concentrations were used, from 2.5 to 500 mM. I recorded the same mutant type on different days in order to avoid any effects caused by difference in experimental conditions, such as preparation of recording solutions or cells. Ethanol IC_{50} values of each mutant type were determined from recording results of 4-7 cells. In order to further compare ethanol sensitivity between wild-type and mutant receptor, concentration-response curves (figure 3.4) as well as bar graphs of ethanol IC_{50} values (figure 3.5) were generated from ethanol concentration-response results. Mutants shown here were all

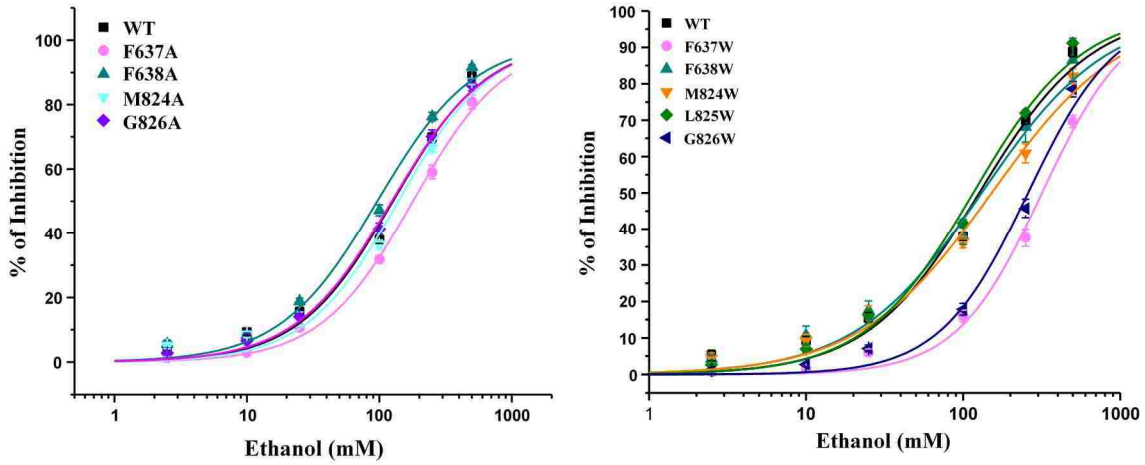

Figure 3.4. Ethanol concentration-response curves of GluN2B mutants

Concentration-response curves show ethanol inhibition of glutamate –activated currents in the presence of 50 µM glycine in cells expressing either wild-type GluN1 and GluN2B subunit or wild-type GluN1 and GluN2B alanine (*left*) and tryptophan (*right*) mutations at Phe637, Phe638, Met824 and Gly826. One-letter amino acid codes are used. Error bars not visible were smaller than the symbols. Curves shown are best fitted to the equation given under "Materials and Methods". Wild-type receptor curve is shown as a black line.

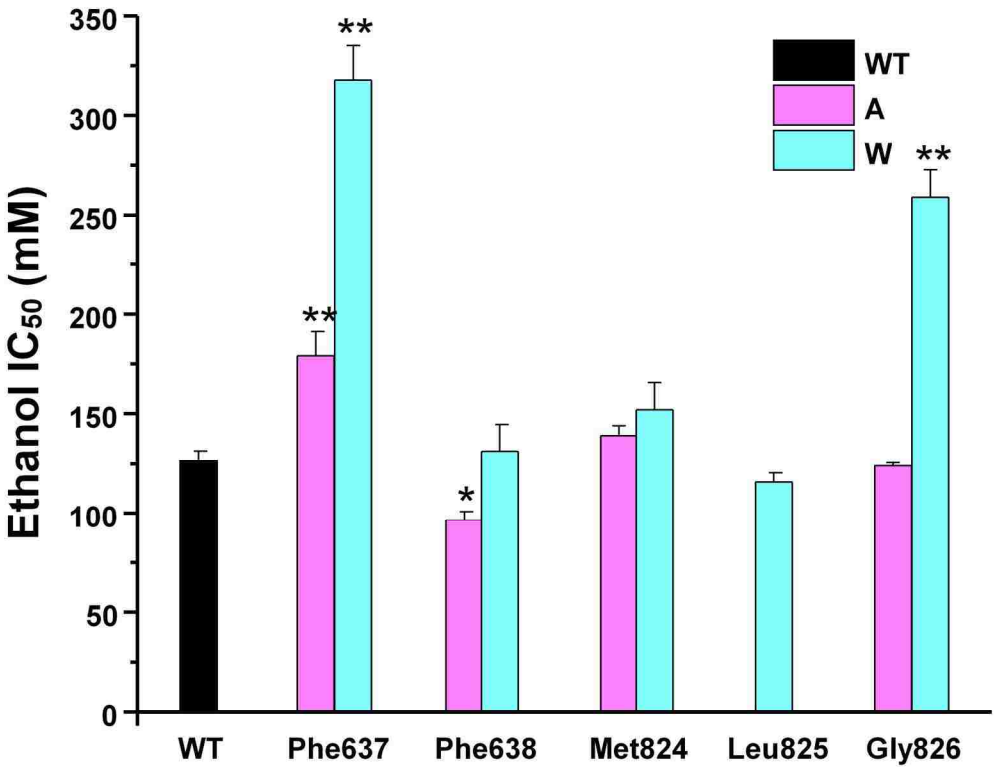

Figure 3.5. Average ethanol IC_{50} of alanine and tryptophan mutants

Bar graphs show average IC_{50} values for ethanol inhibition of glutamate-activated currents in the presence of 50 μM glycine in cells expressing wild-type GluN2B subunit or GluN2B mutants companied with wild-type GluN1 subunit. IC_{50} values that are significantly different from wild-type receptor are indicated by asterisks (*$P < 0.05$; **$P < 0.01$; one-way ANOVA). Results are means ± S.E of 5-7 cells. Pink bars indicate alanine substituents, blue bars are tryptophan substituents, and the black bar shows the ethanol IC_{50} value of wild-type GluN2B-containing NMDA receptor.

inhibited by ethanol in a concentration-dependent manner. However, the manner in which mutations at these positions alter ethanol sensitivity differs from that of the cognate positions in GluN2A subunit. For instance, among alanine mutants, only GluN2B(F637A) and GluN2B(F638A) showed significantly altered ethanol sensitivity

but the other two mutants were unchanged. Whereas among tryptophan mutants, only GluN2B(F637W) and GluN2B(G826W) showed significantly decreased ethanol IC_{50} values compared with wild-type receptors (ANOVA; $p<0.01$). In summary, both alanine and tryptophan mutagenesis at GluN2B(Phe637) significantly altered ethanol sensitivity. Substitutions at this position also showed visible altered desensitization and deactivation. Next, glutamate concentration-response experiments were performed to test ion channel function following mutagenesis at positions shown in this part.

Glutamate potency of alanine and tryptophan substitution mutations at GluN2B M3 and M4 domain residues corresponding to GluN2A ethanol-sensitive positions

Glutamate concentration-response experiments were performed as described in *Materials and Methods*. Both glutamate-activated peak current (I_p) and steady-state current (I_{ss}) were analyzed among wild-type receptors and receptors with either alanine or tryptophan mutation. Results were collected from 4-8 cells at different recording dates in order to avoid side-effects. All constructs recorded here were activated by glutamate in a concentration-dependent manner. Concentration-response curves of glutamate EC_{50} shown here are from either peak current values of alanine mutants as well as tryptophan mutants (Figure 3.6) or steady-state current values of alanine mutants as well as tryptophan mutants (Figure 3.8) activated by various concentrations of glutamate (from 0.01 μM to 300 μM) and maximal concentration of glycine (50 μM). Bar graphs of glutamate EC_{50} values are from either average peak current EC_{50} values (figure 3.7) or average steady-state current EC_{50} values (figure 3.9). As analyzed by ANOVA, only tryptophan mutagenesis at F637 showed both altered glutamate peak current EC_{50} as well as steady-state current EC_{50} (ANOVA; $p<0.01$ or $p<0.05$), which indicates significantly

altered ion channel functions. In addition to altered glutamate EC_{50} on GluN2B(F637W), both alanine and tryptophan mutations at this position showed significant increases in ethanol IC_{50} as described previously. In order to study the role of this position on ethanol action and ion channel function in detail, I made a panel of mutants at this position and tested the ethanol sensitivity of each by whole cell patch-clamp recording, which is shown in the next chapter.

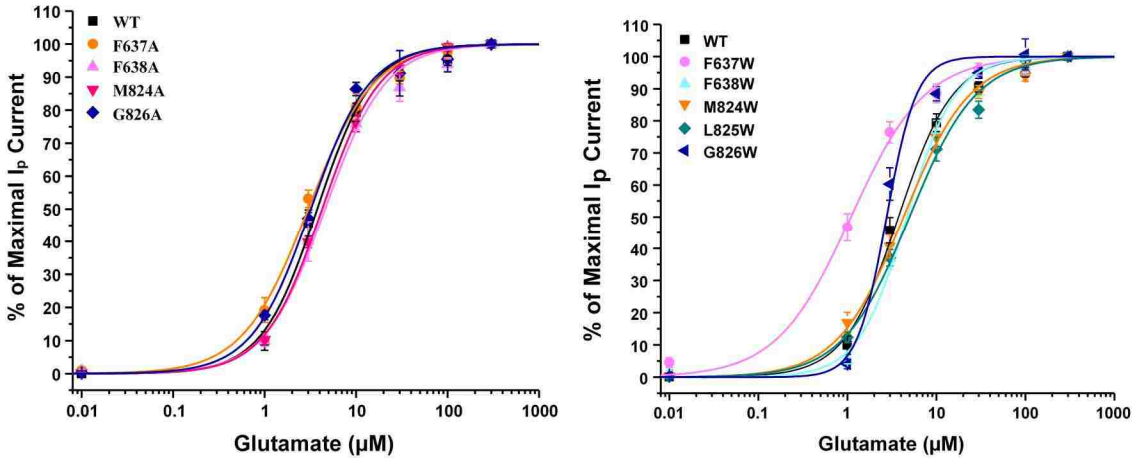

Figure 3.6. Glutamate concentration-response curves of peak currents of GluN2B mutants

Concentration-response curves for glutamate-activated peak currents in the presence of 50 μM glycine in cells expressing either wild-type, Ala-substitution mutation receptors (*left*) or Trp-substitution mutation receptors (*right*). Data points are the means ± S.E of 4-8 cells. Black line shows the fit for the wild-type peak current EC_{50} value. All curves shown here are best fitted to the equation characterized in "*Materials and Methods*".

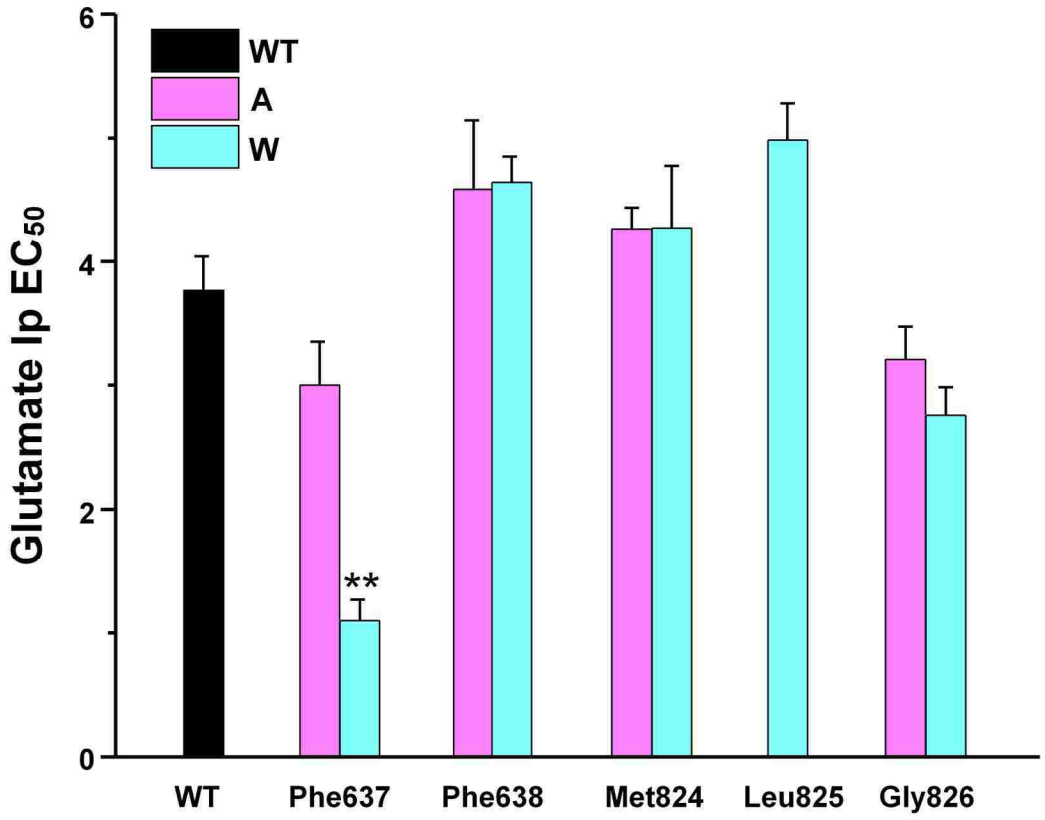

Figure 3.7. Glutamate peak current EC_{50} of GluN2B mutants

Average peak current EC_{50} values recorded on lifted cells expressing either wild-type or mutant receptors. Significantly different from wild-type peak current and steady-state current EC_{50} values were analyzed by one-way ANOVA. EC_{50} values that are significantly different from wild-type receptor are indicated by asterisks (**$P < 0.01$). Data are the means ± S.E of 4-8 cells. Pink bars indicate alanine substituents and blue bars are tryptophan substituents, whereas the black bar shows the ethanol IC_{50} value of wild-type GluN2B-containing NMDA receptor.

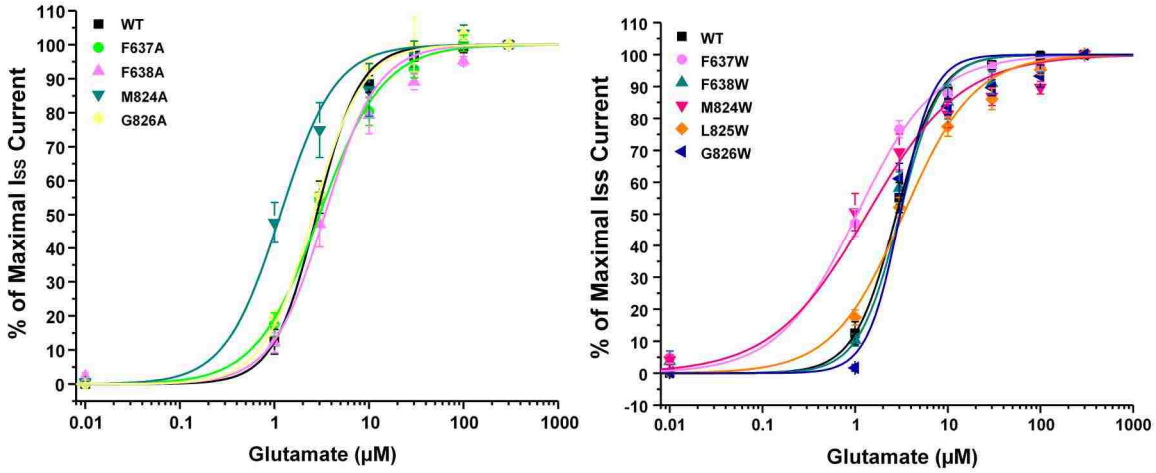

Figure 3.8. Glutamate concentration-response curves of steady-state currents of GluN2B mutants

Concentration-response curves for glutamate-activated steady-state currents in the presence of 50 μM glycine in cells expressing either wild-type, Ala-substitution mutation receptors (*left*) or Trp-substitution mutation receptors (*right*). Data points are the means ± S.E of 4-8 cells, error bars not visible were smaller than the size of symbols. Black line shows the fit for the wild-type steady-state current EC_{50} value. All curves shown here are best fitted to the equation characterized in "*Materials and Methods*".

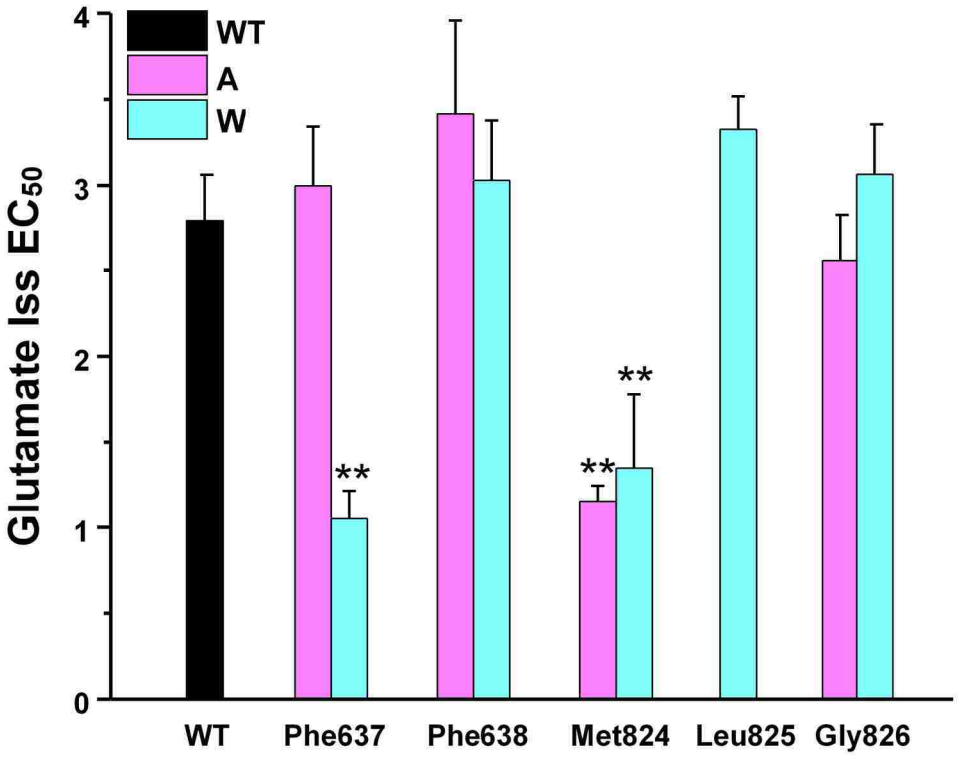

Figure 3.9. Glutamate steady-state current EC_{50} of GluN2B mutants

Average steady-state current EC_{50} values recorded on lifted cells expressing either wild-type or mutant receptors. Asterisks indicate significant difference from EC_{50} value of wild-type receptor (**$P < 0.01$; one-way ANOVA). Data are the means ± S.E of 4-8 cells. Pink bars indicate alanine substituents and blue bars are tryptophan substituents, whereas the black bar shows the ethanol IC_{50} value of wild-type GluN2B-containing NMDA receptor.

Apparent desensitization in mutation at residue in the GluN2B subunit corresponding to GluN2A (Met823) that regulates ion channel gating

A previous study on GluN2A identified that tryptophan mutagenesis at Met823 in the M4 domain significantly altered both the rate and extent of desensitization, when compared with wild-type receptor (Ren *et al*., 2003a). In the current study, although

tryptophan mutagenesis at Met824, the cognate position in the GluN2B subunit, did not affect glutamate EC_{50} and ethanol IC_{50} values, it still showed increased desensitization compared to wild-type GluN1/GluN2B NMDA receptors (Figure 3.3). Apparent desensitization was determined by steady-state current to peak current ratio for current activated by 300 μM glutamate treatment. The importance of the M4 domain in regulating desensitization of NMDA receptor has been previously reported (Ren *et al*., 2003a; Schorge and Colquhoun, 2003), which indicate that residue Met824 in the GluN2B subunit is critical for regulating ion channel gating, even though it is likely that this position is not involved in regulation of ethanol action on the NMDA receptor.

Comparing the role of cognate positions in the GluN2A and GluN2B subunit in regulating ethanol sensitivity of the NMDA receptor

Functional screening of ethanol-sensitive position in the GluN2B subunit is based on the high homology of membrane-associated domains among different GluN2 subunits. In **Figure 3.10** and **Table 3.1**, it is clearly shown that although alanine and tryptophan substitutions at GluN2B(Phe638) and GluN2B(Met824) do not show any ethanol sensitivity changes compared to wild-type GluN2B-containing NMDA receptors, their cognate positions in the GluN2A subunit showed significantly altered ethanol IC_{50} values upon mutagenesis of original amino acid.

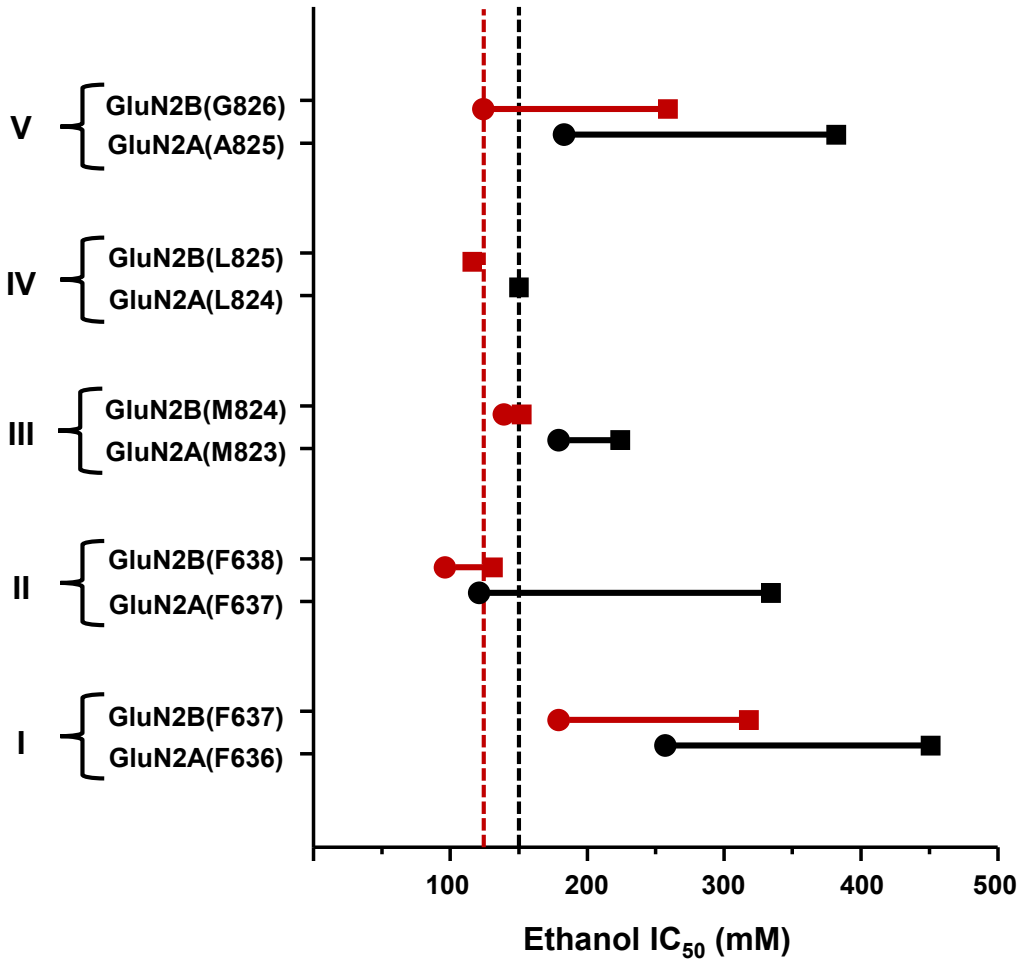

Figure 3.10. Comparison of ethanol sensitivity of either alanine or tryptophan substitution at cognate positions between GluN2A and GluN2B subunit

X axis indicates ethanol IC_{50} values of various mutants. The black dotted line shows ethanol IC_{50} value of wild-type GluN2A-containing NMDA receptor, whereas the red one shows ethanol IC_{50} value of wild-type GluN2B-containing NMDA receptor. Squares indicate ethanol IC_{50} values of tryptophan mutants, and circles indicate ethanol IC_{50} values of alanine mutants (black, GluN2A subunit; red, GluN2B subunit). The longer of distance from dotted line, the more significantly the ethanol sensitivity change is. Ethanol IC_{50} values for alanine and tryptophan substitutions at various positions in GluN2A subunit are from previous studies in our laboratory (Ren *et al*., 2003b; Honse *et al*., 2004; Ren *et al*., 2007; Ren *et al*., 2013).

Mutants	F637A	F637W	G638A	G638W	M824A	M824W	L825A	G826A	G826W
EtOH IC_{50}	Increase	Increase	Decrease	No change	No change	No change	No change	No change	Increase
Glu I_p EC_{50}	No change	Decrease	No change	No change	No change	No change	No change	No change	No change
Glu I_{ss} EC_{50}	No change	Decrease	No change	No change	Decrease	Decrease	No change	No change	No change
I_{ss} : I_p	Increase	Increase	No change	No change	Decrease	Decrease	No change	No change	No change

Table 3.1. Comparison of characteristics of GluN2B mutants with wild-type receptors

Discussion

Based on the highly homologous sequences of the third transmembrane domain (M3) and the fourth transmembrane domain (M4) between the GluN2A and the GluN2B subunit, I initially scanned for ethanol-sensitive position by replacing original amino acids at positions in the M3 and M4 domain of the GluN2B subunit, that correspond to the ethanol-sensitive positions in the GluN2A subunit, with either tryptophan or alanine. Since tryptophan is the largest and most hydrophobic amino acid, substitution of a tryptophan at these positions can significantly increase the molecular volume and hydrophobicity. Alanine is the smallest hydrophobic amino acid that (in contrast to glycine) is unlikely to destabilize α-helical regions. Alanine substitution at an ethanol-sensitive position may alter ethanol sensitivity, as it has been shown that ethanol

sensitivity is related to the molecular volume of certain ethanol-sensitive position (Ren *et al*., 2003b; Smothers and Woodward, 2006; Ren *et al*., 2007; Salous *et al*., 2009). Previous studies in this laboratory identified several ethanol-sensitive positions in both the M3 and M4 domains of the GluN2A subunit: Phe636, Phe637, Met823, and Ala825 (Ren *et al*., 2003b; Honse *et al*., 2004; Ren *et al*., 2007; Ren *et al*., 2013). In this study, a number of tryptophan and alanine substitutions were initially introduced into cognate positions in the GluN2B subunit M3 and M4 subunits, which are Phe637, Phe638, Met824 and Gly826. However, the manner in which mutants altered ethanol sensitivity differs from that of the cognate positions in the GluN2A subunit. For example, tryptophan substitution at GluN2A(Phe637) increases ethanol IC_{50} by over two-fold relative to the wild-type value (Ren *et al*., 2007), whereas in the GluN2B subunit, tryptophan substitution at the cognate position, Phe638, had no effect on ethanol sensitivity. At another position in the M4 domain of the GluN2A subunit, Met823, the tryptophan mutant showed significantly increased ethanol IC_{50}, and further experiments at this position revealed that ethanol sensitivity was dependent on molecular volume and hydrophilicity of the substituent at this position, which is consistent with the volume occupation model (Ren *et al*., 2003b). However, in the present study, neither tryptophan or alanine substitution at the cognate position, GluN2B(Met824), altered the ethanol IC_{50} when compared with the wild-type value. Of the four positions in the GluN2B subunit we tested in this study, only Phe637 showed alterations in ethanol sensitivity following both tryptophan and alanine mutagenesis. We also found no changes in glutamate EC_{50}, maximal I_{ss}:I_p, or deactivation time constant in alanine or tryptophan substitution mutants at Phe638 or Gly826. Interestingly, alanine or tryptophan substitution at Met824 altered

glutamate steady-state EC_{50} and maximal I_{ss}:I_p values, as was observed for the cognate position Met823 in GluN2A (Ren *et al*., 2003a), but did not alter ethanol sensitivity. The tryptophan mutant at Phe637 showed increases in both glutamate EC_{50} and time constant of deactivation, and both tryptophan and alanine mutants at this position showed decreased macroscopic desensitization.

In earlier studies, this laboratory reported that two positions in the GluN2A M3 and M4 domains could interactively regulate ethanol sensitivity (Ren *et al*., 2008), and Smothers *et al*. identified a pair of positions in the GluN1 subunit that can interact with each other with respect to ethanol inhibition. A recent study in this laboratory further demonstrated that positions in the M3 domain of one subunit type could interact with positions in the M4 domain of the other subunit type to regulate ethanol sensitivity and ion channel function (Ren *et al*., 2012). These observations revealed that ethanol inhibition of the NMDA receptor is not determined by a single position in the membrane-associated domains, but rather, multiple ethanol-sensitive positions appear to contribute to two pairs of sites of ethanol action. Therefore, the differences in ethanol action between GluN2A and GluN2B subunits identified in this study, likely arise because positions in the GluN2B subunit interact with positions in the GluN1 differently from the cognate positions in the GluN2A subunit. Furthermore, although transmembrane domains among different GluN2 isoforms are highly conserved, there are clear distinctions in other regions, such as the C-terminal domain and extracellular domain (Fukaya *et al*., 2003; Martel *et al*., 2012). It is possible that distant parts of the protein can affect ethanol action on the positions in transmembrane domains. Based on studies in this laboratory, different ethanol sensitivity patterns have been identified following tryptophan

mutagenesis at cognate positions among different GluN2 subunit types (Figure 3.11) (GluN2C data are from Jasmine Wu). Within these mutants, GluN2A and GluN2C

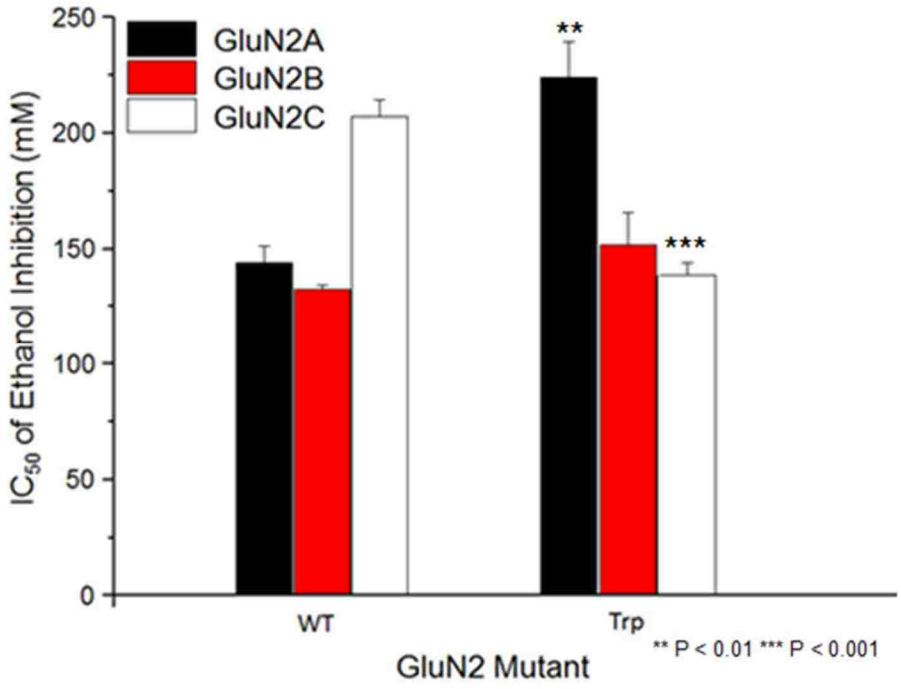

Figure 3.11. Mutagenesis at cognate positions showed different types of ethanol sensitivity change

Bar graphs show ethanol IC_{50} values of either wild-type GluN1/GluN2A, GluN1/GluN2B and GluN1/GluN2C NMDA receptor or mutant receptors with tryptophan substitution at cognate positions among different GluN2 subunits, that are GluN2A(Met823), GluN2B(Met824) and GluN2C(Met821). IC_{50} values that are significantly different from wild-type receptor are indicated by asterisks (** $P < 0.01$; *** $P < 0.001$; two-way ANOVA).

mutants show significantly altered ethanol sensitivity, however, in an opposite manner. As previously mentioned, the GluN2B mutant did not change ethanol sensitivity. This study further indicates that positions in other regions of the protein may contribute to the

indirect regulation of ethanol sensitivity and ion channel functions. Future studies can address the possible mechanism of these differences. For example, a chimeric NMDA receptor can be constructed by overlap extension PCR cloning, by which a C-terminal domain of GluN2A subunit will be fused with the rest of GluN2B subunit containing a tryptophan mutagenesis at Met824. Ethanol sensitivity will be tested on these chimeric receptors to study whether the C-terminal domain plays an important role in regulating ethanol action on the membrane-associated domains.

Besides the possibility of differences in distant regions of GluN2A and GluN2B subunits in regulation of ethanol sensitivity, it has been previously demonstrated that with highly similar sequences (70% identity), they have distinct temporal and spatial expression patterns and channel properties in the brain. For example, GluN2B is expressed early in the prenatal brain, and is soon overtaken by GluN2A during development (Sheng *et al*., 1994). The reason for this compositional switch is still under extensive investigation, though it is clear that there are differences between these "twins" receptor isoform (Matta *et al*., 2011; Gambrill and Barria, 2011). Moreover, GluN2A and GluN2B subunits also show spatial expression differences in the brain, in which the GluN2A subunits are mainly located in synapses, and the GluN2B subunits are in both synapses and the extrasynaptic region (Li *et al*., 1998; Townsend *et al*., 2003; Harris and Pettit, 2007). Previous studies have also reported that the GluN2A and GluN2B subunit-containing NMDAR play different roles in LTP and LTD. For example, de novo LTP can be induced by activation of GluN2A subunit-containing NMDA receptors, whereas LTD required activation of GluN2B subunit-containing NMDA receptors (Massey *et al*., 2004; Morishita *et al*., 2007; Bartlett *et al*., 2007; Tang *et al*., 2009). Previous studies have in

some cases demonstrated distinct ethanol actions on different NMDA receptor subunits. One study found that ethanol inhibition of NMDA receptor synaptic events are dependent on GluN2B-containing NMDAR, but not GluN2A-containing NMDAR (Kash *et al*., 2008). In addition, chronic ethanol treatment upregulated GluN2B subunit expression level in the hippocampus, but left GluN2A subunit levels unchanged (Kash *et al*., 2009). It is in turn possible that these differences in expression pattern and function can lead to distinctly different ion channel functions properties between the GluN2A and GluN2B subunits.

IV. PHE637 IN THE NMDA RECEPTOR GLUN2B SUBUNIT M3 DOMAIN REGULATES ETHANOL SENSITIVITY

Introduction

As described previously, mutation of one position, Phe637, at the M3 domain of the GluN2B subunit altered both ethanol IC_{50} and glutamate EC_{50}, suggesting the importance of this position on regulation of NMDA receptor function and ethanol sensitivity. In order to test the role of this position on ethanol sensitivity in detail, it is important to identify the ethanol sensitivity alteration pattern based on physicochemical parameters of various amino acid substituents at this position. So I initially made a panel of amino acid substitutions at this position and tested ethanol sensitivities on them by conducting ethanol concentration-response experiments.

Studies about the action of alcohol on other neurotransmitter receptors have previously reported that alcohols and anesthetics can produce their actions by occupying a certain molecular volume in a binding cavity (Mihic *et al*., 1997; Wick *et al*., 1998; Mascia *et al*., 2000), which means that the larger the molecular volume of the cavity, the lower the ethanol sensitivity. In our previous studies, these observations were not consistent throughout four ethanol-sensitive positions. Only one of these three residues, Met823, showed an inverse relationship between ethanol potency and molecular volume of the mutant amino acid when the value of tryptophan mutant was excluded from the analysis (Ren *et al*., 2003b). Moreover, a number of studies have demonstrated that alcohols act on NMDA receptor in a manner that involves hydrophobic interactions between alcohol molecule and its action site (Lovinger *et al*., 1989; Fink and Gothert,

1990; Gonzales *et al*., 1991; Peoples and Weight, 1995; Peoples and Ren, 2002; Ren *et al*., 2003b). In order to determine whether observed change in ethanol sensitivity is dependent on physicochemical parameters of the substituent at GluN2B(Phe637), I plotted ethanol IC_{50} values of mutants with molecular volume, hydrophilicity and polarity of substituents at this position to test if there is any correlation between ethanol sensitivity and these parameters.

Results

Presumed location Phe637 in the M3 domain of GluN2B subunit

Previously identified ethanol-sensitive positions in our laboratory exhibit a common motif, that they are all hydrophobic; they do not line the ion channel lumen; they have limited access from cytoplasmic side of membrane; and they are located in regions that influence ion channel gating. Results from the previous chapter show that mutation of one position, GluN2B(Phe637), significantly altered ethanol sensitivity and ion channel function of the NMDA receptor when mutated to tryptophan. **Figure 4.1** shows the presumed location of Phe637 in the M3 domain of the GluN2B subunit, which shares similar elements with ethanol sensitive positions in the GluN2A subunit.

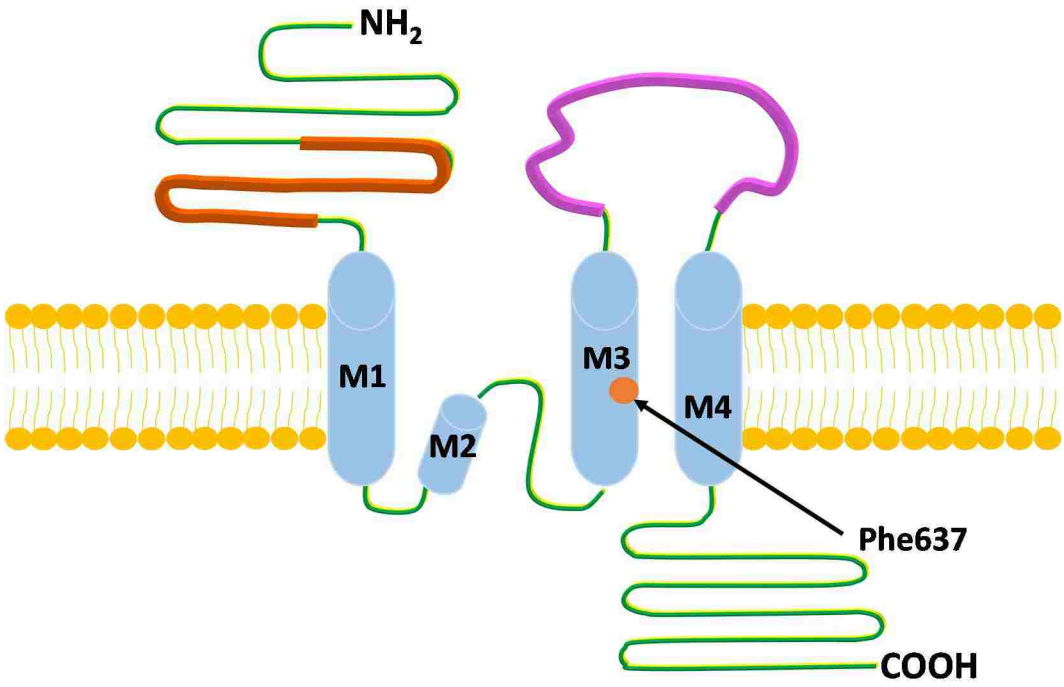

Figure 4.1. Presumed location of GluN2B(Phe637)

Topological model of the GluN2B subunit showing the membrane-associated domains (M1-M4), ligand-binding domains (S1-S2), and the presumed location of Phe637 is noted by a yellow dot.

Mutations at GluN2B(Phe637) could alter ethanol sensitivity

In order to study the role of this position in ethanol action in detail, ten mutants were made by using site-directed mutagenesis (F637A, F637C, F637G, F637I, F637L, F637M, F637S, F637V, F637W, F637Y). This panel of amino acids includes both polar and nonpolar amino acids. Mutants constructed all yielded functional NMDA receptors when co-expressed with the wild-type GluN1 subunit and resulted in measurable currents. **Figure 4.2** shows the inhibition by 100 mM ethanol in the NMDA receptors with various substitutions at position Phe637 in the M3 domain of the GluN2B subunit. Values of peak current activated by 10μM glutamate and 50μM glycine varied

over a large range among individual mutant subunit, which indicates the variable transfection efficiency or single channel conductance. These currents also show that all the mutant subunits constructed here were inhibited by ethanol.

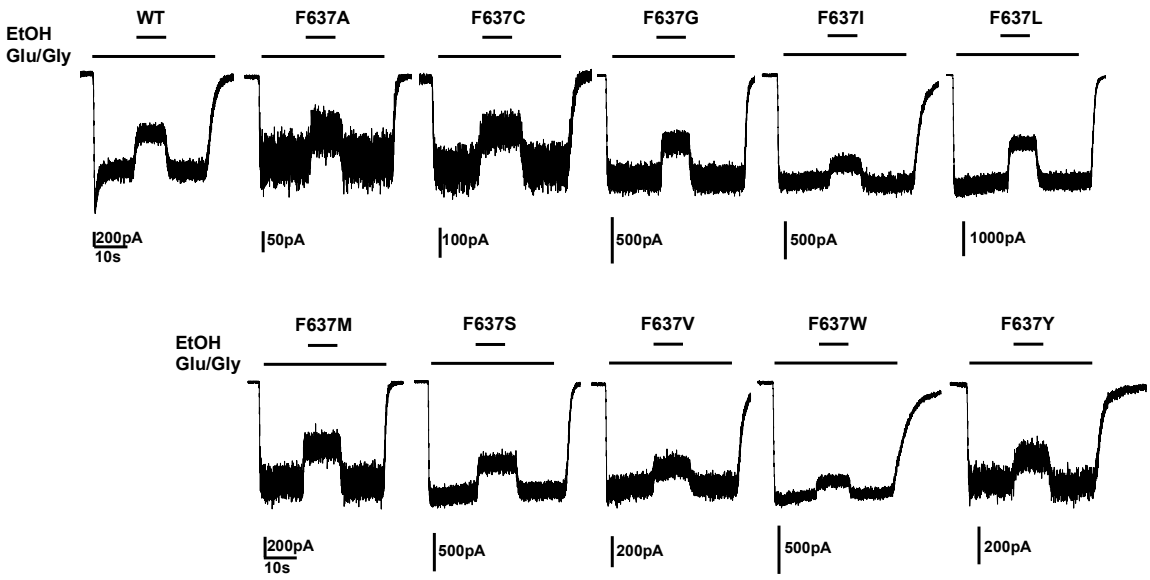

Figure 4.2. Ethanol inhibition traces of cells expressing various GluN2B(Phe637) mutants

Traces show currents activated by 10 μM glutamate and 50 μM glycine and their inhibition by 100 mM ethanol in tsA-201 cells expressing either wild-type receptor or GluN1 subunit combined with various GluN2B(Phe637) mutants. One-letter amino acid codes are used.

Ethanol concentration-response experiments were performed on tsA-201 cells expressing either wild-type or mutant GluN2B-containing NMDA receptor. A series of ethanol concentrations from 2.5 mM to 500 mM were used in these experiments, and results were collected from at least 5 cells on different recording dates. As shown in **Figure 4.3,** all functional mutants constructed here were inhibited by ethanol in a concentration-

dependent manner. Although concentration-response curves for ethanol inhibition were essentially parallel to each other, as the slope factors of these curves did not differ

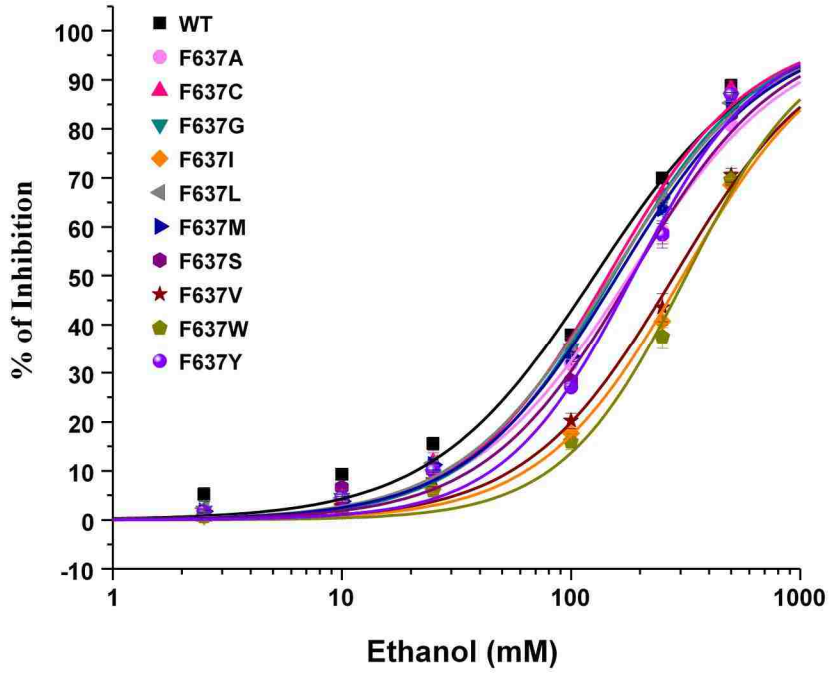

Figure 4.3. Ethanol concentration-response of various GluN2B(Phe637) mutants

Concentration-response curves for ethanol inhibition of glutamate-activated currents in the presence of 50 µM glycine in cells expressing various substitution mutations at GluN2B(F637). Data are the means ± S.E of 5-7 cells, error bars not visible were smaller than the size of symbols. The black line shows the fit for the wild-type receptor. All curves are best fitted to the equation given under "Materials and Methods".

significantly, bar graphs of average ethanol IC_{50} values indicate the statistical difference in ethanol sensitivity among various mutants compared to wild- type receptor (Figure 4.4). Values of ethanol IC_{50} in the substitution mutants ranged from 144 mM to 318 mM, and tryptophan substitution at this position produced the greatest increase in ethanol IC_{50}

value. In turn, six out of ten mutants showed significantly decreased ethanol sensitivity compared to the wild-type receptor (one-way ANOVA; $p<0.05$ or $p<0.01$), however, none of these mutants exhibited increased ethanol sensitivity compared to the wild-type receptor. Interestingly, even isoleucine and leucine are isomeric amino acids, only isoleucine substitution significantly changed ethanol sensitivity (IC_{50} value: 307 ± 9.4 mM; one-way ANOVA; $p<0.01$).

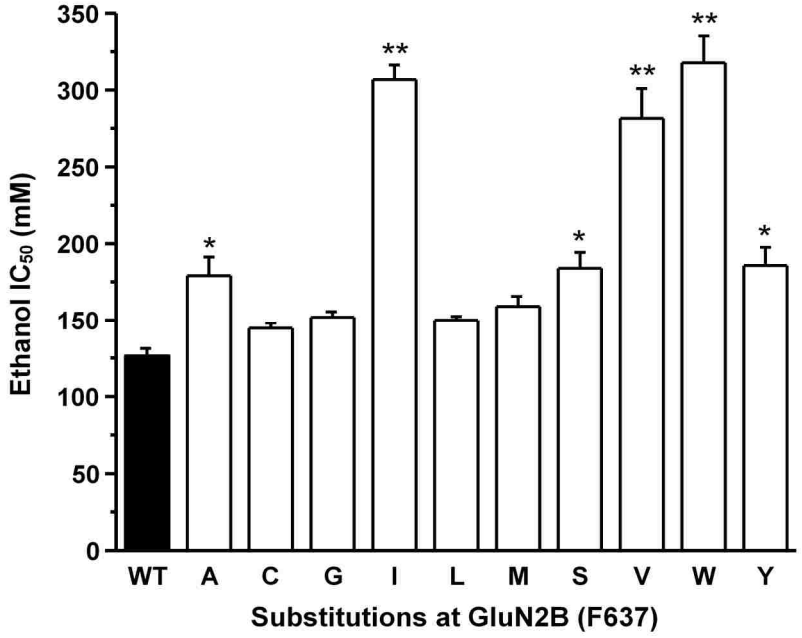

Figure 4.4. Ethanol IC_{50} of various GluN2B(Phe637) mutants

The average IC_{50} values for ethanol inhibition of glutamate-activated current in wild-type or GluN2B(Phe637) mutant receptors were shown by Bar Graphs. Asterisks indicate significant difference from IC_{50} value of wild-type receptor (*$P < 0.05$; **$P < 0.01$; ANOVA and Dunnett's test). Results are the means ± S.E of 5-7 cells. IC_{50} value of wild-type receptor is shown by black bar.

Relationship between ethanol sensitivity and physical-chemical properties of amino acid at GluN2B(Phe637)

To determine whether physical-chemical properties of amino acid substitutions contribute to the variation of ethanol IC_{50} values among these mutants, I tested if the IC_{50} value of each substitution could be linearly related to amino acid polarity, hydrophilicity, or molecular volume respectively. Because if the observed changes in ethanol sensitivity among GluN2B(Phe637) mutants were due to a physical interaction of the ethanol molecule with this position in some manner, linear relationship between ethanol sensitivity and one of these physical-chemical properties should be found from these analysis. In order to evaluate the relative contribution of the physicochemical parameters of the amino acid residue at this position to ethanol action, I performed linear regression analysis between ethanol IC_{50} and molecular volume (Figure 4.5), hydrophilicity (Figure 4.6) as well as polarity (Figure 4.7). However, no significant linear relations were spotted among these measurements ($P > 0.05$).

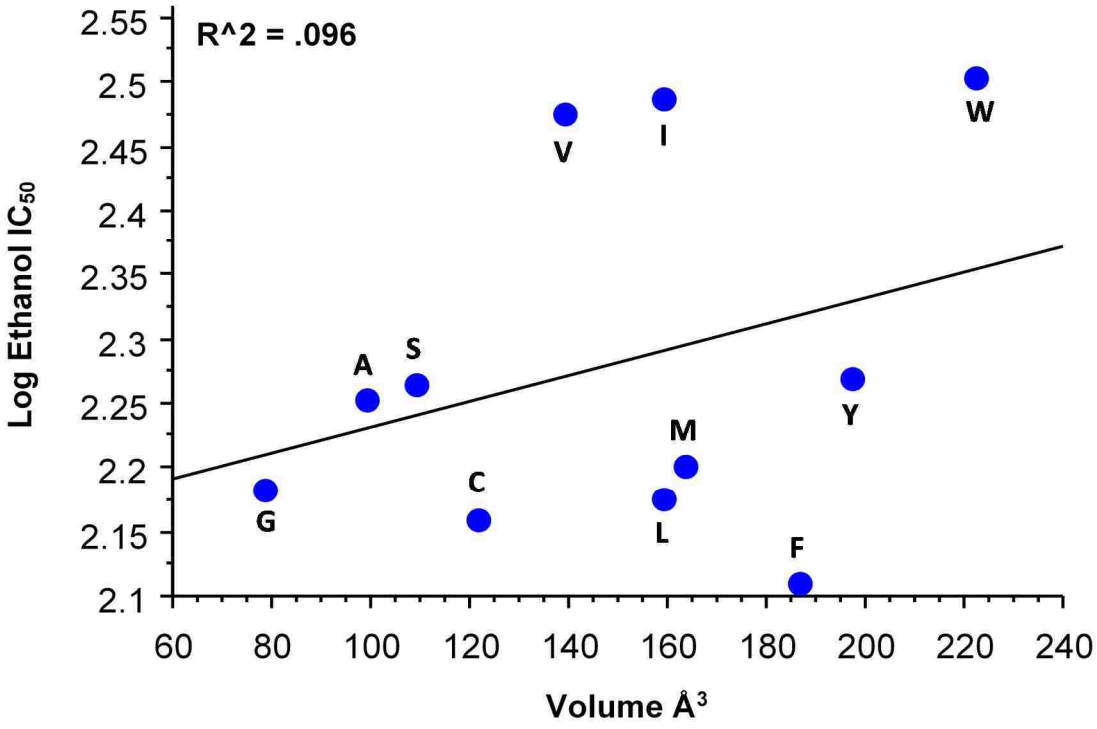

Figure 4.5. Relation of amino acid molecular volume of the substituent at GluN2B(Phe637) to Log-transformed IC_{50}

Log-transformed IC_{50} for ethanol plotted versus molecular volume. The line shown is the least-squares fits to the data. There is no significant linear relationship between ethanol IC_{50} and molecular volume (R^2=0.096; P>0.05).

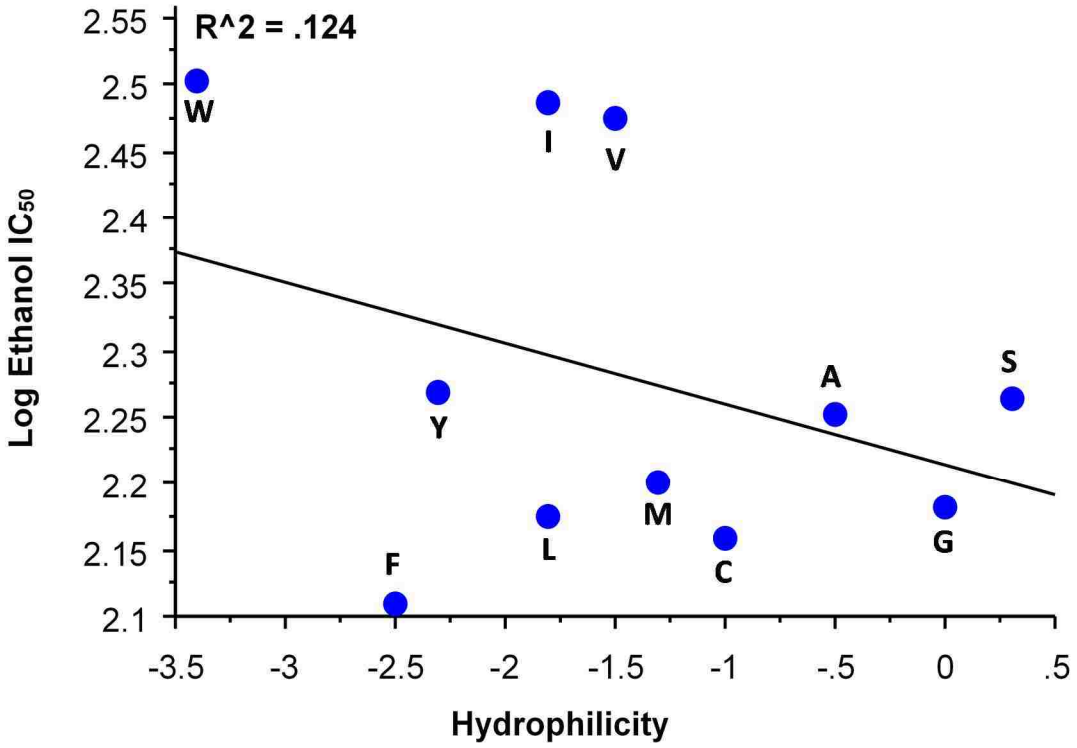

Figure 4.6. Relation of amino acid hydrophilicity of the substituent at GluN2B(Phe637) to Log-transformed IC_{50}

Log-transformed IC_{50} for ethanol plotted versus hydrophilicity. The line shown is the least-squares fits to the data. There is no significant linear relationship between ethanol IC_{50} and hydrophilicity (R^2=0.124; P>0.05).

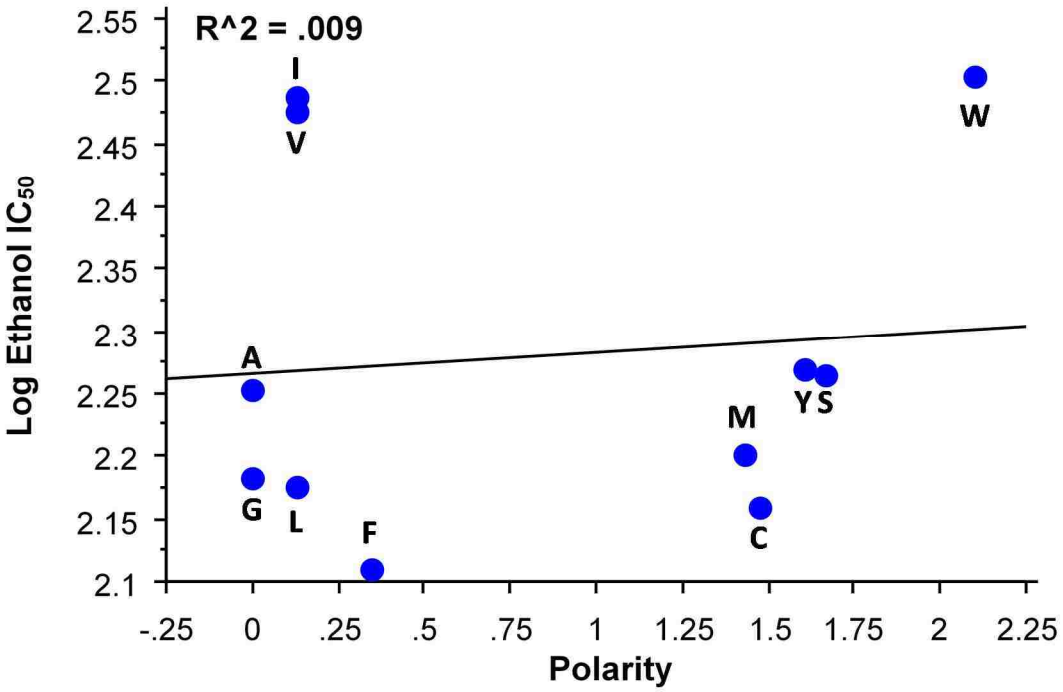

Figure 4.7. Relation of amino acid polarity of the substituent at GluN2B(Phe637) to Log-transformed IC_{50}

Log-transformed IC_{50} for ethanol plotted versus polarity. The line shown is the least-squares fits to the data. There is no significant linear relationship between ethanol IC_{50} and polarity (R^2=0.009; P>0.05).

Ethanol IC_{50} values of GluN2B(Phe637) mutants are correlated with that of GluN2A(Phe636) mutants

Since I noticed different characteristics of cognate positions between the GluN2A and GluN2B subunit (Figure 3.10), and also found ethanol sensitivity alterations among various substitutions at GluN2B(Phe637), which is the cognate position of ethanol-sensitive position in GluN2A subunit, Phe636, I asked next if these two positions are correlated with each other with respect to ethanol sensitivity change upon the same amino acid substitution. To test this hypothesis, I plotted the ethanol IC_{50} values of various

GluN2B(Phe637) mutants against values of corresponding substitutions at GluN2A(Phe636). The ethanol sensitivity among various mutants were highly correlated with each other, which indicates the same functional role of each substituent at different GluN2 subunits on NMDA receptor ethanol sensitivity ($R^2 = 0.98$, $P < 0.0001$; Figure 4.8).

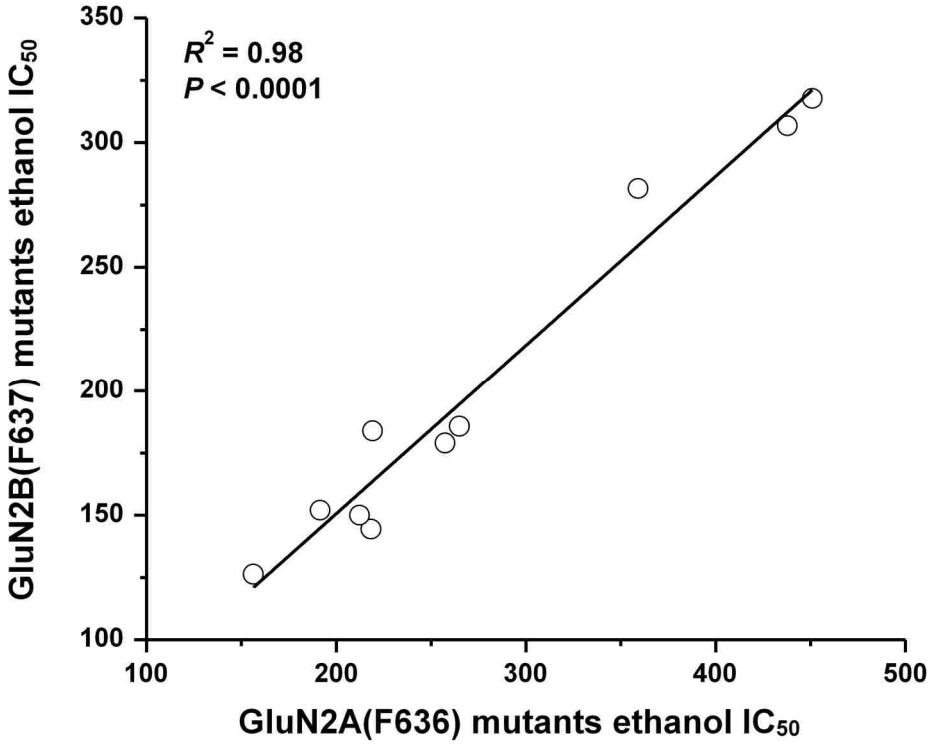

Figure 4.8. Ethanol IC_{50} values of GluN2B(Phe637) mutants are correlated with that of same amino acid substitutions at GluN2A(Phe636)

Log ethanol IC_{50} values of GluN2B(Phe637) are plotted *versus* log ethanol IC_{50} values of GluN2A(Phe636) in a series of mutants, they are significantly correlated ($R^2 = 0.98$, $P < 0.0001$). The line shown here is the least squares fits to the data. Ethanol IC_{50} values of various GluN2A(Phe636) mutants are from previous study in our laboratory (Ren *et al*., 2013).

Discussion

These studies demonstrated that GluN2B(Phe637), the cognate position of the ethanol-sensitive position in the GluN2A subunit, could regulate ethanol sensitivity. I further studied the role of this position on ethanol sensitivity by constructing a panel of amino acid mutations. Substitutions at GluN2B(Phe637) could significantly alter ethanol sensitivity. Although substantial variations of ethanol sensitivity were observed among different amino acid substitutions, ethanol sensitivity was not correlated with any physicochemical parameter of amino acid substituent at this position.

Some investigators have demonstrated that alcohols and anesthetics can bind to residues in $GABA_A$ and glycine receptors and regulate channel functions by occupying a critical volume (Mihic *et al*., 1997; Wick *et al*., 1998; Yamakura *et al*., 1999; Kash et al., 2004). Moreover, for a previously-identified ethanol-sensitive position in M3 domain, Phe639 in GluN1, ethanol sensitivity was correlated with molecular volume of the side-chain at this position (Smothers and Woodward, 2006), and this position is the cognate to GluN2B(Phe637). Previous studies in this laboratory demonstrated that ethanol sensitivity was dependent on molecular volume of the substituent at Met823 in the M4 domain of GluN2A subunit (Ren *et al*., 2003b), and was inversely dependent upon molecular volume of the substituent at GluN2A(Phe637) (Ren *et al*., 2007). In contrast, I was unable to identify this molecular volume occupation model at Phe637 in the M3 domain of the GluN2B. The possible reason for this may be that, the side chain at 637 interacts with adjacent side chains to form critical volume for ethanol action, rather than that the molecular volume of an amino acid at a certain position is important to ethanol action. This assumption also can explain the high correlation of ethanol sensitivity among

various substitutions between GluN2B(Phe637) and GluN2A(Phe636). In this case, the side chain at GluN2B(Phe637) does not directly project into the interior of ethanol-sensitive site, but rather forms part of the outer boundary of this site.

The observation of a relation between ethanol sensitivity and hydrophilicity of the substitution at GluN2A(Met823) suggests that ethanol could interact with certain residues in a manner that involves hydrophobic binding (Ren *et al*., 2003b). However, the results of the present study also did not show any relation between ethanol sensitivity and the hydrophilicity of the substituent at GluN2B(Phe637). The lack of a clear role for molecular volume and hydrophilicity in the present study was particularly apparent when comparing the leucine and isoleucine mutants: there was a striking difference in ethanol sensitivity between two mutants, even though they have identical physicochemical characteristics. Thus, GluN2B(F637) can regulate receptor ethanol sensitivity in a complex manner relaying on the interaction between side chain at this position and other positions or environment. Studies in following chapters will define some of these interactions.

V. PHE637 IN THE NMDA RECEPTOR GLUN2B SUBUNIT M3 DOMAIN REGULATES ION CHANNEL KINETICS

Introduction

It has been demonstrated that ethanol inhibits NMDA receptors by changing ion channel gating, especially decreasing mean open time (Lima-Landman and Albuquerque, 1989; Peoples et al., 1997), leading to the assumption that the ethanol action site also plays an important role for ion channel functions. Previous studies in our laboratory found that mutations at ethanol-sensitive positions in the GluN2A subunit could also influence ion channel kinetic behaviors, such as glutamate EC_{50}, apparent desensitization, deactivation, and single-channel mean open time (Ren *et al*., 2003a; Ren *et al*., 2003b; Honse *et al*., 2004; Ren *et al*., 2007; Ren *et al*., 2013). So it is possible that changes in ethanol sensitivity among various mutants at these positions are secondary to changes in ion channel gating kinetics. GluN2B(Phe637) is within the M3 domain, which has conserved role in NMDA receptor ion channel gating, and is at a considerable distance from the ligand binding domain (Low *et al*., 2003; Yuan *et al*., 2005; Sobolevsky *et al*., 2007; Sobolevsky *et al*., 2009). Furthermore, the importance of this position for ethanol action on the NMDA receptor was demonstrated in previous chapter, it's valuable to study the ion channel functions among various mutants. As a first step to answer this question, I used whole-cell patch clamp recording and glutamate concentration-response experiments to study the apparent potency (glutamate peak current EC_{50} value), apparent affinity (glutamate steady-state current EC_{50} value) as well as apparent desensitization

(maximal steady-state current to peak current ratio) of series of mutations at GluN2B(Phe637).

Mutations of residues in the γ-aminobutyric acid type A ($GABA_A$) and glycine receptors showed that physicochemical parameters, such as molecular volume, of the substituent could influence ion channel gating behavior (Wick *et al*., 1998; Koltchine *et al*., 1999; Yamakura *et al*., 1999; Jenkins *et al*., 2001). Previous studies in our laboratory have also demonstrated the relationship between physicochemical parameters of substituent amino acid and either glutamate EC_{50} or desensitization of mutant receptor (Ren *et al*., 2003a). In order to study the relationship between physicochemical parameters of the substituent amino acid and ion channel functions, I also plotted the value of each physicochemical parameter against each ion channel function.

Results

Mutations at GluN2B(Phe637) alter glutamate peak current EC_{50} and glutamate steady-state EC_{50}

In **Chapter III**, I first compared EC_{50} values for glutamate-activated peak and steady-state currents between wild-type and mutant NMDA receptor by co-transfecting GluN1 and GluN2B (either wild-type or alanine and tryptophan mutant) into tsA-201 cells and performing glutamate concentration-response experiments in lifted cells. I found that tryptophan substitution at GluN2B(Phe637) significantly decreased glutamate EC_{50} values on both peak (EC_{50} value: 1.1 ± 0.17 μM; ANOVA and Dunnett's test; $p < 0.01$) (Figure 3.7) and steady-state (EC_{50} value: 1.05 ± 0.16 μM; ANOVA and Dunnett's test; $p < 0.01$) (Figure 3.9) glutamate-activated currents. Besides that, this mutant also

showed significantly decreased apparent desensitization (I_{ss}:I_p) (WT: 0.76 ± 0.02; F637W: 0.96 ± 0.01; two-way ANOVA; $p < 0.01$) as well as an increased time constant of deactivation (τ) following removal of agonist (WT: 920.20 ± 3.30 ms; F637W: 2167.78 ± 2.84 ms; ANOVA; $p < 0.0001$) (Figure 5.1).

Figure 5.1. Desensitization and deactivation of GluN2B (F637W) mutant

Normalized traces show the desensitization (left) and deactivation (right) of currents activated by 300 μM glutamate in the presence of 50 μM glycine in lifted tsA-201 cells expressing GluN1/GluN2B or GluN1/GluN2B(F637W) subunit.

Based on initial results of how tryptophan substitution at GluN2B(Phe637) altered ion channel kinetics, I decided to further determine whether other mutations at this position could also influence NMDA receptor physiological characteristics. Results from this study can provide a more complete understanding about the role of this position on ion channel function. Values of current activated by maximal glutamate concentration (300 μM) and 50 μM glycine varied among mutants, which may indicate variable transfection efficiency. I performed glutamate concentration-response experiments in all mutants I tested for ethanol sensitivity using a rapid solution exchange apparatus in lifted

cells. It is noticeable that at least three out of ten mutants exhibit prolonged channel deactivation (figure 5.2).

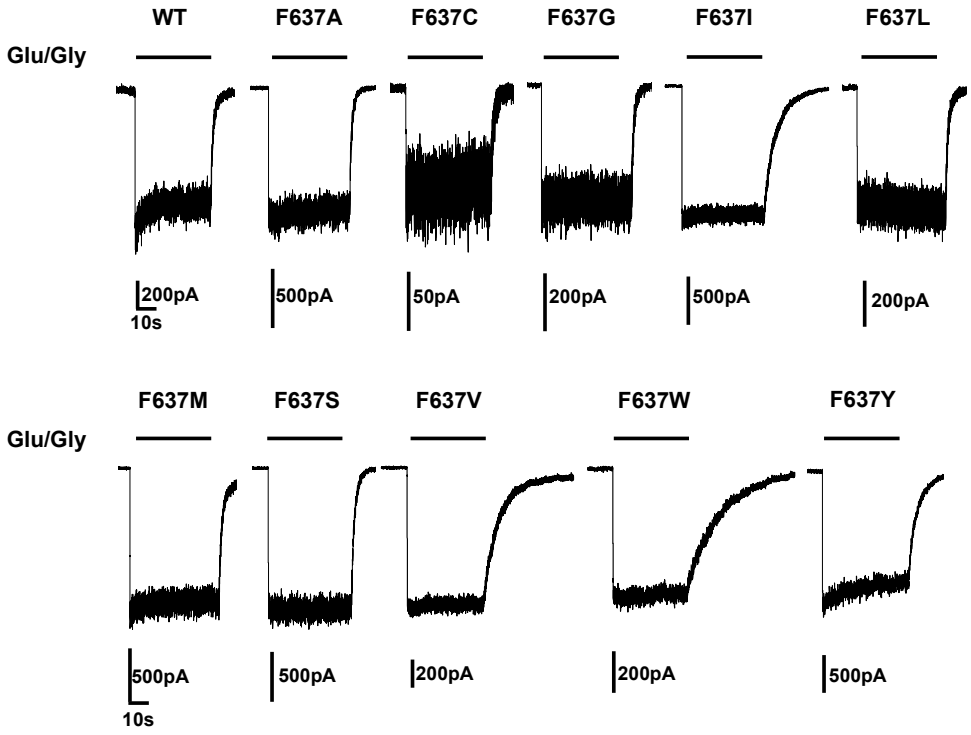

Figure 5.2. Glutamate-activated currents in cells expressing various GluN2B(Phe637) mutants

Traces show 300 μM glutamate-activated current in the presence of 50 μM glycine in lifted cells expressing wild-type receptors or receptors containing various substitutions at GluN2B(F637). One-letter amino acid codes are used.

Hill coefficients of the glutamate concentration- response curves for various mutants did not differ significantly from the wild-type peak current (Figure 5.3) as well as steady-state current value (Figure 5.5). However, highly significant differences were observed among the peak current EC_{50} (ANOVA; $P < 0.01$; Figure 5.4) and the steady-

state current EC_{50} (ANOVA; $p < 0.01$; Figure 5.6) of the series of substitutions at GluN2B(Phe637). Eight out of ten mutants showed significantly decreased glutamate peak current EC_{50} values, but only four of them showed decreased glutamate steady-state current EC_{50} values. Interestingly, the difference between isoleucine and leucine was also present here, as only isoleucine substitution at this position showed significantly altered glutamate steady-state current EC_{50} (0.85 ± 0.08 μM; ANOVA; $p < 0.01$), whereas substitution with leucine did not (2.5 ± 0.24 μM; ANOVA; $p > 0.05$) (Figure 5.6).

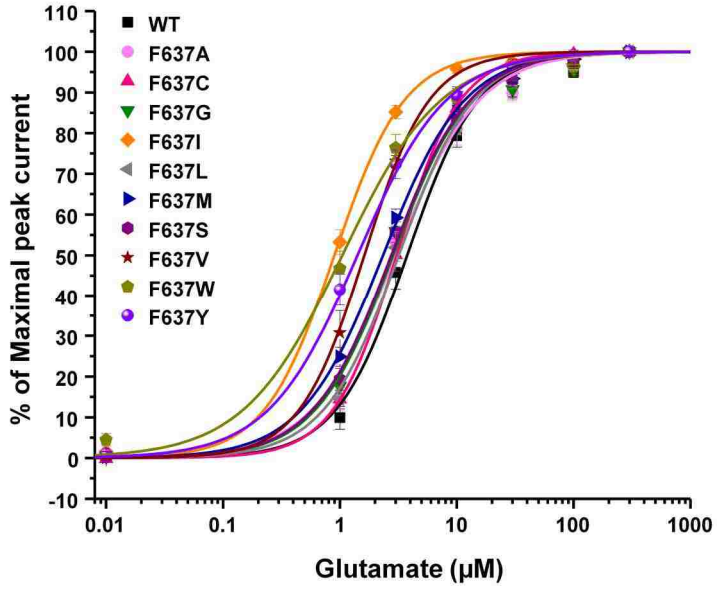

Figure 5.3. Glutamate concentration-response curves of peak currents of GluN2B(Phe637) mutants

Concentration-response curves for glutamate-activated peak current EC_{50} recorded from lifted cells expressing wild-type or mutant receptors. Data points are the means ± S.E of 4-6 cells, error bars not visible were smaller than the size of symbols. All curves here are best fitted to the equation described in "Materials and Methods". The black curve indicates the fit for wild-type receptors.

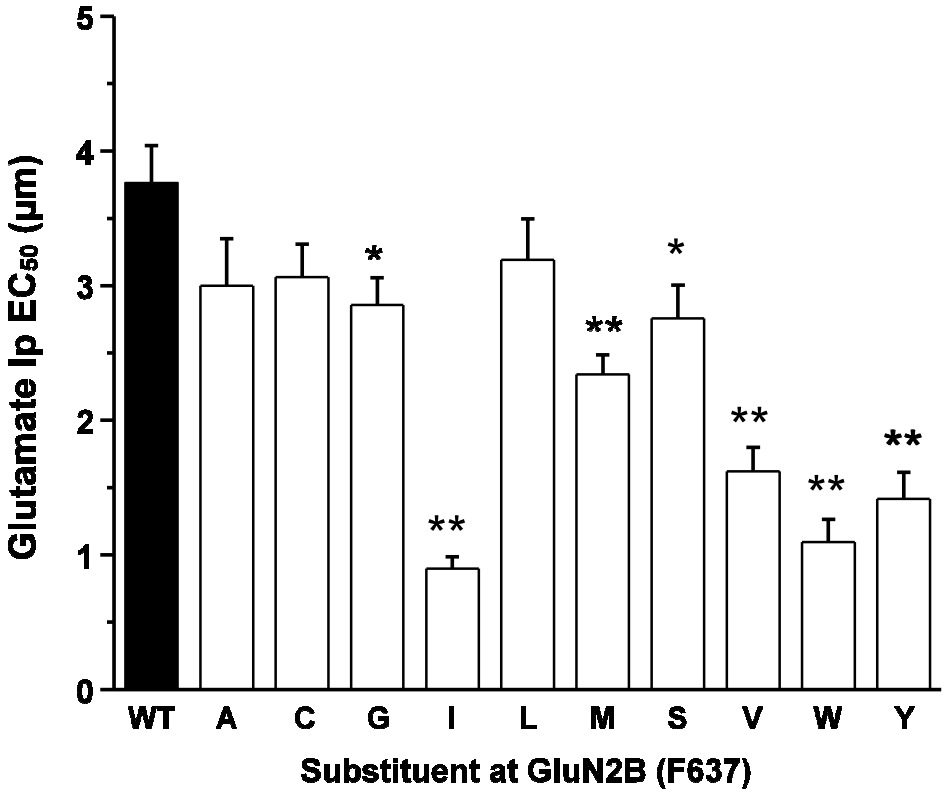

Figure 5.4. Glutamate peak current EC50 of GluN2B(Phe637) mutants

Bar graphs show the average EC_{50} values for glutamate activated peak currents recorded from cells expressing GluN1 and wild-type GluN2B or cells expressing GluN1 and GluN2B(F637) mutants. Asterisks indicate EC_{50} values that differ significantly from that of the wild-type GluN1/GluN2B subunit (**P < 0.01; ANOVA and Dunnett's test). Results are the means ± S.E of 4-6 cells. The EC_{50} value for wild-type receptor is shown by black bar.

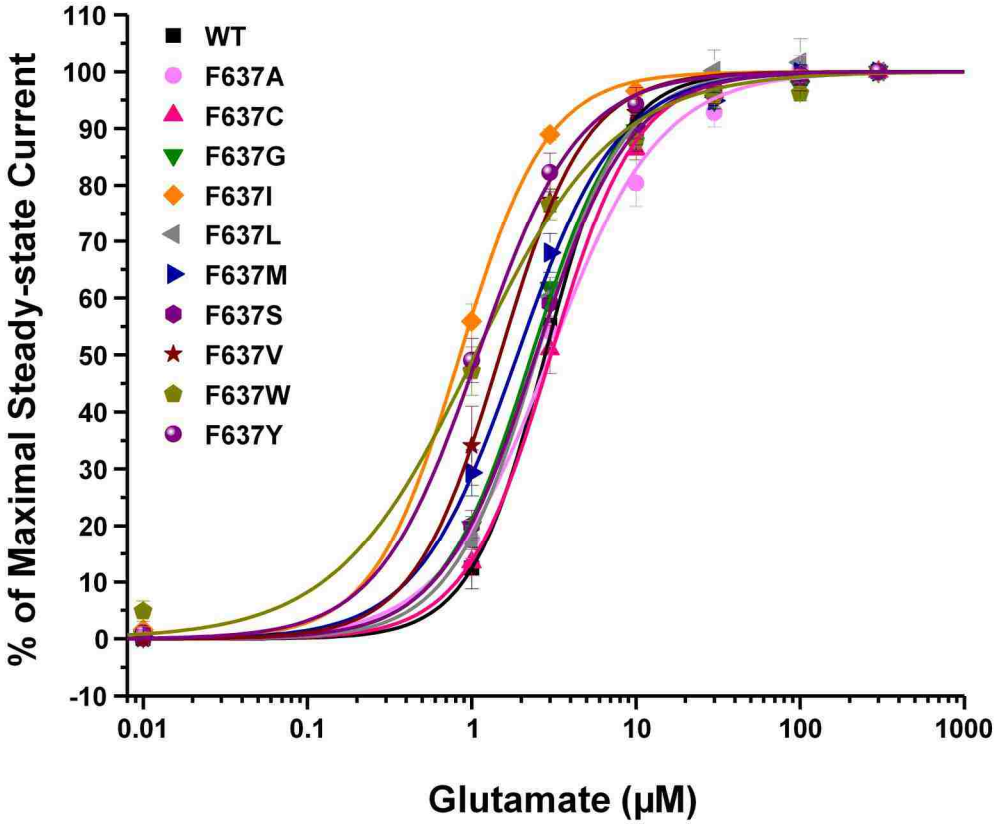

Figure 5.5. Glutamate concentration-response curves of steady-state currents of GluN2B(Phe637) mutants

Concentration-response curves for glutamate-activated steady-state current EC_{50} recorded from lifted cells expressing wild-type or mutant receptors. Data points are the means ± S.E of 4-6 cells; error bars not visible were smaller than the size of symbols. All curves here are best fitted to the equation described in "Materials and Methods". The black curve indicates the fit for wild-type receptors.

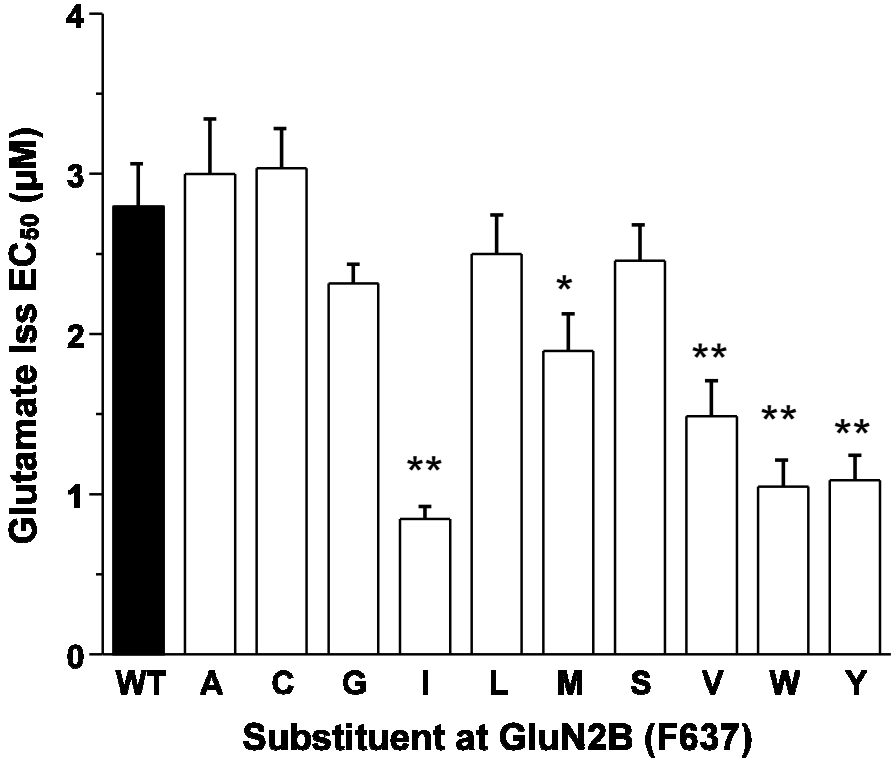

Figure 5.6. Glutamate steady-state EC_{50} of GluN2B(Phe637) mutants

Bar graphs show the average EC_{50} values for glutamate steady-state currents recorded from cells expressing GluN1 and wild-type GluN2B or cells expressing GluN1 and GluN2B(F637) mutants. Asterisks indicate EC_{50} values that differ significantly from that of the wild-type GluN1/GluN2B subunit (**$P < 0.01$; ANOVA and Dunnett's test). Results are the means ± S.E of 4-6 cells. The EC_{50} value for wild-type receptor is shown by black bar.

Mutations at GluN2B(Phe637) of NMDA receptor could alter steady-state to peak current ratio (I_{ss}:I_p)

In whole-cell recordings from lifted cells, besides altered glutamate EC_{50}, pronounced decreases were also obtained in apparent desensitization (maximal steady-state to peak current ratio) of all mutants, which indicates significantly altered ion channel gating in mutant receptors (ANOVA; $p < 0.01$; figure 5.7). Moreover, these

results combined with altered glutamate EC_{50} values reveal the importance of this position on NMDA receptor ion channel gating.

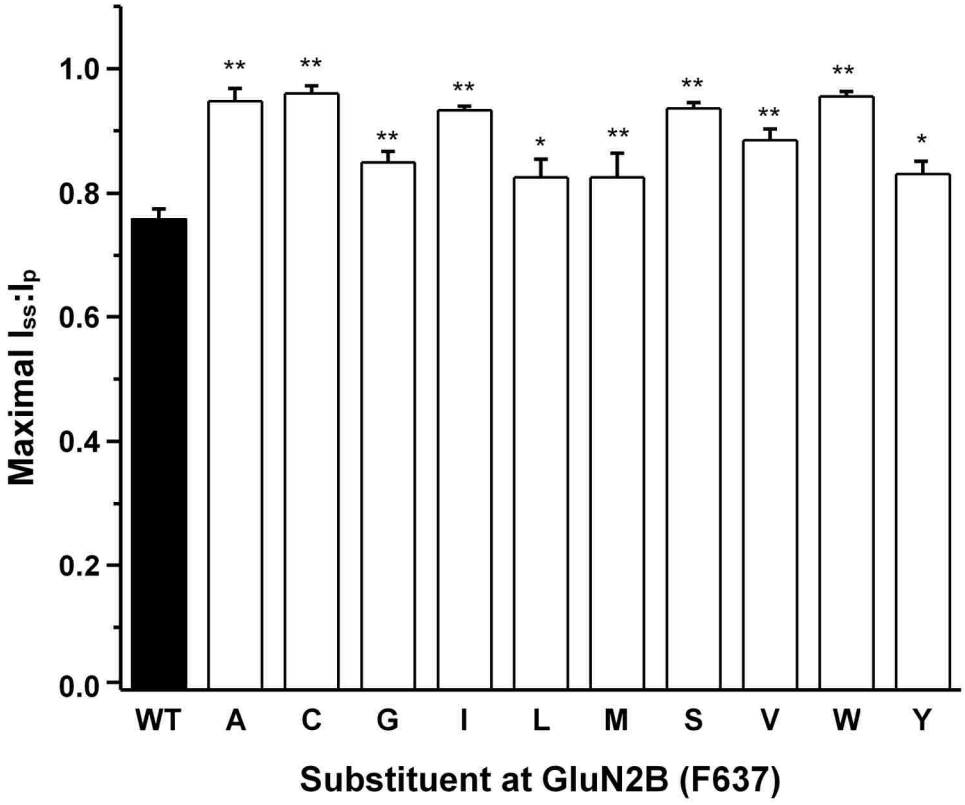

Figure 5.7. Apparent desensitization of GluN2B(Phe637) mutants

The average values of maximal steady-state to peak current ratio (I_{ss}:I_p) in lifted cells co-expressing GluN1 and GluN2B(wild-type) subunits or GluN2B subunits containing various substitutions at F637. Currents were activated by 300 μM glutamate in the presence of 50 μM glycine. Asterisks indicate I_{ss}:I_p values that are significantly different from the value for the wild-type GluN1/GluN2B subunits (*$P < 0.05$; **$P < 0.01$; ANOVA and Dunnett's test). Results are the means ± S.E of 4-6 cells. The value for wild-type receptor is shown by the black bar.

GluN2B(Phe637) mutant glutamate EC_{50} and physical-chemical properties of the amino acid at the substituted position

To determine whether physical-chemical properties of amino acid substituents contribute to the changes of peak current EC_{50} values, steady-state current EC_{50} values, and apparent desensitization, I tested if they could be linearly related to amino acid polarity, hydrophilicity or molecular volume. However, no significant linear relation was observed among these measures (Table 5.1).

	Glutamate I_p EC_{50}		Glutamate I_{ss} EC_{50}		Maximal I_{ss}:I_p	
	R^2	*P*	R^2	*P*	R^2	*P*
Molecular Volume	0.196	>0.05	0.33	>0.05	0.07	>0.05
Hydrophilicity	0.173	>0.05	0.276	>0.05	0.05	>0.05
Polarity	0.079	>0.05	0.056	>0.05	0.07	>0.05

Table 5.1. Relation of NMDA receptor ion channel functions with the physicochemical parameters of the substituent at GluN2B(Phe637)

Relation of peak current glutamate EC_{50}, steady-state current EC_{50}, as well as maximal steady-state to peak current ratio, among various substitutions at GluN2B(Phe637)

In one previous study, our lab found that, by introducing mutated residues at GluN2A(Phe636), there was a strong correlation between peak and steady-state glutamate EC_{50}, and both of them were altered in a manner that was linked to desensitization (Ren *et al*., 2013). Moreover, one study at Met823 in the M4 domain of the GluN2A subunit demonstrated that for a series of substitutions at this position, changes in steady-state glutamate EC_{50} were attributed to changes in desensitization (Ren *et al*., 2003a). In this study, I observed that the peak current glutamate EC_{50} values were

strongly correlated with steady-state current glutamate EC_{50} values ($R^2 = 0.95$, $P < 0.0001$; figure 5.8). However, when I plotted maximal I_{ss}:I_p ratios against EC_{50} values of glutamate- activated peak and steady-state current, neither of them showed significant correlation with apparent desensitization ($R^2 = 0.01$, $P > 0.05$ for peak and $R^2 = 0.008$, $P > 0.05$ for steady-state; Fig 5.9).

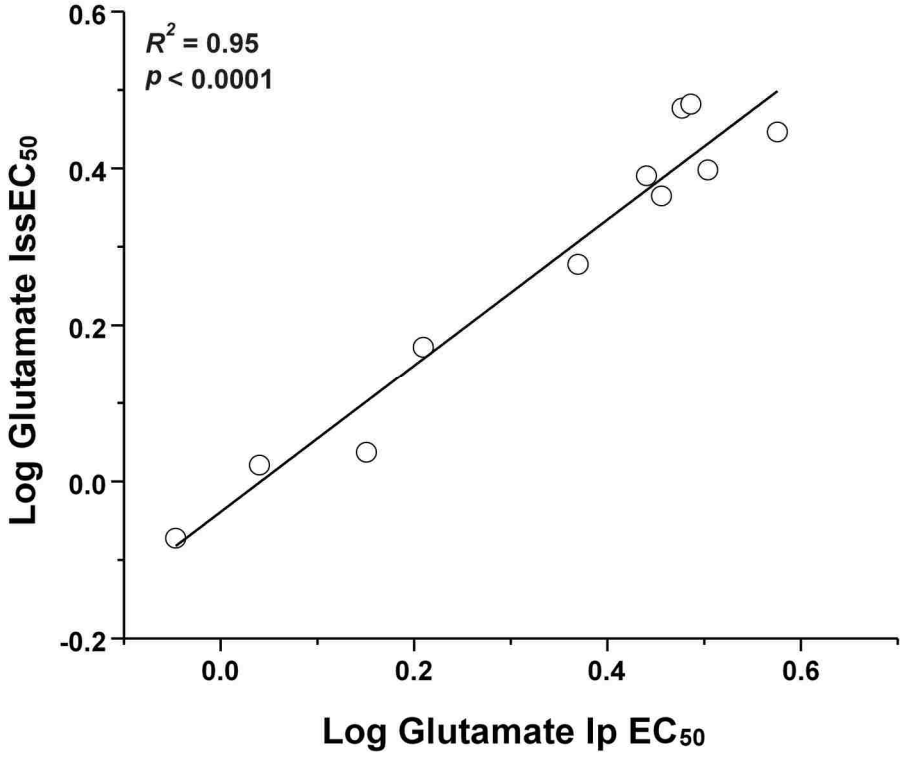

Figure 5.8. Relationship between I_p EC_{50} and I_{ss} EC_{50}

Graph plots values of glutamate log EC_{50} values for steady-state current *versus* peak current in a series of GluN2B mutants. Glutamate EC_{50} values for peak and steady-state current are significantly correlated ($R^2 = 0.95$, $P < 0.0001$). The line shown is the least squares fits to the data.

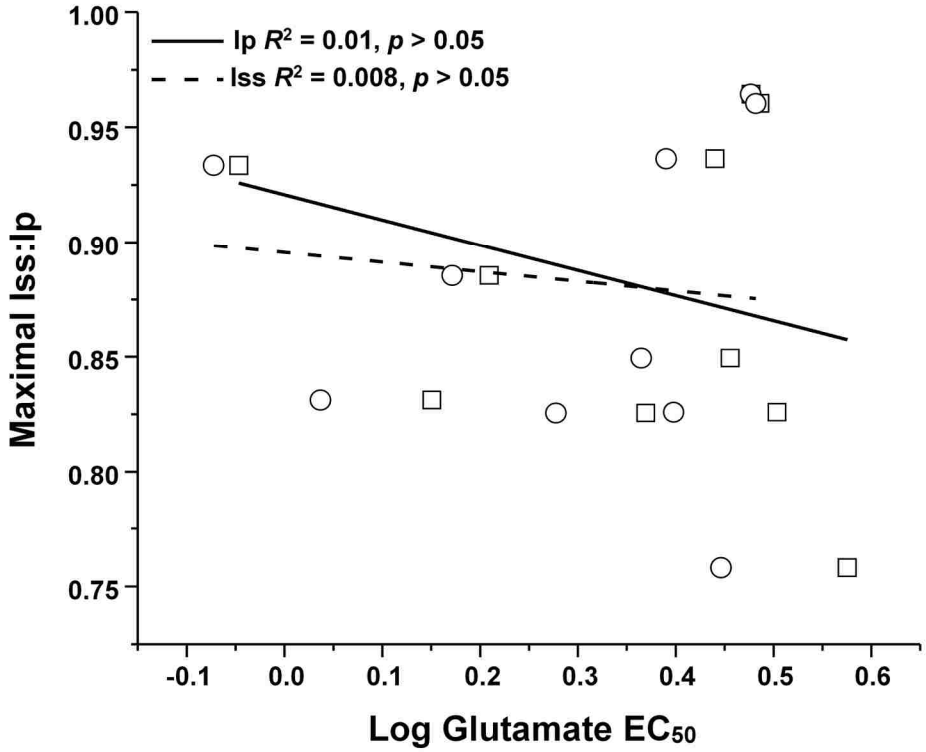

Figure 5.9. Relationship between apparent desensitization and either I_p EC_{50} or I_{ss} EC_{50}

Graph plots log maximal steady-state to peak current ratio (I_{ss}:I_p) *versus* either glutamate peak current log EC_{50} values or glutamate steady-state current log EC_{50} values from various GluN1/GluN2B(F637) mutant receptors. Log maximal I_{ss}:I_p for glutamate was not correlated with either of them (p > 0.05). The lines shown are the least squares fits to the data. Open circles show values from glutamate peak current and open squares show values from glutamate steady-state current.

GluN2B(F637) mutant ethanol sensitivity and receptor function

Since mutagenesis at GluN2B(Phe637) altered both ethanol sensitivity and NMDA receptor function, I asked if observed changes in ethanol sensitivity among mutants result from changes in agonist potency or ion channel gating kinetics. By plotting ethanol IC_{50} values against glutamate peak current, steady-state current glutamate EC_{50}

values or maximal steady-state to peak current ratios, I noticed that ethanol sensitivity of mutants was significantly negatively correlated with both peak ($R^2 = 0.78$, $p < 0.001$) and steady-state current EC_{50} ($R^2 = 0.63$, $p < 0.01$) (Figure 5.10). As expected, ethanol IC_{50} was not correlated with maximal I_{ss}:I_p ($p > 0.05$; figure 5.11), since glutamate EC_{50} values were not correlated with maximal I_{ss}:I_p ($p > 0.05$; figure 5.9).

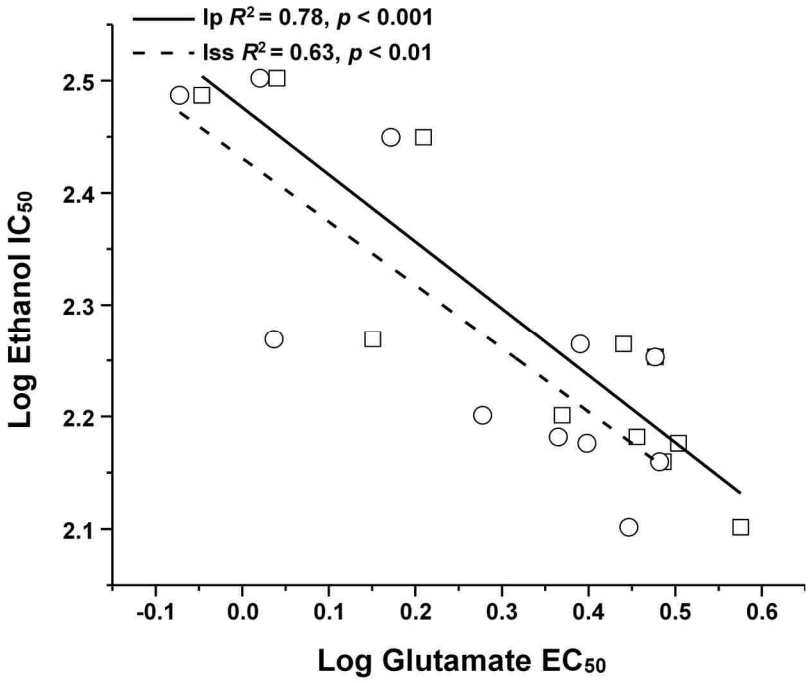

Figure 5.10. Relationship between ethanol sensitivity and glutamate potency

Graph plots log ethanol IC_{50} values in a series of mutants *versus* log values of glutamate EC_{50} for peak or steady-state current. Ethanol IC_{50} values are correlated with glutamate EC_{50} for peak ($R^2 = 0.78$, $p < 0.001$) and steady-state current ($R^2 = 0.63$, $p < 0.01$). The lines shown are the least squares fits to the data. Open circles show values from glutamate peak current and open squares show values from glutamate steady-state current.

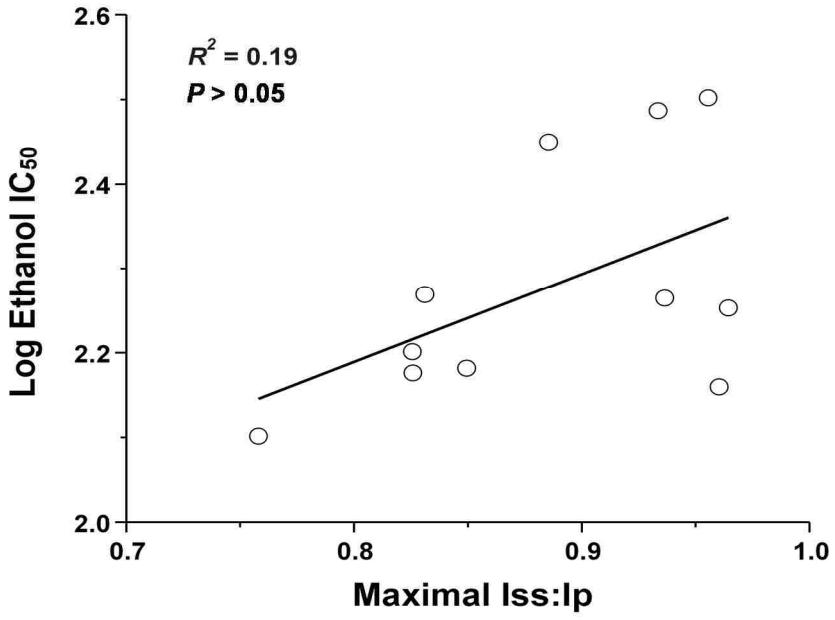

Figure 5.11. Relationship between ethanol sensitivity and apparent desensitization

Graph plots log ethanol IC_{50} values in a series of mutants versus the log I_{ss}:I_p values. Ethanol IC_{50} values are not correlated with I_{ss}:I_p values ($R^2 = 0.19$, $p > 0.05$). The line shown is the least squares fits to the data.

Glutamate EC_{50} and maximal steady-state current to peak current values of GluN2B(Phe637) mutants are correlated with those of GluN2A(Phe636) mutants

Since amino acid substitutions altered channel ethanol sensitivity in the same manner between two cognate positions, GluN2A(Phe636) and GluN2B(Phe637), it is possible that these two positions could regulate ion channel function in a similar manner. In order to study this possibility, I plotted glutamate Ip EC_{50}, glutamate I_{ss} EC_{50}, as well as maximal I_{ss}:I_p values, of various GluN2B(Phe637) mutants against values of each parameter of the same substitutions at GluN2A(Phe636), and noticed that their peak current EC_{50} ($R^2 = 0.84$, $P < 0.0001$) and steady-state current EC_{50} changes ($R^2 = 0.76$, P

< 0.001) (Fig. 5.12) are also highly correlated among various substitutions. When comparing their apparent desensitization changes among mutants, we found that they are also correlated with each other ($R^2 = 0.49$, $P < 0.05$; Fig. 5.13).

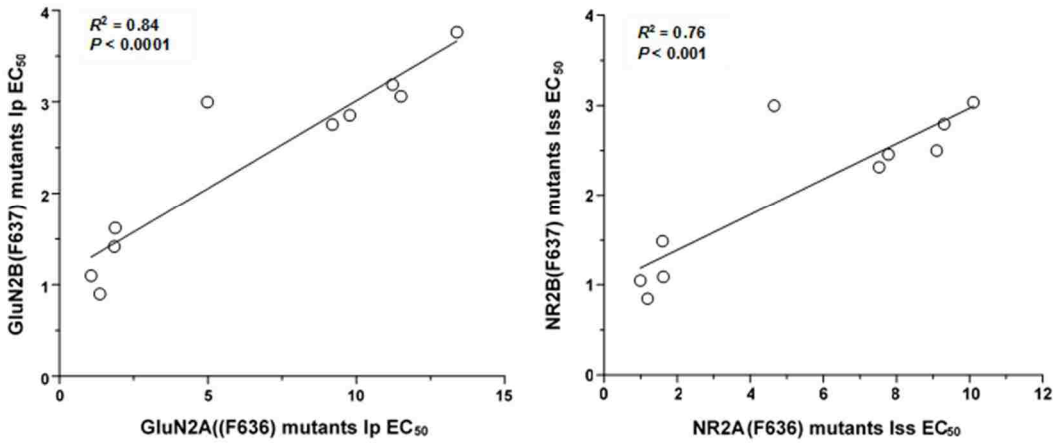

Figure 5.12. Glutamate EC_{50} values of GluN2B(Phe637) mutants are correlated with those of the same amino acid substitutions at GluN2A(Phe636)

Graphs plot log values of I_p EC_{50} (*left*) or I_{ss} EC_{50} (*right*) of GluN2B(Phe637) mutants *versus* each of these two parameters of GluN2A(Phe636) mutants. Both of them are significantly correlated ($R^2 = 0.84$, $P < 0.0001$ and $R^2 = 0.76$, $P < 0.001$). The lines shown are the least squares fits to the data. Glutamate EC_{50} values of GluN2A(Phe636) mutants are from a previous study in our laboratory (Ren *et al*., 2013).

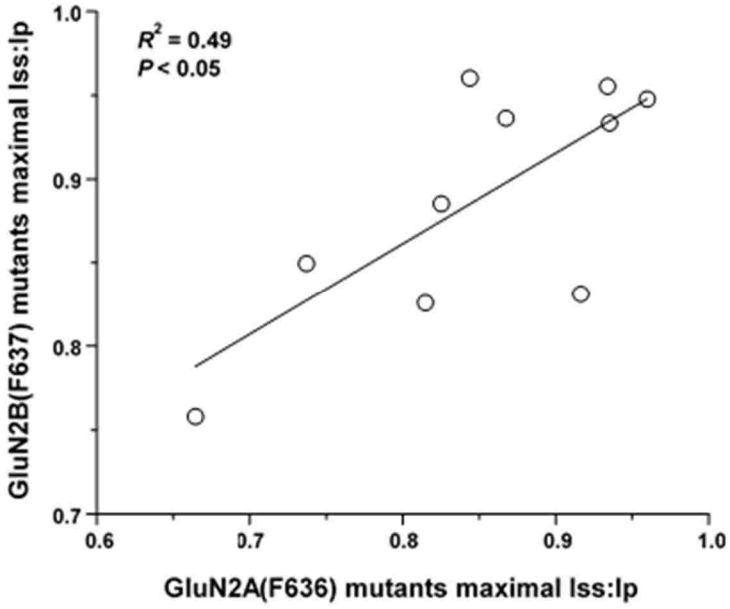

Figure 5.13. Steady-state to peak current ratios of GluN2B(Phe637) mutants are correlated with those of the same amino acid substitutions at GluN2A(Phe636)

Graph plots maximal steady-state to peak current ratio (I_{ss}:I_p) of GluN2B(Phe637) *versus* I_{ss}:I_p values of GluN2A(Phe636) among various mutant receptors. Maximal I_{ss}:I_p for glutamate of GluN2B(Phe636) mutants were also correlated with apparent desensitization values of its cognate position, GluN2A(Phe636) ($R^2 = 0.49$, $P < 0.05$). The line shown is the least squares fit to the data. Maximal I_{ss}:I_p values of GluN2A(Phe636) mutants are from a previous study in our laboratory (Ren *et al*., 2013).

Discussion

At the four positions in the GluN2B subunit tested in this study, both tryptophan and alanine mutagenesis at Phe637 showed significantly decreased ethanol sensitivity. Subsequent glutamate concentration-response experiments demonstrated that tryptophan mutagenesis at Phe637 also showed altered glutamate EC_{50} values for glutamate-

activated peak and steady-state current. Moreover, this tryptophan mutant also showed increased deactivation tau, and both tryptophan and alanine mutants showed decreased apparent desensitization compared with the wild-type receptor. I further studied the role of Phe637 on channel gating by constructing a panel of amino acid substitutions at this position. All mutants constructed at GluN2B(Phe637) showed decreased peak glutamate EC_{50} values and increased maximal steady-state to peak current ratios (I_{ss}:I_p), and only three of them showed significantly decreased steady-state glutamate EC_{50} values. Since Phe637 is in the M3 domain, which has an important role in NMDA receptor ion channel gating, and is at a considerable distance from ligand binding domain (Low *et al*., 2003; Yuan *et al*., 2005; Sobolevsky *et al*., 2007; Sobolevsky *et al*., 2009), the changes in glutamate EC_{50} values among mutants at this position could be a result of modifications in ion channel gating. Among all mutants, isoleucine and leucine were also different from each other with respect to glutamate EC_{50}, such that only isoleucine mutagenesis increased peak and steady-state glutamate EC_{50}. This indicates that not only ethanol sensitivity of the NMDA receptor, but also ion channel function, is sensitive to alterations in the side chain of amino acid at position 637 in the GluN2B subunit. A previous study on GluN2A(Met823) identified that EC_{50} values for glutamate-activated steady-state current in mutants were highly correlated with I_{ss}:I_p ratios, which can indicate apparent desensitization. The mechanism for the increased glutamate affinity in this case appeared to be increased dwell times of the receptor in one or more desensitized states, resulting in a greater degree of trapping of the agonist (Ren *et al*., 2003a). Among a series of amino acid substitutions at Phe636 in the GluN2A subunit, which is the cognate position of GluN2B(Phe637), I_{ss}:I_p ratios had an inverse relationship with peak glutamate EC_{50}

values, and a parallel trend with steady-state glutamate EC_{50} values (Ren *et al*., 2013). Results of the present study, however, appear to show different characteristics of GluN2B(Phe637) from those previously characterized ethanol-sensitive positions. There was a strong correlation between glutamate peak and steady-state EC_{50} values, but neither EC_{50} values for glutamate-activated peak nor steady-state current were correlated with maximal I_{ss}:I_p ratios, indicating that agonist potency can be influenced by the substituent at this position in a manner that is independent of changes in desensitization. The mechanism for this is still unclear at this time, but could be explained by the long-distance modulation of agonist-binding domain resulted from the substitutions at this present position. Thus, although GluN2A(Phe636) and GluN2B(Phe637) are cognate positions, and both affect ethanol sensitivity, their actions on ion channel function differ. Precise ion channel kinetic differences between them can be addressed by single-channel recording studies and detailed kinetic modeling. By performing single-channel recording in outside-out patches at very low glutamate concentrations (but a saturating concentration of glycine), effects of mutations at GluN2B(Phe637) on ion channel gating can be fully characterized.

It has been shown that ethanol inhibits NMDA receptors by altering ion channel gating, primarily by decreasing mean open time (Wright *et al*., 1997; Lima-Landman and Albuquerque, 1989). Previous studies in this laboratory showed that ethanol sensitivity of the NMDA receptor was inversely correlated with glutamate EC_{50}. In these studies, we observed that mutations at GluN2A(Phe636) and GluN2A(Phe637) which had higher agonist potency yielded lower ethanol sensitivity (Ren *et al*., 2007; Ren *et al*., 2013). Similar inverse correlations between ethanol sensitivity and glutamate peak or steady-

state EC_{50} were observed in this current study at GluN2B(Phe637), and a correlation between ethanol sensitivity and apparent desensitization among mutants was also observed at this position. Studies in several ligand-gated ion channels showed that ethanol could influence desensitization in these ion channels (Moykkynen *et al*., 2009; Dopico and Lovinger, 2009). Because ethanol-sensitive positions in different subunits of the NMDA receptor are also important for ion channel function, based on the correlations between ethanol sensitivity and ion channel gating characterized from studies about GluN2B(Phe637), the possibility was raised that changes in ethanol sensitivity are secondary to alterations of ion channel functions in GluN2B subunit-containing NMDA receptors. Previous findings initially suggested that ethanol inhibition of NMDA receptor does not involve changes in desensitization (Peoples *et al*., 1997; Woodward, 2000; Ren *et al*., 2003b). Furthermore, single-channel recording results involving three ethanol-sensitive positions in the GluN2A subunit, Phe637, Met823, or Ala825, revealed that tryptophan substitution mutations differentially affect ethanol sensitivity and mean open time. Although tryptophan substitution at all three positions showed significantly increased ethanol IC_{50} values, mean open time was decreased in the Phe637Trp mutant, increased in the Met823Trp mutant, and unchanged in Ala825Trp mutant. Thus, effects of mutations on mean open time in positions in the GluN2A subunit are not sufficient to explain decreased ethanol sensitivity. In general, these studies cannot support the possibility that changes in ethanol sensitivity are secondary to changes in open-time of the ion channel. As a first step to study this possibility in the GluN2B subunit-containing NMDA receptor, cells expressing GluN1/GluN2B(F637W) subunits were used for performing ethanol concentration-response experiments. However, ethanol sensitivity

was not altered when ethanol was either pre-applied for 10 s before receptor was activated or when ethanol was applied during steady-state current. So far, it appears most likely that similar factors may influence both ion channel kinetics and ethanol sensitivity in parallel. I also recorded from outside-out patches by using the single-channel recording method to compare mutagenesis-induced changes in ethanol sensitivity and mean open time. However, it was difficult to get a large enough number of open events for subsequent analysis. A previous study showed that GluN2A-containing NMDA receptors have a higher open probability than GluN2B-containing NMDA receptors (Erreger *et al*., 2005). Further studies should be conducted to resolve this question, such as optimizing transfection efficiency of GluN2B-containing NMDA receptors.

We showed that the role of cognate positions in the GluN2A and GluN2B subunits in determining ethanol sensitivity differs. The position we characterized in this chapter, GluN2B(Phe637), showed significant correlations with its cognate position in the GluN2A subunit for values of ethanol IC_{50}, glutamate I_p and I_{ss} EC_{50}, and maximal I_{ss}:I_p. This suggests that there are both important similarities and differences in the action of ethanol on the GluN2A and GluN2B subunits, and in the contributions of ethanol-sensitive positions to ion channel gating.

VI. INTERACTIONS AMONG POSITIONS AT THE INTERSUBUNIT INTERFACES OF GLUN2B-CONTAINING NMDA RECEPTOR FORM SITES OF ETHANOL ACTION

Introduction

Previous studies in our and other laboratories have identified and characterized a number of amino acid positions in the GluN1 and GluN2A subunit membrane-associated (M) domains of the NMDA receptor that influence both gating and alcohol sensitivity of the ion channel. These studies indicate that ethanol action sites may be located in one or more membrane-associated (M) domains (Ronald *et al*., 2001; Ren *et al*., 2003a; Ren *et al*., 2003b; Honse *et al*., 2004; Ren *et al*., 2007). Besides the importance of a single position on the NMDA receptor ethanol sensitivity, an earlier study in our laboratory also found that two ethanol-sensitive positions, Phe637 and Met823 in the GluN2A subunit, could interact to regulate ethanol sensitivity, but do not appear to form a common site of ethanol action (Ren *et al*., 2008). Based upon the crystal structure of the GluA2 AMPA receptor, a putative model describing the arrangement of M domains of the NMDA receptor suggest the presence of unitary sites of alcohol action on the NMDA receptor. By using both two-way ANOVA and mutant cycle analysis methods, we found that there are four sites of alcohol action: two sites at the GluN1 M3/GluN2A M4 interfaces, and the other two at the GluN1 M4/GluN2A M3 interfaces (Ren *et al*., 2012).

Although the GluN2A subunit predominates in the mammalian brain, a number of studies suggest a major role for the GluN2B subunit in the action of alcohol in the brain and a therapeutic importance of GluN2B subunit for alcohol addiction (Chazot, 2004; Nagy, 2004; Boyce-Rustay and Holmes, 2005; Gogas, 2006; Kash *et al*., 2008).

However, information about the molecular mechanism of alcohol modulation of the GluN2B subunit is still limited. Recent studies from two laboratories presented the X-ray crystal structures of GluN1/GluN2B NMDA receptor. They characterized that the arrangement of M domain helices is the similar to that of the GluA2 AMPA receptor, following a 1-2-1-2 fashion (Lee *et al*., 2014; Karakas and Furukawa, 2014). So the orientation of M3 domains of one subunit type appear to face the M4 domain of the adjacent subunit of the other type, which also suggests the interactions between M3 and M4 domain of different subunit types. Here, I tested for the specific ethanol-sensitive sites in GluN1/GluN2B NMDA receptor, which are composed of multiple amino acids in different subunits (Figure 6.1), as well tested some adjacent residues. In the present study, three pairs of positions in the GluN1/GluN2B NMDA receptor were identified, whose interactions could affect ethanol inhibition and ion channel functions. Based upon the results found previously that the GluN2B subunit contains different ethanol-sensitive positions compared with the GluN2A subunit, I also proposed here that the GluN1/GluN2B NMDA receptor shows different intersubunit interactions with respect to both ethanol sensitivity and ion channel kinetics, compared with the GluN2A-containing NMDA receptor, although they do share highly conserved M domains sequences (89% identity) (Ryan *et al*., 2013).

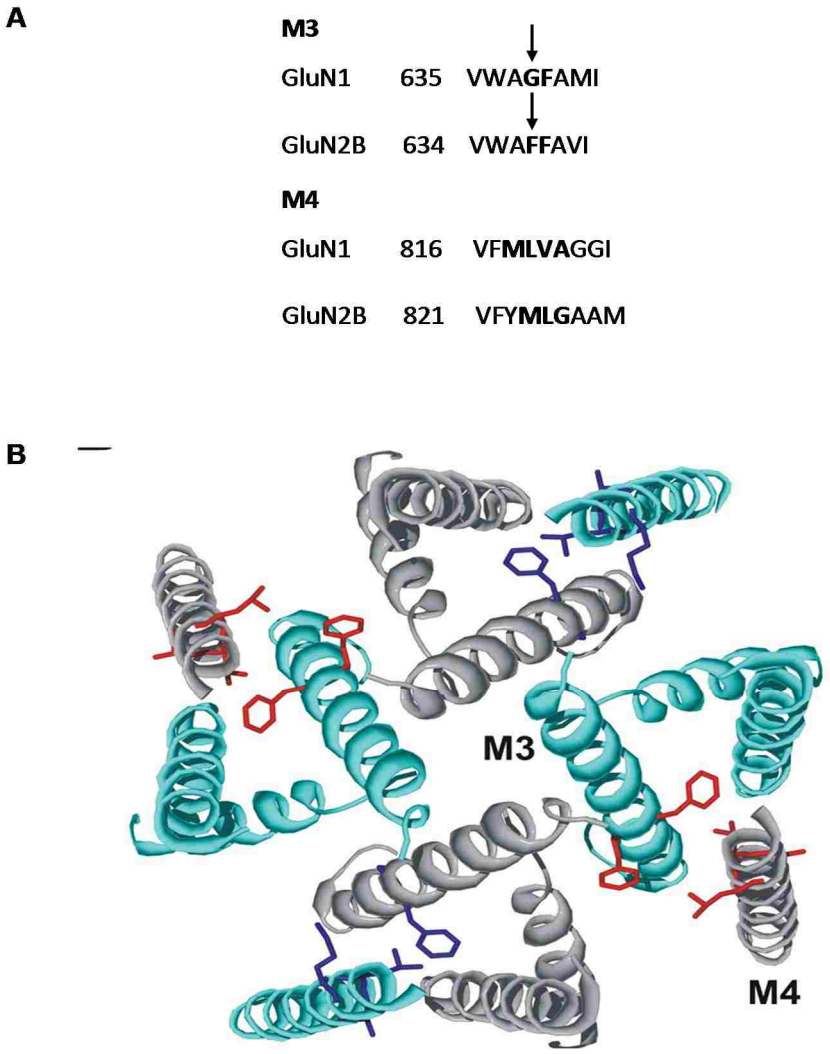

Figure 6.1. Putative interacting positions between M3 and M4 domains of adjacent subunit types

A, Alignment of M3 and M4 domain residues in GluN1 and GluN2B subunits. Alcohol-sensitive positions identified previously are noted by arrows; residues proposed in this study to contribute to interactions are denoted by bold lettering. Single letter abbreviations are used to indicate amino acids. *B*. Model of the NMDA receptor M domains from Sobolevsky *et al*., 2009. M domains of the GluN1 subunit are shown in gray and those of the GluN2B subunit are shown in cyan. Side-chains of the amino acids noted by bold letters in *A* are illustrated in red for the GluN1 M4/GluN2B M3 interface and in blue for the GluN1 M3/GluN2B M4 interface.

Results

Single mutations in the M3 and M4 domains of GluN1 and GluN2B subunits alter ethanol inhibition of NMDA receptors.

Previous work in this laboratory identified significant interactions at four pairs of positions in the GluN1/GluN2A interfaces with respect to ethanol inhibition and receptor deactivation (Ren *et al*., 2012). In the current study, putative ethanol action sites in the M3-M4 intersubunit interfaces between GluN1 and GluN2B subunits were initially tested, and each of these sites is formed by group of 4-6 residues corresponding to interacting positions in the GluN1/GluN2A subunit described in Ren *et al*. (2012). One previous study described a residue in the M3 domain of the GluN1 subunit, Phe639, as an ethanol-sensitive position (Ronald *et al*., 2001). In this study, I tested possible interactions between this position and positions in the M4 domain of the GluN2B subunit. Since previous studies showed that tryptophan substitution has the greatest modulation of ion channel behavior without loss of normal channel function (Ren *et al*., 2003a; Ren *et al*., 2003b; Honse *et al*., 2004; Ren *et al*., 2007; Ren *et al*., 2008; Salous *et al*., 2009; Ren *et al*., 2012; Ren *et al*., 2013), I first tested whether single tryptophan mutagenesis at each residue of these putative interacting sites could alter ethanol sensitivity. All tryptophan substitution mutants tested in this study yielded functional NMDA receptors. Current recorded from GluN1(G638W) mutant showed altered ethanol inhibition and delayed deactivation compared with wild-type receptors (Figure 6.2). These mutants

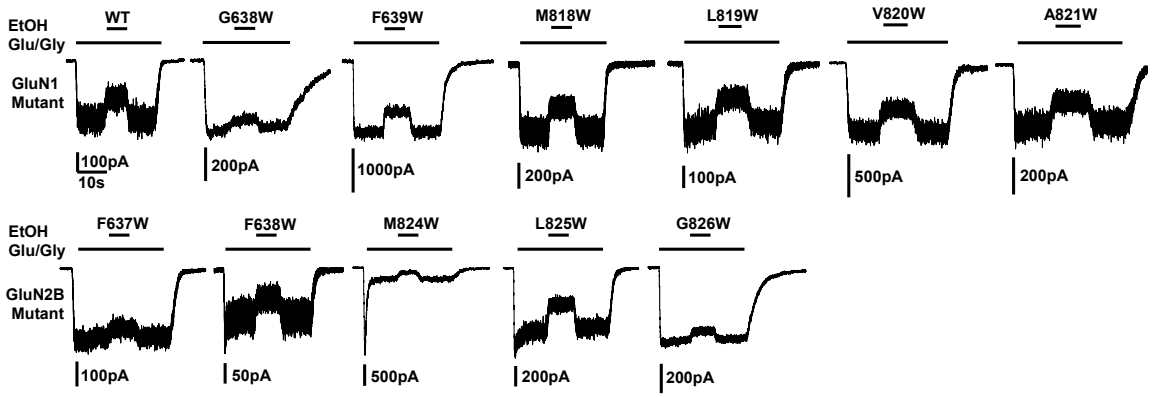

Figure 6.2. Ethanol inhibition traces of cells expressing tryptophan substitutions at various positions in GluN1 and GluN2B subunit

Traces are currents activated by 10 μM glutamate in the presence of 50 μM glycine and their inhibition by 100 mM ethanol in cells expressing various single tryptophan substitution mutations in GluN1 (*upper*) and GluN2B (*lower*) subunits. One-letter amino acid codes are used.

all showed concentration-dependent ethanol inhibition (Figure 6.3). Only GluN1(G638W) and GluN1(F639W) showed shifted concentration-response curves compared with the wild-type receptor, which indicates that they may exhibit altered ethanol IC_{50} values compared with wild-type. Bar graphs of ethanol IC_{50} values of the wild-type receptor and mutants showed that all tryptophan substitutions in the GluN1 subunit M3 domain exhibited significantly decreased ethanol sensitivity, while tryptophan substitution at any of the four positions in the GluN1 subunit M4 domain did not have any effect on receptor ethanol IC_{50} value (Figure 6.4). Among tryptophan substitutions in GluN2B subunit, only two of them exhibited significantly decreased ethanol sensitivity (Figure 3.5), which was shown previously in **Chapter III**.

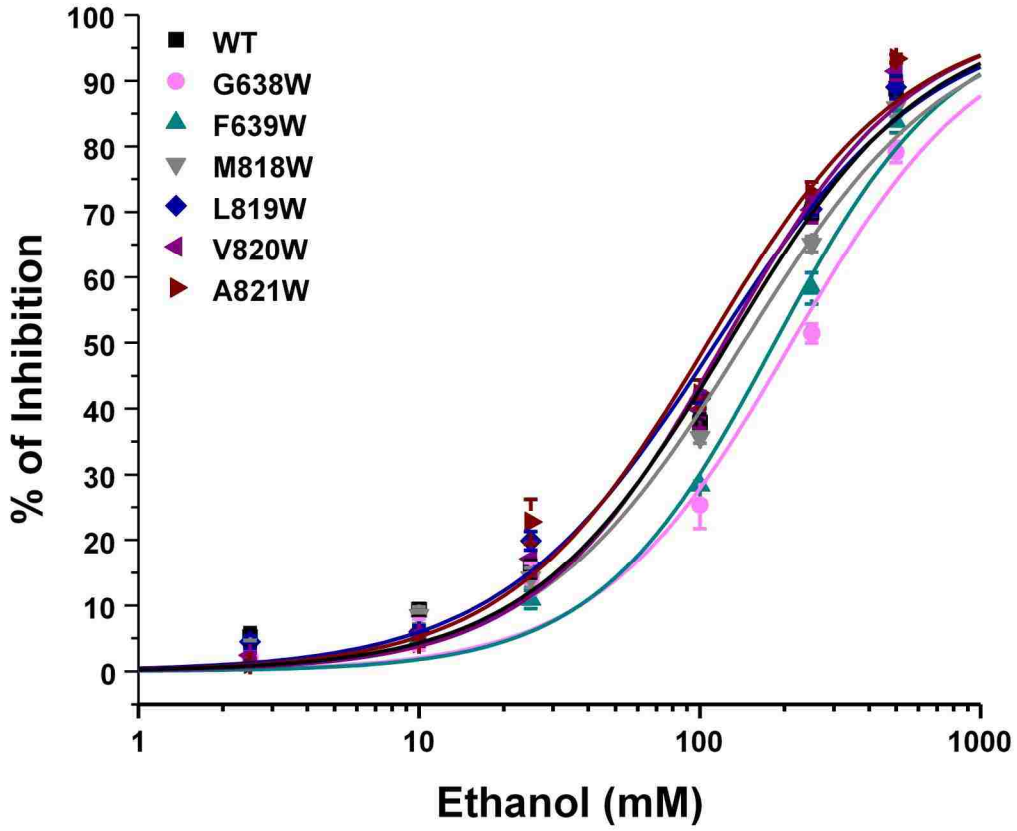

Figure 6.3. Ethanol concentration-response of various GluN1 mutants

Concentration-response curves for ethanol inhibition of glutamate-activated currents in the presence of 50 μM glycine in cells expressing various substitution mutations in GluN1 subunit. Data are the means ± S.E of 5-6 cells, error bars not visible were smaller than the size of symbols. The black curve shows the fit for the wild-type receptor. All curves are best fitted to the equation given under "Materials and Methods".

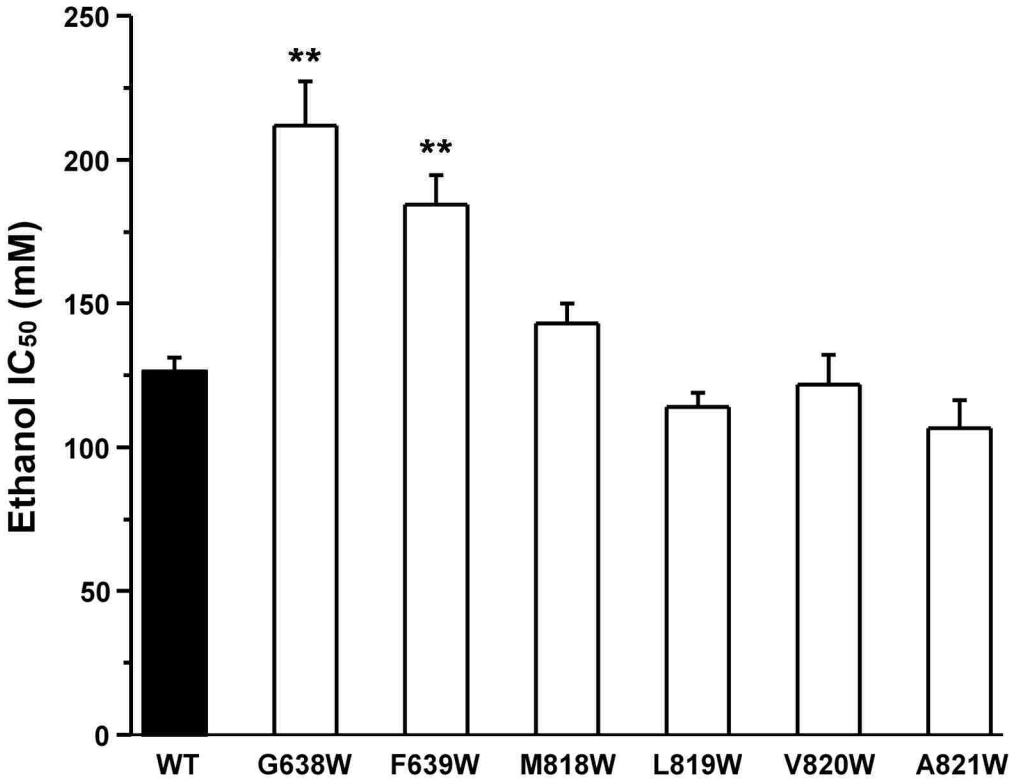

Figure 6.4. Ethanol IC_{50} of tryptophan substitutions at various positions in GluN1 subunit

Bar graphs show average IC_{50} values for ethanol in cells expressing either wild-type receptor or the various single mutants at M3 and M4 domains of GluN1 subunits. *Asterisks* indicate IC_{50} values that differed significantly from the IC_{50} value for wild-type GluN1/GluN2B subunits (**$P < 0.01$; ANOVA and Dunnett's test). Black bar shows average ethanol IC_{50} value for wild-type receptor. Results are means ± S.E. of 5-7 cells.

Dual mutations in the M3 domain of the GluN1 subunit and the M4 domain of the GluN2B subunit could alter ethanol inhibition of the NMDA receptors.

Next GluN1 and GluN2B tryptophan mutant subunits were co-transfected into tsA-201 cells, and I tested if the dual mutant could show any alteration of ethanol

inhibition. Mutant pairs used here correspond to interacting positions shown previously in the GluN1/GluN2A NMDA receptor (Ren *et al*., 2012). Currents recorded from cells expressing either wild-type or mutant receptors show that all mutant combinations tested formed functional NMDA receptors (Figure 6.5). It is noticeable that the current in the GluN1(G638W)/GluN2B(M824W) dual mutant showed obvious increased desensitization compared to that in the wild-type receptor. Two other dual mutants, GluN1(G638W)/GluN2B(L825W) as well as GluN1(F639W)/GluN2B(G826W), exhibited changes in current features, with the former showing altered deactivation and the latter exhibiting almost no inhibition by 100 mM ethanol. However, two single mutations of the latter pair, GluN1(F639W) and GluN2B(G826W), both showed evident ethanol inhibition as characterized in **Figure 6.2**, which indicates a possible interaction between these two positions.

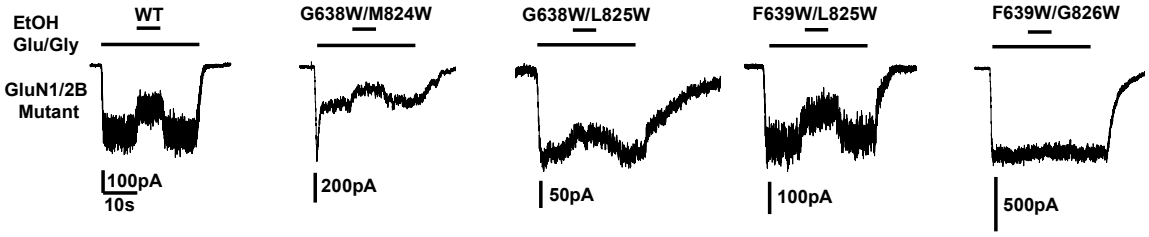

Figure 6.5. Ethanol inhibition traces of cells expressing tryptophan substitutions at dual positions in M3 of GluN1 and M4 of GluN2B subunit

Records are currents activated by 10 μM glutamate and 50 μM glycine with 100 mM ethanol inhibition in cells expressing dual mutations in the GluN1 M3 / GluN2B M4 domains. One-letter amino acid codes are used.

These dual mutants showed typical concentration-response curves for ethanol inhibition (Figure 6.6), and these curves are essentially parallel to each other, as the slope factors did not differ significantly. Among these dual mutants, two showed obvious shifted curves compared with the wild-type receptor. Statistical analysis of ethanol IC_{50} values of wild-type and mutant receptors revealed a significant decrease in ethanol inhibition of two mutants with shifted ethanol concentration-response curves (Figure 6.7).

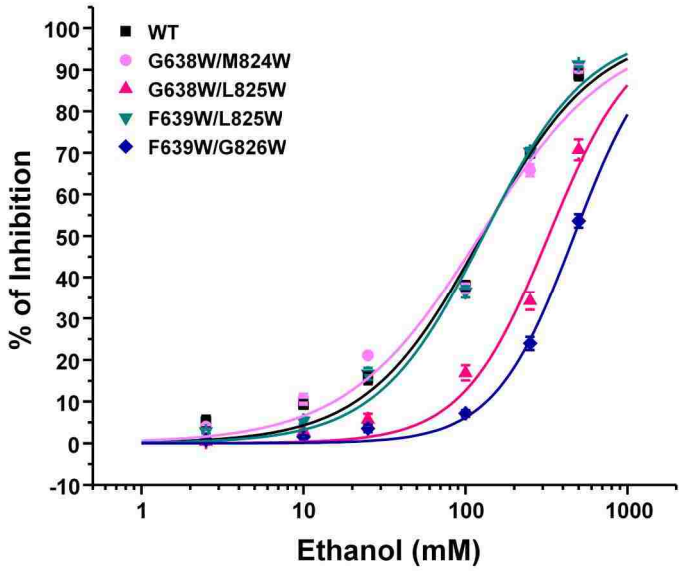

Figure 6.6. Ethanol concentration-response of dual tryptophan substitutions at M3 of GluN1 subunit and M4 of GluN2B subunit

Concentration-response curves for ethanol inhibition of glutamate-activated currents in the presence of 50 μM glycine in cells expressing various dual tryptophan substitution mutations in GluN1 M3 and GluN2B M4. Data are the means ± S.E of 6-7 cells, error bars not visible were smaller than the size of symbols. The black curve shows the fit for the wild-type receptor. All curves are best fitted to the equation given under "Materials and Methods".

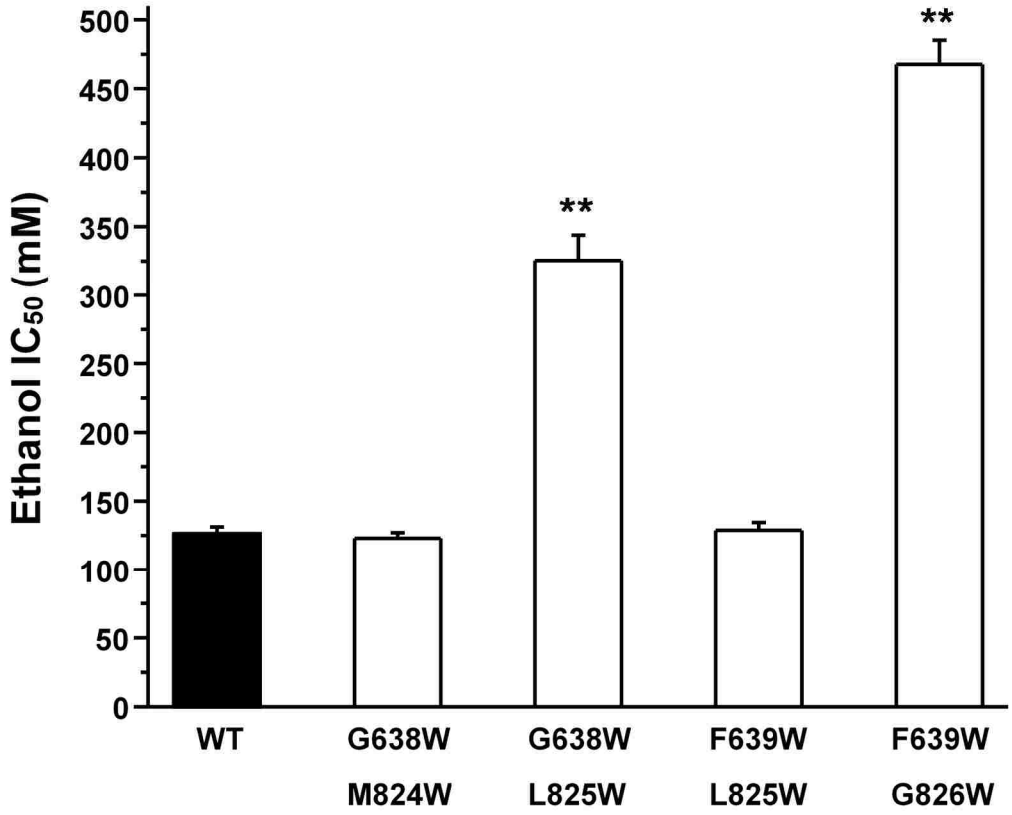

Figure 6.7. Ethanol IC_{50} of dual tryptophan substitutions at positions in the M3 of GluN1 subunit and M4 of GluN2B subunit

Graphs plot average IC_{50} values for ethanol in dual mutations in the GluN1 M3 / GluN2B M4. *Asterisks* indicate IC_{50} values that differed significantly from the IC_{50} value for wild-type GluN1/GluN2B subunits (**$P < 0.01$; ANOVA and Dunnett's test). Black bar shows average ethanol IC_{50} value for wild-type receptor. Results are means ± S.E. of 6-7 cells.

Dual mutations in the M3 of GluN2B and M4 of GluN1 subunit alter ethanol inhibition of NMDA receptors.

Following these results, the ethanol sensitivity pattern of mutants with dual tryptophan substitutions in the M3 domain of the GluN2B subunit and the M4 domain of

the GluN1 subunit were also studied. As in previously tested mutants, these mutants all formed functional receptors and exhibited typical ethanol inhibition of current upon activation by glutamate and glycine (Figure 6.8).

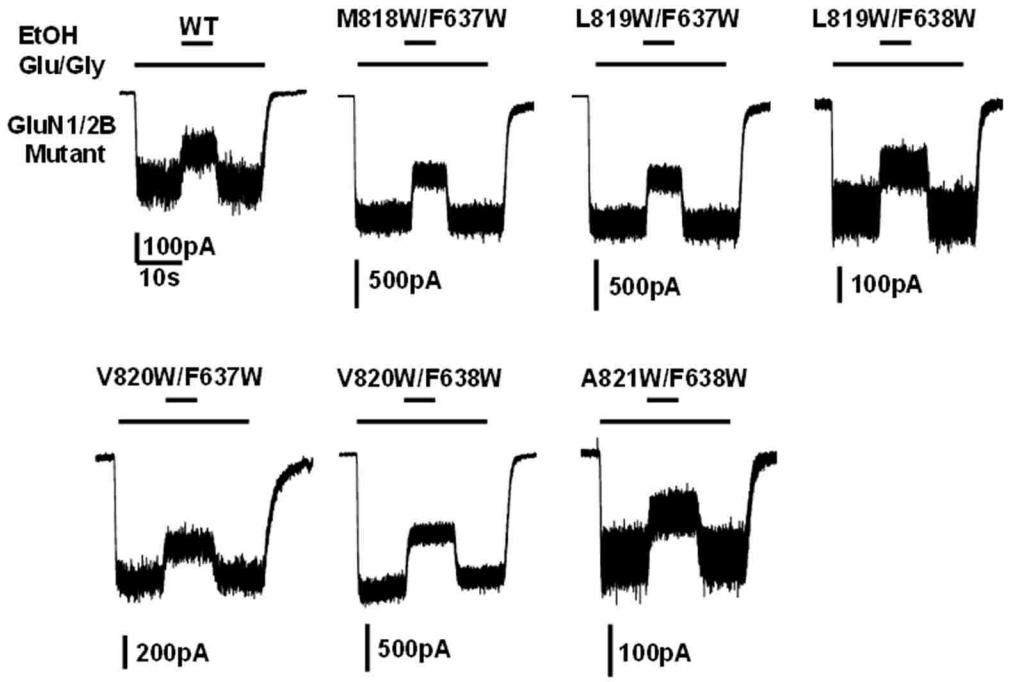

Figure 6.8. Ethanol inhibition traces of cells expressing tryptophan substitutions at dual positions in M3 of GluN2B and M4 of GluN1 subunit

Records are currents activated by 10 μM glutamate and 50 μM glycine with 100 mM ethanol inhibition in cells expressing dual mutations in the GluN2B M3 / GluN1 M4 domains. One-letter amino acid codes are used.

These mutant receptors also show typical ethanol concentration-response curves as other mutants tested in this thesis (Figure 6.9). Analysis of ethanol IC_{50} values showed that only two of six mutants exhibited significantly increased ethanol $IC_{50,}$ and both are GluN1 mutant subunits combined with the GluN2B(F637W) mutant (Figure 6.10).

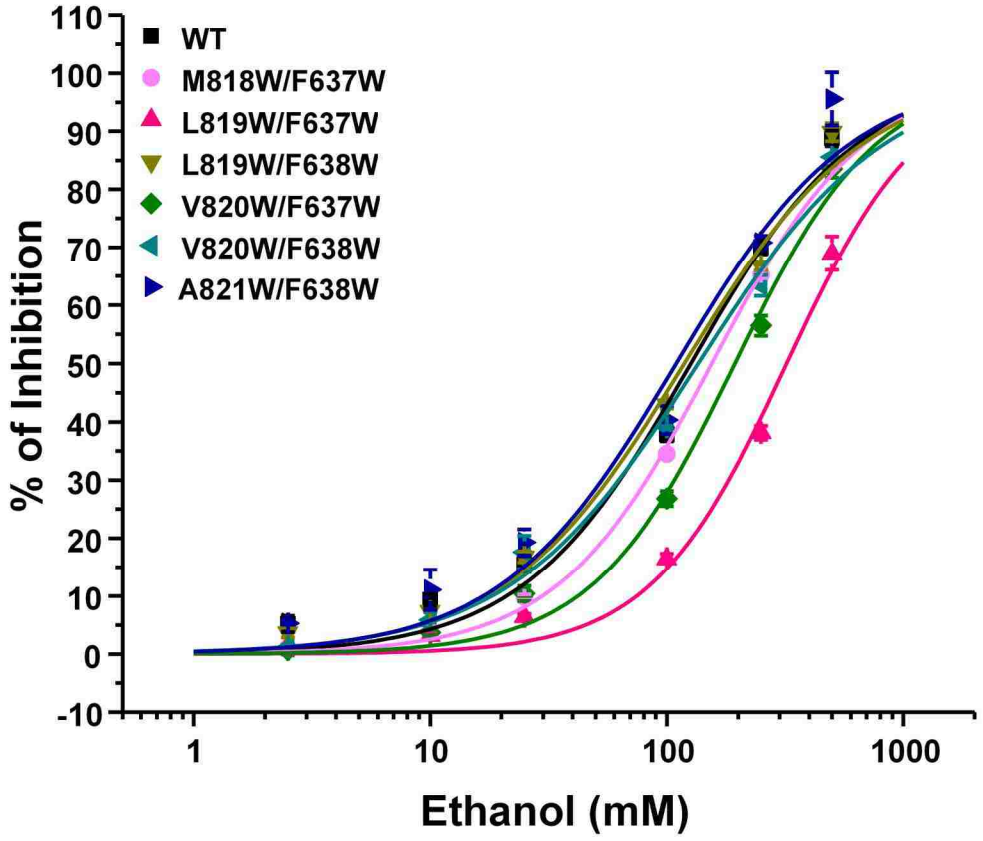

Figure 6.9. Ethanol concentration-response of dual tryptophan substitutions at M4 of GluN1 subunit and M3 of GluN2B subunit

Concentration-response curves for ethanol inhibition of glutamate-activated currents in the presence of 50 µM glycine in cells expressing various dual tryptophan substitution mutations in GluN1 M4 and GluN2B M3. Data are the means ± S.E of 4-7 cells, error bars not visible were smaller than the size of symbols. The black curve shows the fit for the wild-type receptor. All curves are best fitted to the equation given under "Materials and Methods".

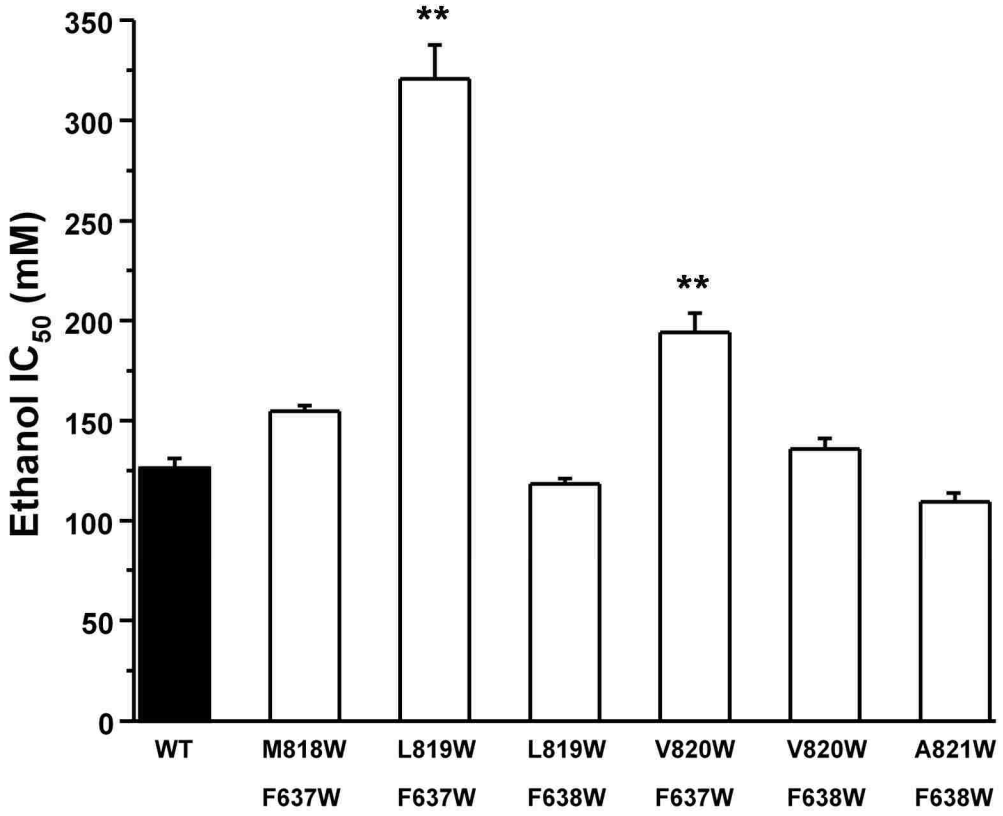

Figure 6.10. Ethanol IC_{50} of dual tryptophan substitutions at positions in the M4 of GluN1 subunit and M3 of GluN2B subunit

Graphs plot average IC_{50} values for ethanol in dual mutations in the GluN1 M4 / GluN2B M3. *Asterisks* indicate ethanol IC_{50} values that differed significantly from the IC_{50} value for wild-type GluN1/GluN2B subunits (**$P < 0.01$; ANOVA and Dunnett's test). Black bar shows average ethanol IC_{50} value for wild-type receptor. Results are means ± S.E. of 4-7 cells.

In some cases, compared with single mutagenesis in the M3 and M4 domains of either the GluN1 or GluN2B subunit, dual mutations influenced ethanol sensitivity in a manner that is non-additive, which indicates the possibility that amino acids at these

positions could interactively mediate the action of ethanol on the receptor (Figure 6.4, Figure 3.16, Figure 6.7 and 6.10).

Positions in the M3 and M4 domains of the GluN1 and GluN2B subunits interact to regulate ethanol inhibition of NMDA receptors.

In order to test possible interactions between positions characterized in the previous part, I used both two-way analysis of variance (ANOVA) on log-transformed ethanol IC_{50} values and mutant cycle analysis (characterized in the *Materials and Methods*). For the GluN1 M3 / GluN2B M4 mutant combinations, I found significant interactions between GluN1(Gly638) and GluN2B(Met824). As indicated in the figure on the right, the apparent interaction free energy $\Delta\Delta G_{INT}$ for mutations at two positions is the free energy difference between the parallel energies in this cycle (i.e., from the wild-type to either G638W or M824W, and from either single mutant to the dual mutant, G638W/M824W). Because non-interacting positions should have an apparent interaction free energy of zero, mean values of $\Delta\Delta G_{INT}$ ± S.E.M. were tested for statistically significant differences from zero energy using one sample *t* tests (Figure 6.11). I also found interactions between GluN1(Gly638) and GluN2B(Leu825), GluN1(Phe639) and GluN2B(Leu825), as well as GluN1(Phe639) and GluN2B(Gly826) with respect to ethanol sensitivity, as indicated by both types of analysis (Figure 6.12 and Table 6.1).

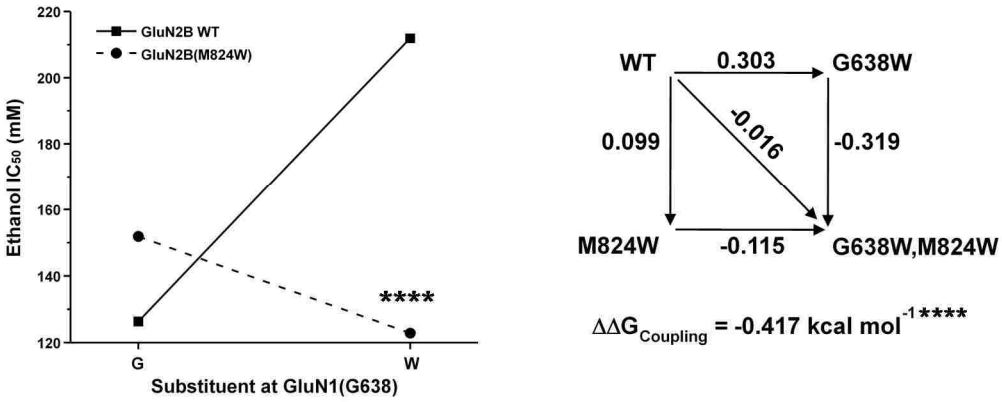

Figure 6.11. GluN1(Gly638) and GluN2B(Met824) interactively regulate NMDA receptor ethanol sensitivity

Graph (left) plots ethanol IC_{50} values *vs*. the tryptophan substitution at GluN1(Gly638) for tryptophan substitution at GluN2B(Met824), as indicated. A significant interaction detected using log-transformed IC_{50} values between two positions is indicated by asterisks (****$P < 0.0001$; two-way ANOVA). One-letter amino acid codes are used. Mutant cycle analysis of ethanol IC_{50} values (right) for the combination GluN1(Gly638)/GluN2B(Met824), which showed the most significant interaction with respect to ethanol sensitivity. Apparent free energy values associated with various mutations (ΔG_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 (****$P < 0.0001$).

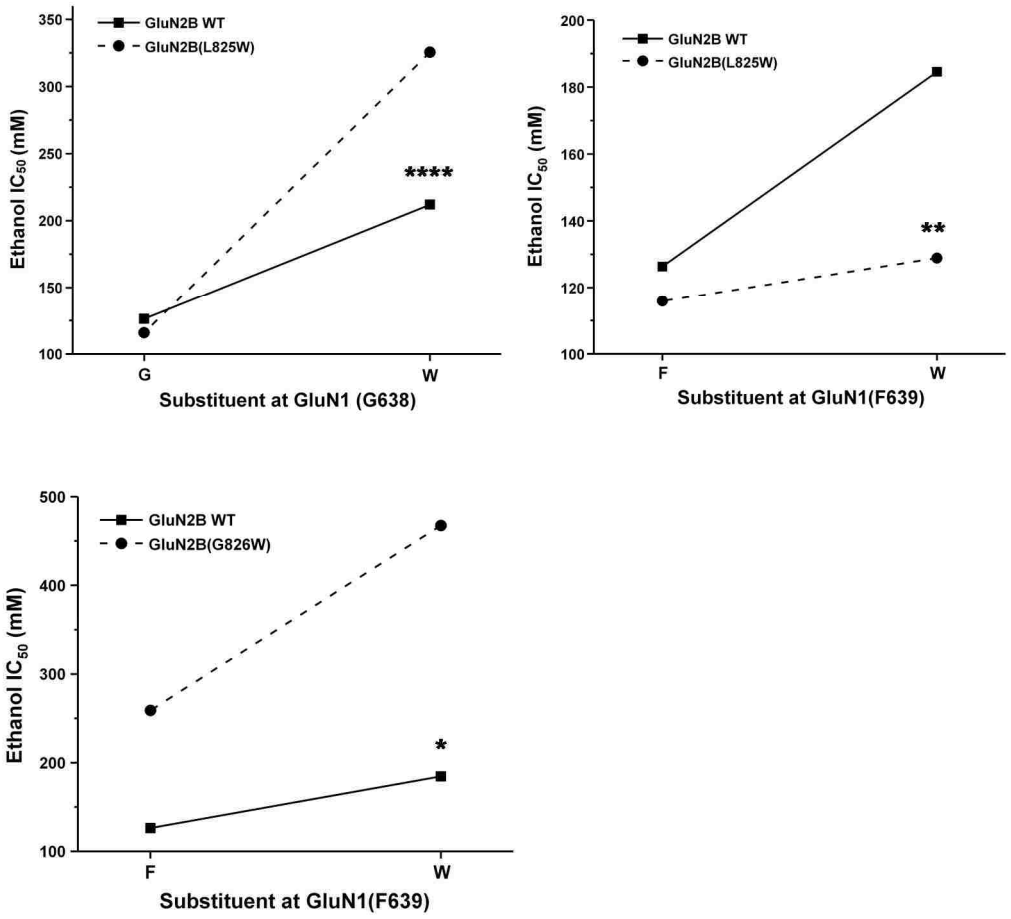

Figure 6.12. GluN1(Gly638) and GluN2B(Met824) interactively regulate NMDA receptor ethanol sensitivity

Graphs plot ethanol IC_{50} values *vs*. the substituent at GluN1(Gly638) or GluN1(Phe639) for mutants at GluN2B(Leu825) or GluN2B(Gly826), as indicated. Significant interactions detected using log-transformed IC_{50} values between positions are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$; two-way ANOVA). One-letter amino acid codes are used.

Mutant Pair (GluN1/GluN2B)	ΔG_1 WT→N1	ΔG_2 WT→N2B	ΔG_3 WT→N1/ N2B	ΔG_4 N1→N1/ N2B	ΔG_5 N2B→N1/ N2B	$\Delta\Delta G_{INT}$	df	Significance
G638W/M824W	0.303	0.0987	-0.0162	-0.319	-0.115	-0.417	20	P < 0.0001
G638W/L825W	0.303	-0.0511	0.558	0.255	0.609	0.307	19	P = 0.0001
F639W/L825W	0.222	-0.0511	0.0108	-0.212	0.0620	-0.160	21	P < 0.01
F639W/G826W	0.222	0.422	0.775	0.552	0.353	0.130	21	P < 0.05
M818W/F637W	0.0722	0.543	0.122	0.0503	-0.420	-0.493	21	P < 0.0001
L819W/F637W	-0.0605	0.543	0.551	0.611	0.00792	0.0684	20	P > 0.05
V820W/F637W	-0.0275	0.543	0.253	0.280	-0.290	-0.262	20	P < 0.005
L819W/F638W	-0.0605	0.0112	-0.0352	0.0253	-0.0471	0.0134	16	P > 0.05
V820W/F638W	-0.0275	0.0119	0.0667	0.0941	0.0548	0.0823	14	P > 0.05
A821W/F638W	-0.177	0.0119	-0.0829	0.0942	-0.0947	0.0823	14	P > 0.05

Table 6.1. Mutant cycle analysis of ethanol concentration-response

Values of ΔG_X in kcal mol^{-1} are RT [ln(R1 IC_{50} – ln(R2 IC_{50})], where R1 and R2 refer to the NMDA receptor subunit combinations on the left and right sides, respectively, of the column headings (WT, wild-type; N1, GluN1 mutant/GluN2B wild-type; N2, GluN1 wide-type/GluN2B mutant; N1/N2B, GluN1 mutant/GluN2B mutant). Values of apparent free energy $\Delta\Delta G_{INT}$ in kcal mol^{-1} are means ± S.E.M. $\Delta\Delta G_{INT}$. Degrees of freedom (df) and S.E.M were determined as described in *Materials and Methods*. Statistical significance of $\Delta\Delta G_{INT}$ values were determined by using one sample *t* tests.

Of the GluN1 M4 / GluN2B M3 mutant combinations, I tested interactions of three positions in GluN1 subunit with GluN2B(Phe637). Interestingly, I found significant interactions between GluN1(Met818) and GluN2B(Phe637), GluN1(Val820) and

GluN2B(Phe637), but not between GluN1(Leu819) and GluN2B(Phe637) (Figure 6.13; Table 6.1).

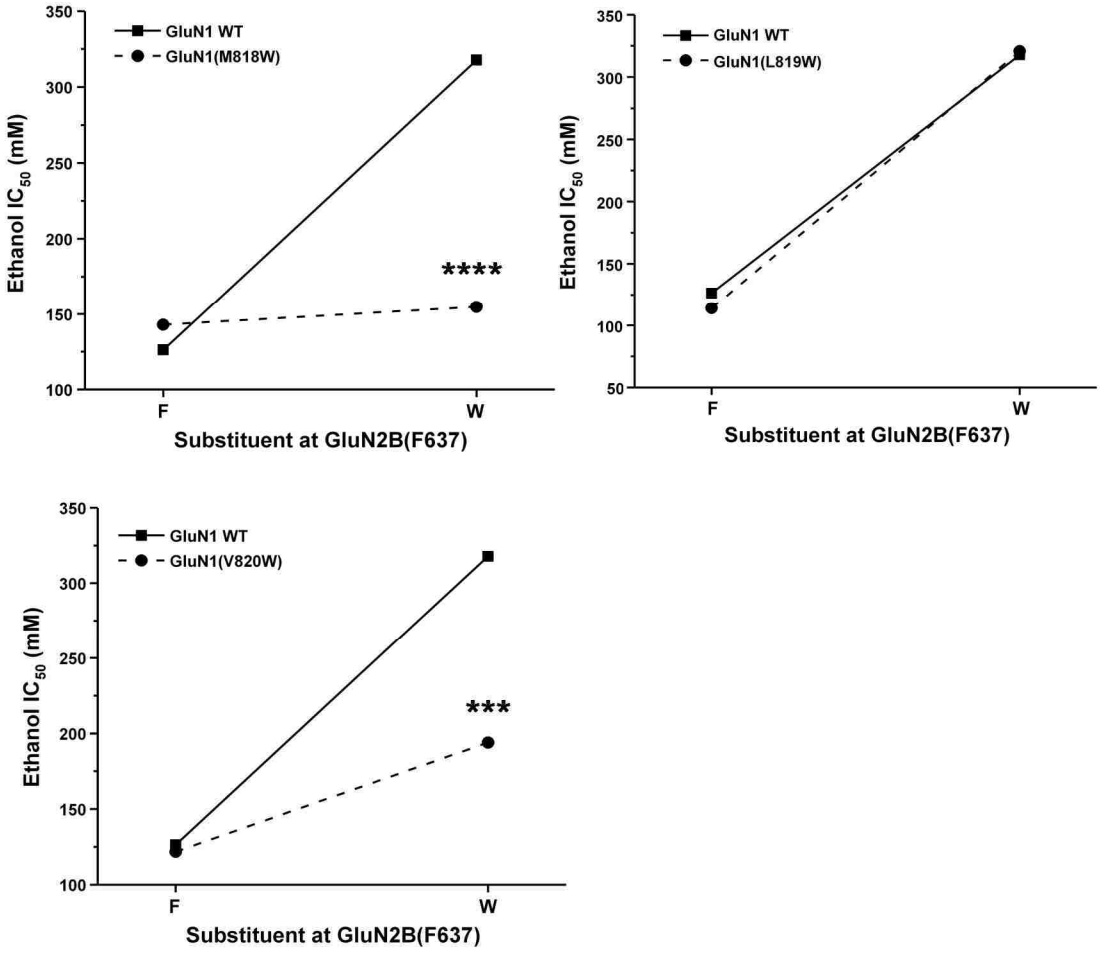

Figure 6.13. GluN2B(Phe637) interacts with positions in the M4 domain of GluN1 subunit to regulate NMDA receptor ethanol sensitivity

Graphs plot ethanol IC_{50} values *vs*. the tryptophan substitution at GluN2B(Phe637) for mutants at GluN1 positions 818, 819 and 820, as indicated. Significant interactions detected by log-transformed IC_{50} values are indicated by asterisks (***$P < 0.005$, ****$P < 0.0001$; two-way ANOVA).

Interactions between three positions in the GluN1 subunit with GluN2B(Phe638) were tested. Both types of analysis indicated that there are no significant interactions between GluN2B (Phe638) and three positions at the GluN1 subunit tested in this study (Figure 6.14; Table 6.1). However, the cognate position of GluN2B (Phe638) in the GluN2A subunit, Phe637, expressed a strong interaction with GluN1 (Leu819) in a previous study in our laboratory (Ren *et al*., 2012).

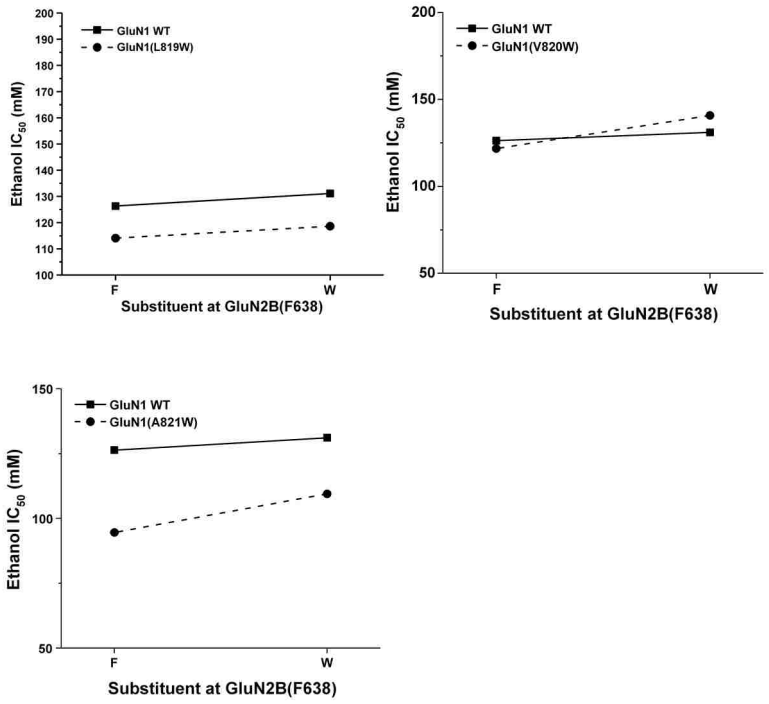

Figure 6.14. GluN2B(Phe638) does not show interaction with positions in the M4 domain of GluN1 subunit with respect to ethanol sensitivity

Graphs plot ethanol IC_{50} values *vs*. the tryptophan substitution at GluN2B(Phe638) for mutants at GluN1 positions 819, 820 and 821, as indicated. There is no significant interaction detected by log-transformed IC_{50} values among these three pairs of residues.

Two sites of ethanol action are formed by residues in the GluN1 and GluN2B subunit.

Results of studies on putative ethanol-sensitive positions in the M3 and M4 domain of the GluN1 and GluN2B subunit, taken together with functional interactions between these positions with respect to ethanol action, point to two types of sites of ethanol action: two sites at the GluN1 M3/GluN2B M4 interfaces and the other two at the GluN1 M4/GluN2B M3 interfaces. The former type is composed of five amino acids, however, the latter one is only composed of three amino acids (Figure 6.15).

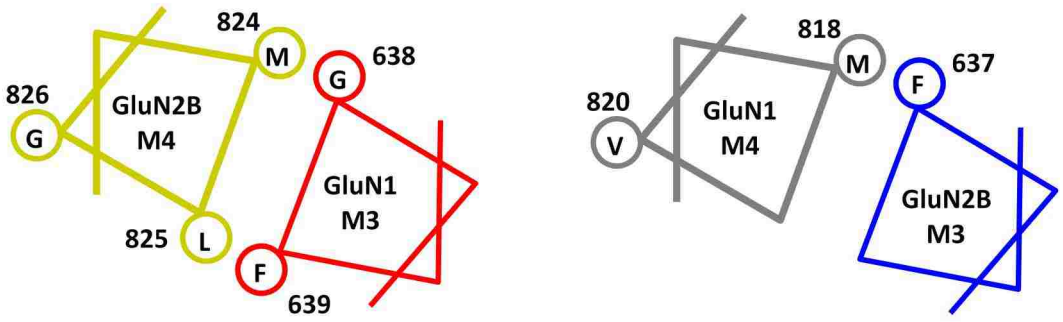

Figure 6.15. Helical wheel plots of the ethanol sites formed by residues in different subunits

Helical wheel plots of the ethanol sites formed by the GluN1 subunit M3 domain (*red*) and GluN2B subunit M4 domain (*yellow*), and formed by the GluN1 subunit M4 domain (*grey*) and GluN2B subunit M3 domain (*blue*).

Positions in the M3 and M4 domains of the GluN1 and GluN2B subunits interactively regulate glutamate potency.

Previous studies showed that ethanol inhibits NMDA receptors primarily by decreasing mean open time of the ion channel, and studies from our laboratory reported that mutations at ethanol-sensitive positions in the NMDA receptor strongly influenced ion channel gating kinetics (Ronald *et al*., 2001; Ren *et al*., 2003a; Ren *et al*., 2003b; Smothers and Woodward, 2006; Ren *et al*., 2007; Ren *et al*., 2013). Studies from our laboratory also showed that interacting positions in different subunits with respect to ethanol sensitivity also exhibited interactive regulation of desensitization and deactivation (Ren *et al*., 2008; Ren *et al*., 2012). In this study, I tested three pairs of residues, which expressed significant interactions on ethanol action, to see if they could interact with each other to regulate glutamate potency. In addition, another pair of positions, GluN1(Leu819)/GluN2B(Phe638), was also studied. Although they did not show interaction with respect to ethanol sensitivity, it is not impossible that their effect on ethanol action and channel function differ.

Among these combinations, I tested interactions with respect to effects on both glutamate peak current and steady-state current EC_{50} by using two-way analysis of variance on either peak EC_{50} values or steady-state EC_{50} values as well as mutant cycle analysis. Results of these studies showed that the GluN1(M818W) mutant and GluN2B(F637W) mutant showed significantly altered Ip EC_{50}. However, dual mutations did not cause any alteration of glutamate Ip EC_{50} values. This indicates that there might be an interaction between these two positions with respect to Ip EC_{50} (Figure 6.16). By using both two-way ANOVA as well as the mutant cycle analysis method, significant

interactions with respect to glutamate peak current EC_{50} between GluN1(Gly638) and GluN2B(Met824), GluN1(Phe639) and GluN2B(Leu825), as well as GluN1(Met818) and GluN2B(Phe637), were observed (Table 6.2). Two of these also showed significant interaction with respect to steady-state glutamate EC_{50} (Table 6.3).

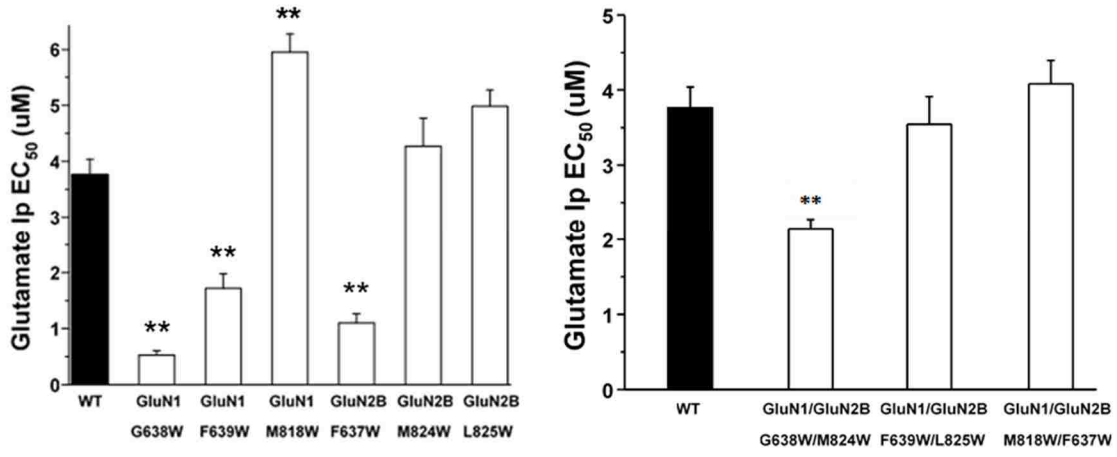

Figure 6.16. Glutamate peak current EC_{50} of either single substitution or dual substitution mutants

Bar graphs show the average EC_{50} values for glutamate activated peak currents recorded from cells expressing either wild-type, single tryptophan mutant receptors (upper) or dual tryptophan mutant receptors (lower). Asterisks indicate EC_{50} values that differ significantly from that of the wild-type GluN1/GluN2B subunit (**$P < 0.01$; ANOVA and Dunnett's test). Results are the means ± S.E of 5-8 cells. The EC_{50} value for the wild-type receptor is shown by the black bar.

Mutant Pair (GluN1/GluN2B)	ΔG_1 WT→N1	ΔG_2 WT→N2B	ΔG_3 WT→N1/ N2B	ΔG_4 N1→N1/ N2B	ΔG_5 N2B→N1/ N2B	$\Delta\Delta G_{INT}$	df	Significance
G638W/M824W	-1.18	0.0540	-0.326	0.853	-0.380	0.799	20	P < 0.0001
F639W/L825W	-0.486	0.170	-0.0457	0.440	-0.216	0.270	20	P < 0.05
M818W/F637W	0.276	-0.769	0.0468	-0.230	0.816	0.540	21	P < 0.001
L819W/F638W	0.000881	0.131	0.241	0.240	0.110	0.109	17	P > 0.05

Table 6.2. Mutant cycle analysis of glutamate peak current (I_p) EC_{50}

Values of ΔG_X in kcal mol^{-1} are RT [ln(R1 I_pEC_{50} – ln(R2 I_pEC_{50})], where R1 and R2 refer to the NMDA receptor subunit combinations on the left and right sides, respectively, of the column headings (WT, wild-type; N1, GluN1 mutant/GluN2B wild-type; N2, GluN1 wide-type/GluN2B mutant; N1/N2B, GluN1 mutant/GluN2B mutant). Values of apparent free energy $\Delta\Delta G_{INT}$ in kcal mol^{-1} are means ± S.E.M. $\Delta\Delta G_{INT}$. Degrees of freedom (df) and S.E.M were determined as described in *Materials and Methods*. Statistical significance of $\Delta\Delta G_{INT}$ values were determined by using one sample *t* tests.

Mutant Pair (GluN1/GluN2B)	ΔG_1 WT➔N1	ΔG_2 WT➔N2B	ΔG_3 WT➔N1/ N2B	ΔG_4 N1➔N1/ N2B	ΔG_5 N2B➔N1/ N2B	$\Delta\Delta G_{INT}$	df	Significance
G638W/M824W	-1.06	-0.641	-0.984	0.0765	-0.343	0.717	21	P < 0.05
F639W/L825W	-0.380	0.112	-0.0998	0.280	-0.212	0.168	20	P > 0.05
M818W/F637W	0.397	-0.618	0.183	-0.214	0.801	0.404	21	P < 0.05
L819W/F638W	-0.0842	0.0426	-0.0293	0.0549	-0.0719	0.0123	17	P > 0.05

Table 6.3. Mutant cycle analysis of glutamate steady-state current (I_{ss}) EC_{50}

Values of ΔG_X in kcal mol^{-1} are RT [ln(R1 $I_{ss}EC_{50}$ – ln(R2 $I_{ss}EC_{50}$)], where R1 and R2 refer to the NMDA receptor subunit combinations on the left and right sides, respectively, of the column headings (WT, wild-type; N1, GluN1 mutant/GluN2B wild-type; N2, GluN1 wide-type/GluN2B mutant; N1/N2B, GluN1 mutant/GluN2B mutant). Values of apparent free energy $\Delta\Delta G_{INT}$ in kcal mol^{-1} are means ± S.E.M. $\Delta\Delta G_{INT}$. Degrees of freedom (df) and S.E.M were determined as described in *Materials and Methods*. Statistical significance of $\Delta\Delta G_{INT}$ values were determined by using one sample *t* tests.

Interaction of GluN1(Gly638) and GluN2B(Met824) in regulation of channel desensitization.

Glutamate concentration-response experiments identified that tryptophan mutagenesis at GluN2B(Met824) significantly altered apparent desensitization, as assessed by using steady-state to peak current ratio, and tryptophan mutagenesis at GluN1(Gly638) did not. However, dual mutagenesis at these two positions significantly altered apparent desensitization, which is less than that of the single mutation at GluN2B subunit (Figure 6.17). Next, both two-way analysis of variance on steady-state to

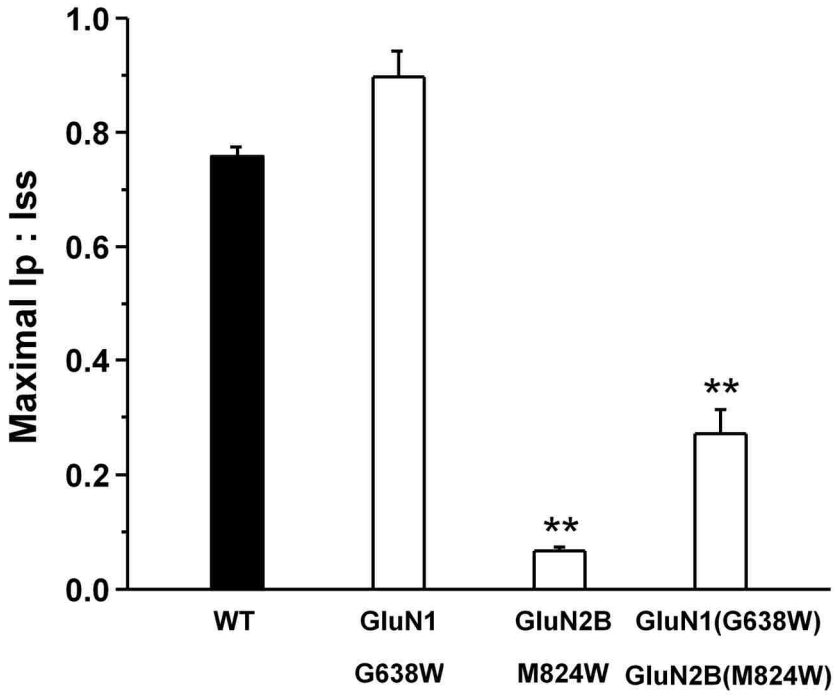

Figure 6.17. Apparent desensitization values of single mutant and dual mutants

Bar graph shows maximal steady-state to peak current ratios (I_{ss}:I_p) for current activated by 300 μM glutamate and 50 μM glycine recorded from cell expressing wild-type, single substituent at GluN1(Gly638), GluN2B(Met824) and dual substitutions at GluN1(Gly638)/GluN2B(Met824) subunits. Statistically significant differences of maximal apparent desensitization from wild-type receptor are indicated by asterisks (**$P < 0.01$; ANOVA and Dunnett's test).

peak current ratios and mutant cycle analysis were used to study a possible interaction between these two positions in regulating ion channel desensitization. Both types of analysis indicated a significant interaction between them with respect to apparent desensitization (Figure 6.18).

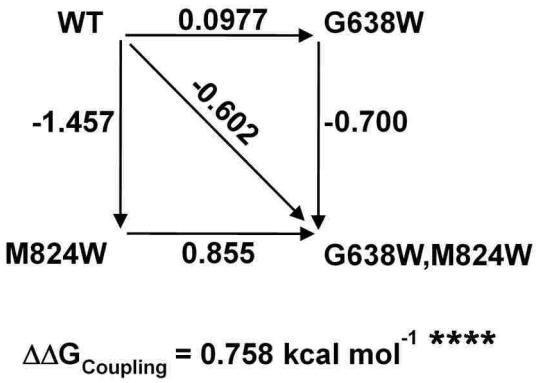

Figure 6.18. Mutant cycle analysis of I_{ss}:I_p for GluN1(Gly638) and GluN2B(Met824)

Mutant cycle analysis of maximal steady-state to peak current ratios for dual mutant combination GluN1(Gly638)/GluN2B(Met824). Apparent free energy values associated with various mutations (ΔG_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 (****$P < 0.0001$).

Discussion

Functional interactions with respect to ethanol sensitivity between pairs of residues in the M3 domain of one subunit type and the M4 of the other subunit type were characterized in GluN2B-containing NMDA receptors. These pairs of residues could also interactively regulate ion channel function (agonist affinity and apparent desensitization). **Does the GluN2 subunit type alter the effect of mutations at positions in the GluN1 subunit on ethanol sensitivity?**

Studies showing similar subjective effects between alcohol and NMDA receptor antagonists support a direct inhibitory role of alcohol on NMDA receptor activity at

relevant pharmacological concentrations (Grant *et al*., 1991; Krystal *et al*., 1998, 2010; Hodge and Cox, 1998), and suggest the importance of determining the molecular sites of alcohol action on the NMDA receptor on developing therapies of alcohol addiction and alcoholism. Over years of studies, previous work in our and other laboratories have previously identified a number of residues in GluN1 and GluN2A M domains which could influence ethanol sensitivity on NMDA receptor. The GluN1(Phe639) position was first reported as an ethanol-sensitive position and substitution at this position did not affect agonist potency (Ronald *et al*., 2001). These authors reported the functional role of this position in not only GluN1/GluN2A NMDA receptors, but also GluN1/GluN2B NMDA and GluN1/GluN2C NMDA receptors. Our previous work also characterized the importance of this position on ethanol sensitivity of GluN1/GluN2A NMDA receptor (Ren *et al*., 2012). However, these two studies reported different results about the ethanol sensitivity change following tryptophan substitution at this position. Recent studies in this laboratory demonstrated significantly decreased ethanol sensitivity in the tryptophan mutant; however, the previous study showed either unchanged or slightly increased ethanol sensitivity by tryptophan substitution. The reason for this discrepancy is still not clear. It is possible that since recombinant NMDA receptors were expressed in different cell types, in some case they can yield different responses. The prior study was carried out in *Xenopus* oocytes, whereas, the study in our laboratory used HEK-293 cells. A more recent study in the Woodward laboratory transfected mutant receptors into HEK-293 cells, and reported that alanine mutagenesis at GluN1(Phe639) significantly decreased ethanol sensitivity. They did not report the results of tryptophan mutagenesis,

but showed that other amino acids with large side chains, such as tyrosine, had no effect on receptor sensitivity to ethanol action (Smothers and Woodward, 2006).

In this present study, a tryptophan mutation was introduced into two positions at the M3 domain of the GluN1 subunit, Gly638 and Phe639, and four positions in the M4 domain of GluN1 subunit, Met818, Leu819, Val820 as well as Ala821. All of these GluN1 mutants were transfected into tsA201 cells combined with wild-type GluN2B subunits. Interestingly, only mutants with tryptophan substitution in the M3 domain showed significantly decreased ethanol sensitivity. Tryptophan substitution at positions in the GluN1 M4 domain had no effect on ethanol sensitivity, which was surprising given that their cognate positions are ethanol-sensitive in the GluN2A subunit (Ren *et al*., 2003b; Honse *et al*., 2004). Further analysis of NMDA receptors carrying other amino acid substitutions at these positions may clarify whether they are ethanol-sensitive positions. In turn, results of ethanol sensitivity changes recorded from GluN1 mutants combined with the GluN2B wild-type subunits in this study agree with our previous study on GluN1 mutant subunit combined with GluN2A wild-type subunit, indicating that although there are differences in ethanol-sensitive positions between GluN2A and GluN2B subunits, ethanol–sensitive positions at GluN1 subunit perform the same functional role in ethanol inhibition between GluN2A and GluN2B-containing NMDAR (Ren *et al*., 2012). Further study should be conducted on other subtypes of NMDAR considering this observation, such as GluN2C and GluN2D-containing NMDA receptors.

Is there any interaction with respect to ethanol sensitivity between positions in the M3 and M4 domain of adjacent subunits in the GluN2B-containing NMDA receptor?

Since ethanol can exert its action at multiple positions in the NMDA receptor as these previous studies have shown, it's likely that those positions would interact functionally to regulate ethanol sensitivity. Smothers and Woodward (2006) demonstrated in their study that single GluN1(Phe639) alanine mutant significantly reduced NMDA receptor sensitivity to ethanol inhibition; however, when tryptophan substitutions at certain positions of GluN1 subunit M4 domain were individually added to the GluN1(Phe639) alanine mutant, the altered ethanol sensitivity caused by the 639 mutant returned to that of the wild-type. These findings suggest that these residues may interact with each other to form ethanol action sites within the same subunit. Furthermore, our laboratory demonstrated that Phe637 and Met823 in the GluN2A subunit appear to functionally interact with each other to regulate ethanol sensitivity as well as ion channel function (Ren *et al*., 2008). All these interactions were reported within the same subunit type. We recently reported significant interactions with respect to ethanol action between pairs of residues in the M3 domain of one subunit type and the M4 domain of the other in the GluN2A subunit-containing NMDA receptor (Ren *et al*., 2012), which were based on the proximity of each pair of residues in the structural model of the GluA2 subunit (Sobolevsky *et al*., 2009). This structural model also suggests putative interactions between positions in the M3 and M4 domains of the GluN1 and GluN2B subunit. After introducing tryptophan mutations into these positions, individually or in pairs, dual mutations at some putative interacting positions did not show altered ethanol sensitivity, despite the changes of ethanol sensitivity caused by single mutations at the M3 and M4 domains of the GluN1 and GluN2B subunits. These results suggest that mutations at these pairs of positions influence ethanol sensitivity in a

non-additive manner. Although this does not provide evidence about direct interactions between these positions to form ethanol action sites, observations in this study imply functional interactions between positions in M3 and M4 domain of GluN1 and GluN2B subunit that can influence ethanol sensitivity.

Previous studies reported the suitability of mutant cycle analysis to indicate functional interactions between sets of residues on the basis that the free energy change for the double mutant is not equal to the sum of the changes in free energy of the two single mutations. Moreover, mutant-cycle analysis can be used to characterize structures that are inaccessible to X-ray crystallography (Horovitz, 1996; Kash *et al*., 2003; Venkatachalan and Czajkowski, 2008; Laha and Wagner, 2011; Ren *et al*., 2012). By using both two-way ANOVA and mutant cycle analysis of log-transformed ethanol IC_{50} values, three putative ethanol action sites in GluN1/GluN2B interfaces were identified. The interaction pattern of the GluN1/GluN2A NMDA receptor and the GluN1/GluN2B NMDA receptor differ. We previously reported that GluN1(Leu819) and GluN2A(Phe637) could interactively regulate ethanol sensitivity (Ren *et al*., 2012); however, results in this thesis did not show an interaction between GluN1(Leu819) and the cognate position GluN2B(Phe638) with respect to ethanol sensitivity. This result is consistent with the observation that although GluN2A(Phe637) can regulate ethanol action on NMDA receptor (Ren *et al*., 2007), GluN2B(Phe638) does not. Moreover, GluN2A(Phe637) could interact with GluN1(Ala821) to regulate ethanol sensitivity, however, GluN2B(Phe638) does not. These differences further support the hypothesis that although the GluN2A and GluN2B subunit share highly homologous M3 and M4 domain sequences, the action of ethanol on the two subunits differs. The ethanol-sensitive

position in the GluN2B subunit, Phe637, showed significant interaction with two positions in the M4 domain of the GluN1 subunit, Met818 and Val820, as determined by both analysis methods, but not with the position in between, Leu819. Furthermore, by comparing the two interactions, the apparent free energy of interaction for the former pair was twofold greater than the latter pair. These results suggest that the weaker interaction observed here may reflect not a direct side-chain interaction, but a functional interaction between two residues resulting from conformational restriction caused by long-distance interaction (Ren *et al*., 2008; Ren *et al*., 2012).

Results of a previous study demonstrated two types of sites of ethanol action: two in the GluN1 M3/GluN2A M4 interfaces, and two in the GluN1 M4/GluN2B M3 interfaces. Each of site is composed of five residues, two in the M3 domain and three in the M4 domain (Ren *et al*., 2012). In this current study, two sites of ethanol action at the GluN1 M3/GluN2B M4 interfaces were identified, each of them consisting of two amino acids in the M3 domain of the GluN1 subunit and three amino acids in the M4 domain of the GluN2B subunit. This is consistent with the previous study in the GluN2A subunit-containing NMDA receptor. Furthermore, two ethanol action sites at the GluN1 M4/GluN2B M3 were also found. However, compared to the two ethanol action sites in the GluN1 M4/GluN2A M3 interfaces, each of them consists of only three residues, Phe637 in the M3 domain of the GluN2B subunit, and two in the M4 domain of the GluN1 subunit. GluN2B(Phe638) is not an ethanol-sensitive position as described in the previous chapter, and did not interact with any of residues that are located closely in GluN1 subunit as well. The inability to detect any interaction between this position and other positions in the GluN1 subunit could account for the finding that tryptophan

mutagenesis at GluN2B(Phe638) did not alter ethanol sensitivity of NMDA receptor, despite having an ethanol-sensitive cognate position in the GluN2A subunit. The results of the current thesis also indicate that contributions of amino acids to ethanol action within these ethanol-sensitive sites are not equivalent. Mutations at residues in the M3 domain of either subunit significantly altered NMDA receptor sensitivity to ethanol inhibition, except for GluN2B(F638W), whereas mutations of the M4 domain residues in either subunit did not affect ethanol sensitivity. This indicates that although the GluN1 and GluN2B subunit contribute to both types of ethanol-sensitive sites, the roles of the M3 domain and the M4 domain in forming these sites are not equal. The greater effect of residues in the M3 domain on ethanol action compared to residues in the M4 domain may reflect the importance of the M3 domain in ion channel gating (Jones *et al*., 2002). Based on this assumption, residues in the M4 domain do not directly influence ion channel gating, but influence it indirectly by interaction with residues in the M3 domain (Ren *et al*., 2003a).

Recent studies reported the crystallized structures of the GluN1/GluN2B NMDA receptor with either the allosteric inhibitor ifenprodil or partial agonists (Lee *et al*., 2014; Karakas and Furukawa, 2014). Based on these structures, it is not possible to form direct side-chain interactions between one pair of residues characterized previously, GluN1(Met818) and GluN2B(Phe637), that can functionally interact to regulate ethanol inhibition of the receptor. The distance between them after replacement with tryptophan is ~12 Å. One possible explanation can be that structures of GluN2B-containing NMDA receptor described in these studies were generated when the ion channel is closed, so they cannot represent the composition of the receptor when the ion channel is activated and

open. In order to resolve this possibility, cross-linking studies were performed and are presented in the next chapter.

Can pairs of ethanol-sensitive positions interactively regulate ion channel functions?

A number of studies have shown that mutations at certain ethanol-sensitive positions in the M3 and M4 domains of the GluN1 and GluN2A subunits can alter ion channel kinetics, such as agonist affinity, desensitization, and mean open time (Ronald *et al*., 2001; Ren *et al*., 2003a; Ren *et al*., 2003b; Smothers and Woodward, 2006; Ren *et al*., 2007; Ren *et al*., 2008; Ren *et al*., 2013). The M3 and M4 domains are both important for ion channel gating in the NMDA receptor (Jones *et al*., 2002; Sobolevsky *et al*., 2002; Ren *et al*., 2003a; Schorge and Colquhoun, 2003; Yuan *et al*., 2005; Blank and VanDongen, 2008; Chang and Kuo, 2008), and results in this thesis show that EC_{50} values for glutamate-activated peak current and steady-state current were altered following the introduction of tryptophan residues into single positions or pairs of positions studied for ethanol sensitivity. Previous studies confirmed that mutant-cycle analysis can be used for analyzing the influence of interactions between pairs of residues on ion channel function (Venkatachalan and Czajkowski, 2008; Ren *et al*., 2012). In this thesis, by using both mutant-cycle analysis and two-way ANOVA, three pairs of residues characterized as interactively regulating ethanol sensitivity also appear to interact with each other to regulate glutamate peak current EC_{50}, and two out of three pairs of residues could also interactively regulate glutamate steady-state current EC_{50}.

In the present study, an interaction with respect to maximal steady-state current to peak current ratio (I_{ss}:I_p), a measure of macroscopic desensitization, was found between one pair of residues in adjacent subunits, GluN1(Gly638) and GluN2B(Met824).

Tryptophan substitution at GluN2B(Met824) markedly increased macroscopic desensitization, but desensitization of the GluN1(Gly638) tryptophan mutant was not statistically different from that of the wild-type receptor. However, co-expressing these two mutant subunits into cells largely reversed the effect of the GluN2B(M824W) mutant on the maximal steady-state current to peak current ratio, indicating the presence of a functional interaction between these two positions with respect to ion channel desensitization. A significant interaction between these positions with respect to desensitization was also observed by using mutant cycle analysis of maximal steady-state current to peak current ratio. These results suggest that the side chains of these two residues are able to functionally interact, and at least when tryptophan is introduced into both positions, the interaction between them can regulate ion channel gating. Unlike other glutamate-gated ion channels, NMDA receptor desensitization reflects multiple distinct processes and appears to be more complex (Dingledine *et al*., 1999). In this present study, experimental conditions were designed to minimize the influence of other forms of current decay relative to the true ion channel desensitization based on our previous studies (Ren *et al*., 2003a; Ren *et al*., 2007; Ren *et al*., 2012; Ren *et al*., 2013). Therefore, the results reported here could reflect the regulation of ion channel gating by a functional interaction between the side chains of these two residues.

VII. CROSS-LINKING OF POSITIONS THAT INTERACT TO REGULATE ETHANOL SENSITIVITY AND ION CHANNEL FUNCTIONS

Introduction

In the previous chapter, functional interactions with respect to ethanol sensitivity and ion channel functions between pairs of residues in the M3 domain of one subunit type and the M4 domain of the other were characterized by using two-way ANOVA and mutant cycle analysis. However, these observations are not sufficient to unveil direct interactions between the side chains of residues at these positions, and whether these residues contribute to a unitary alcohol binding cavity. Recent studies have presented X-ray crystal structures of the GluN1/GluN2B NMDA receptor with either an allosteric inhibitor or partial agonists (Lee *et al*., 2014; Karakas and Furukawa, 2014). Based on these structures, at least of one pair of residues characterized in the previous chapter are not close enough to each other to directly interact to regulate ethanol sensitivity and channel function. However, an ion channel blocker, allosteric antagonists, and partial agonists of the NMDA receptor were used to obtain the crystal structures of these receptors, so the structures reported are of the receptor in the resting state, but not in the activated state. It is likely that the distance between proposed interacting residues can change upon the activation of the NMDA receptor.

In order to resolve this question, cysteine substitutions were introduced into this pair of positions, and whether disulfide bonds can form between the position in the M3 domain of one subunit type and the position in the M4 domain of the other subunit type was tested by applying redox-active agents. Previous studies have demonstrated

modifications of NMDA receptor channel kinetics by redox-active agents. Reducing agents potentiated the response of the receptor to agonist, while oxidation with certain agents decreased the magnitude of the response (Aizenman *et al*., 1989; Tang and Aizenman, 1993). Studies also showed that by replacing certain residues in the glycine receptor with cysteine, application of redox agents changed the glycine response of these mutant receptors compared to wild-type receptors (Lobo *et al*., 2008). Since the positions I studied in this chapter are localized in the membrane-associated regions, which are vital to ion channel gating (Low *et al*., 2003; Yuan *et al*., 2005; Sobolevsky *et al*., 2007; Sobolevsky *et al*., 2009), formation of disulfide bonds in these regions may change ion channel function. However, whether oxidizing agents can reduce or potentiate the responses of mutant NMDA receptors to glutamate is still unknown. After application of either the oxidizing agents hydrogen peroxide (H_2O_2) or copper phenanthroline (CuPhen), or the reducing agent dithiothreitol (DTT), I studied their effects on the response of the receptor to agonist and on the ethanol sensitivity of the receptors.

Results

Oxidizing agents significantly reduce glutamate-activated current amplitude in cells expressing NMDA receptors with dual cysteine substitution at a position in the M3 domain of one subunit and a position in the M4 domain of the other subunit, and a reducing agent could exert opposite effects

In the previous interaction study, three pairs of positions, GluN1(Phe638)/GluN2B(Met824), GluN1(Phe639)/GluN2B(Leu825), and GluN1(Met818)/GluN2B(Phe637), were found to functionally interact to affect both

ethanol sensitivity and channel function. In this study, cysteine substitutions were initially introduced into one of these pairs, GluN1(Met818)/GluN2B(Phe637), by site-directed mutagenesis, and whether application of redox agents could form disulfide bonds between these positions and further alter channel function was studied. Dual cysteine substitutions at this pair of positions yielded functional receptors after transfection into tsA-201 cells. The function of a putative disulfide bond formed between them on glutamate-activated NMDA receptor current was tested initially with 0.1% H_2O_2 application. Pronounced decreases in current amplitude were noticed after applying the oxidizing agent. This may indicate the formation of a disulfide bond between these two positions, which changed ion channel function. In order to further test the formation of a disulfide bond, 10 mM DTT was subsequently applied to break disulfide bonds. The result demonstrated that glutamate-activated current amplitude was increased by DTT treatment (Figure 7.1).

Figure 7.1. Redox modulation of GluN1(M818C)/GluN2B(F637C)

Current traces show reduction due to oxidizing function of H_2O_2 in GluN1(M818C)/GluN2B(F637C), and potentiation due to reducing function of DTT in this mutant. Reducing and oxidizing agents were applied for 2 min to allow them to exert their effects.

Redox agents did not exert any change of current amplitude in GluN1(C744A/M818C) /GluN2B(F637C) receptors

Although the results in **Figure 7.1** suggest that a disulfide bond was formed between GluN1(M818C) and GluN2B(F637C), these changes could be a result of endogenous redox sites in the GluN1 subunit. By using site-directed mutagenesis, a previous study determined that two cysteine residues in the GluN1 subunit of the NMDA receptor, Cys744 and Cys798, were responsible for the majority of redox modulation of GluN1/GluN2B NMDA receptors (Sullivan *et al*., 1994). In order to exclude this possible source of redox effect in our results, one position was replaced by alanine. Currents in cells expressing dual cysteine mutagenesis at GluN1(Met818) and GluN2B(Phe637) combined with alanine mutagenesis at GluN1(Cys744) were recorded after extracellular application of either of the oxidizing agents (H_2O_2, CuPhen) or the reducing agent (DTT). However, neither H_2O_2 nor DTT treatment caused significant changes in glutamate-activated current amplitude (Figure 7.2).

Figure 7.2. Redox agents do not exert any effect on current amplitude of GluN1(C744A/M818C)/GluN2B(F637C)

Current traces show no change in current amplitude of GluN1(C744A/M818C)/GluN2B(F637C) upon application of either the oxidizing agent H_2O_2, 0.1% (left) or the reducing agent DTT, 10 mM (right). Reducing and oxidizing agents were applied for 2 min to allow them to exert their effects.

Intracellular application of DTT did not exert any effect on either channel function or ethanol sensitivity of GluN1(M818C)/GluN2B(F637C) NMDA receptors

Recent studies from our laboratory provided evidence that the positions in GluN2C subunit corresponding to the positions in GluN2B subunit are sufficiently proximal to interact, and that they appear to constitutively form disulfide bonds in dual cysteine mutants following intracellular application of DTT (*unpublished data*). It is thus possible that extracellularly-applied DTT can not reach the dual cysteine substitution site from the extracellular space, and if these positions could constitutively form disulfide bonds, an oxidizing agent could not exert any effect on them. To rule out this possibility, I applied DTT intracellularly while GluN1(M818C)/GluN2B(F637C) NMDA receptors were activated with maximal concentration of glutamate. Since DTT was applied intracellularly, it was not necessary to study the receptor with the GluN1(C744A) substitution. I measured current change within 8 minutes and recorded current once per minute based on the diffusion rate of DTT from the pipet to the targeting site at receptor.

However, DTT did not exert any effect on current amplitude (Figure 7.3). I also tested whether DTT could alter ethanol sensitivity of the NMDA receptor by measuring the percentage inhibition by 100 mM ethanol. However, there was no direct evidence to demonstrate alteration of ethanol sensitivity induced by DTT application (Figure 7.4).

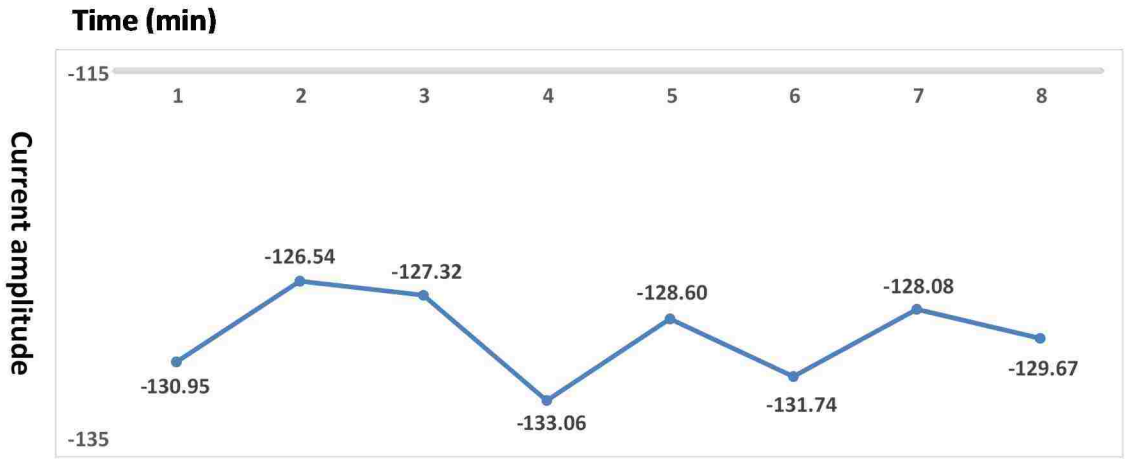

Figure 7.3. Changes in current amplitude of GluN1(M818C)/GluN2B(F637C) by intracellular application of DTT within 8 minutes

A cell containing GluN1(M818C)/GluN2B(F637C) mutant receptors was activated by 10 μM glutamate and 50 μM glycine and recorded once per minute from 1 min to 8 min. DTT was applied intracellularly via the patch-pipet.

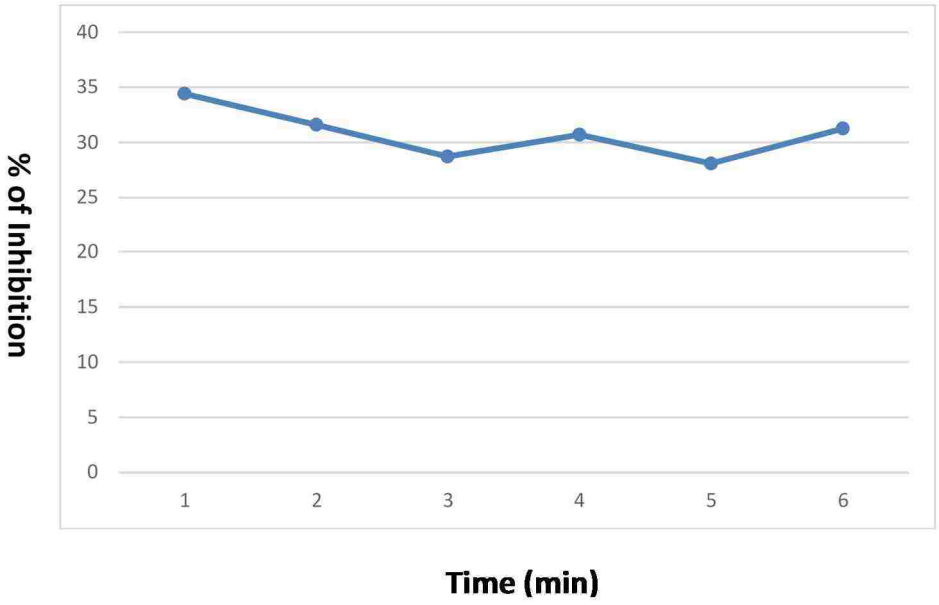

Figure 7.4. Changes in 100 mM ethanol inhibition of GluN1(M818C)/GluN2B(F637C) by intracellular application of DTT within 8 minutes

A cell containing GluN1(M818C)/GluN2B(F637C) mutant receptors was activated by 10 μM glutamate and 50 μM glycine and inhibited by 100 mM ethanol, then recorded once per minute from 1 min to 6 min. DTT was applied intracellularly via the patch-pipet.

Discussion

The functional interaction with respect to ethanol sensitivity and ion channel function between pairs of residues in the M3 domain of one subunit type and the M4 of the other subunit type were characterized by using two-way ANOVA and mutant cycle analysis in **chapter VI**. In this chapter, I tested if they are physically interacting with each other. GluN1(Met818) and GluN2B(Phe637) are ~12 Å apart from each other,

which indicates that it is possible for them to form a direct side-chain interaction. In order to test this possibility, cross-linking studies were performed by introduction of cysteine residues in dual positions. However, we could not obtain clear evidence that redox agents formed disulfide bonds between them, because neither oxidizing agents nor reducing agents were able to alter NMDA receptor currents.

Previous studies in our laboratory have demonstrated interactions between positions in different membrane-associated (M) domains of the GluN2A subunit, and between positions in the M3 domain of one subunit type and the M4 of the other subunit type (Ren *et al*., 2008; Ren *et al*., 2012). In a previous study on the interaction between GluN2A(Phe637) and GluN2A(Met823), the observations did not indicate a direct interaction between the side chains at these positions (Ren *et al*., 2008). So one plausible explanation for the results of cross-linking studies described in this thesis could be that rather than direct side-chain interaction between these positions, functional interactions happened between them. It is possible that the wild-type methionine at position 818 in the GluN1 subunit may form a contact with other residues in its environment to place a conformational constraint on the M4 domain movement. This constraint was then released after tryptophan substitution at this position. This may also explain why the GluN2B(F637W) mutant showed significantly altered ethanol sensitivity, but the addition of the GluN1(M818W) mutant restored regular ethanol inhibition seen in the wild-type receptor. It may also be possible that these two positions can still directly interact with each other, since the crystal structure only represents the receptor structure in the closed state. The inability to observe altered ion channel gating following cysteine mutations and redox agent application may also have resulted from impaired delivery of these

agents. It is possible that the oxidizing agent was not able to achieve an effective concentration in the environment surrounding the side chains. Future studies can be performed using other agents.

VIII. EFFECTS OF NMDA RECEPTOR MUTANTS ON ION CHANNEL GATING AND ETHANOL SENSITIVITY IN CENTRAL NERVOUS SYSTEM NEURONS

Introduction

Expressing NMDA receptors in different cell types can in some cases yield different responses. In one study, it has been demonstrated that zinc could augment NMDA receptor-mediated synaptic responses in the CA1 region of rat hippocampal slices, and this process is mediated by Src family tyrosine kinases (Kim *et al*., 2002). However, another study showed the opposite result in spinal neurons (Xiong *et al*., 1999). As the majority of studies in this thesis were conducted by using tsA-201 cells, a transformed cell line derived from kidney fibroblasts, as the cell model, it is important to exclude the possibility that the characteristics of mutants demonstrated in our studies are cell-specific and may not be relevant to the actual function of these mutants in the central nervous system. The first step to study this question in our laboratory was to use neurons cultured from GluN2A subunit knockout mice; however, this approach proved to be unnecessary, since neurons cultured from wild-type animals early in development have low levels of GluN2A subunit (Mitani *et al*., 1998; Liu *et al*., 2010). In this study, cerebral cortical neurons from P2/P3 rats were used for expression of constructed receptors. Following delivery of recombinant mutant NMDA receptors into these neurons via electroporation, they were used in ethanol concentration-response experiments.

Results

Apparent desensitization of GluN1/GluN2A(M823W) mutant NMDA receptor expressed in central nervous system (CNS) neurons

As described in a previous study, the GluN2A(M823W) mutant NMDA receptor showed significantly increased apparent desensitization compared with wild-type receptors (Ren *et al*., 2003b). The initial study on the function of mutant receptors in neurons used this mutant. GluN1 and GluN2A(M823W) subunits were transfected into P2 or P3 rat cortical neurons by electroporation. In order to avoid the possibility that application of glutamate activated other endogenous glutamate-gated receptor types, such as AMPA receptors and kainate receptors, a maximal concentration of NMDA was used in this study as the agonist instead of glutamate. To validate that neurons at this developmental period do not express NMDA receptors, neurons transfected with GFP but not any exogenous NMDA receptor were also recorded under the same conditions. These neurons did not show any NMDA-activated current. Strychnine, 1 μM, was added in order to eliminate any current from glycine receptor. Currents due to native GluN2B subunit-containing NMDA receptors were blocked using the selective antagonist ifenprodil. As shown in **Figure 8.1**, neurons following transfection expressed GFP.

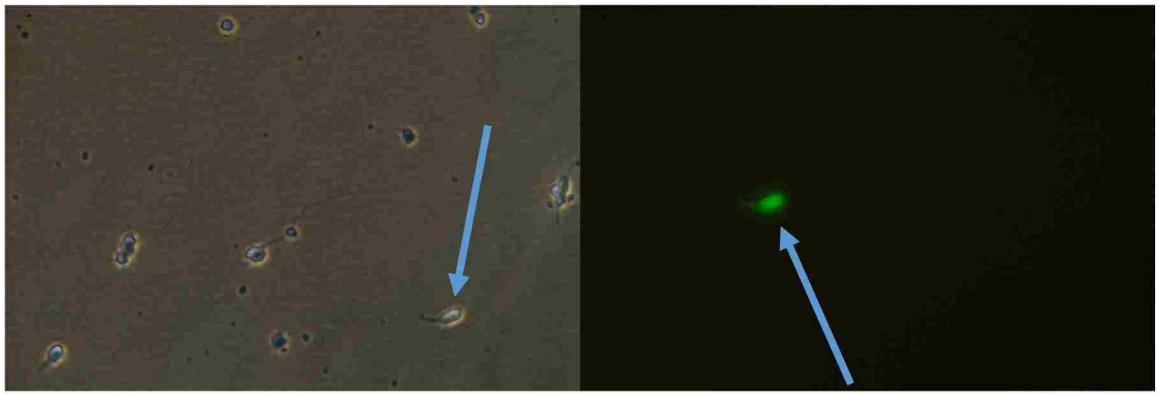

Figure 8.1. Expression of GFP indicates successful transfection of constructed NMDA receptors into a cortical neuron

Arrow points to a cortical neuron which exhibits different features compared with glial nearby in this view (left). This neuron was successfully transfected with GFP since it glows green under fluorescent illumination (right), indicating likely co-expression of exogenous NMDA receptors.

Comparison of wild-type GluN1/GluN2A NMDA receptor expressed in HEK293 cells and in CNS neurons

I first compared the basic features of wild-type NMDA receptors expressed in non-neuronal cells as well as in neurons. As shown in Figure 8.2, wild-type NMDA receptors expressed in different cell types show similar ion channel characteristics, such as a small degree of desensitization upon activation by 300 μM glutamate and 50 μM glycine, as well as concentration-dependent ethanol inhibition (100 mM: ~35%; 250 mM: ~65%).

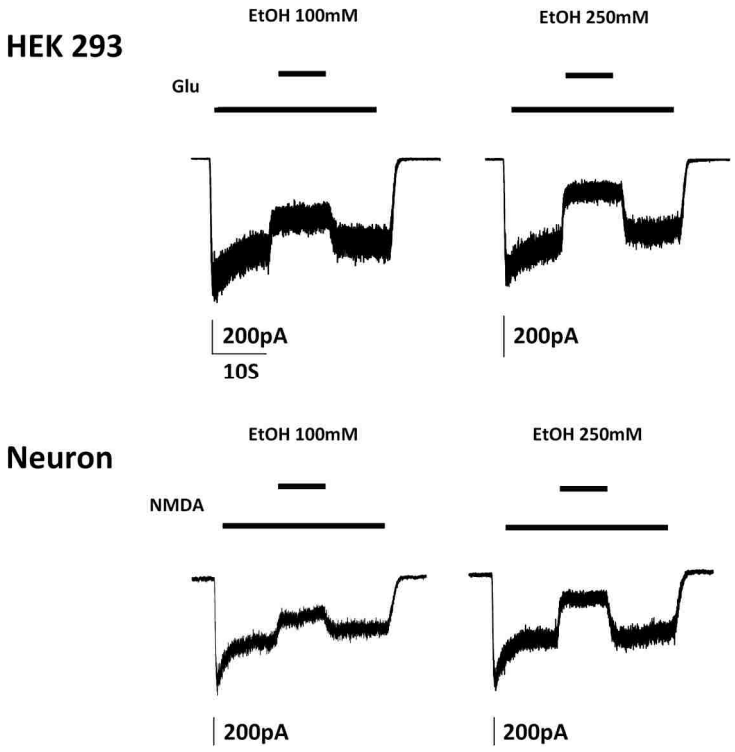

Figure 8.2. The characteristics of wild-type GluN1/GluN2A NMDA receptor observed in HEK 293 cells are also present in neurons

Records are currents activated by 300 μM glutamate and 50 μM glycine with 100 mM ethanol inhibition in either HEK293 cells (upper) or cortical neurons (lower) expressing wild-type NMDA receptors.

Comparison of GluN2A(M823W) mutant NMDA receptor expressed in HEK293 cells and in CNS neurons

Mutant receptors expressed in cortical neurons showed the most prominent feature characterized in HEK 293 cells in our previous study (Ren *et al*., 2003a), that is significantly increased desensitization compared to the wild-type receptor (wild-type receptor: ~20%; GluN2A mutant: ~95%; Figure 8.2, 8.3).

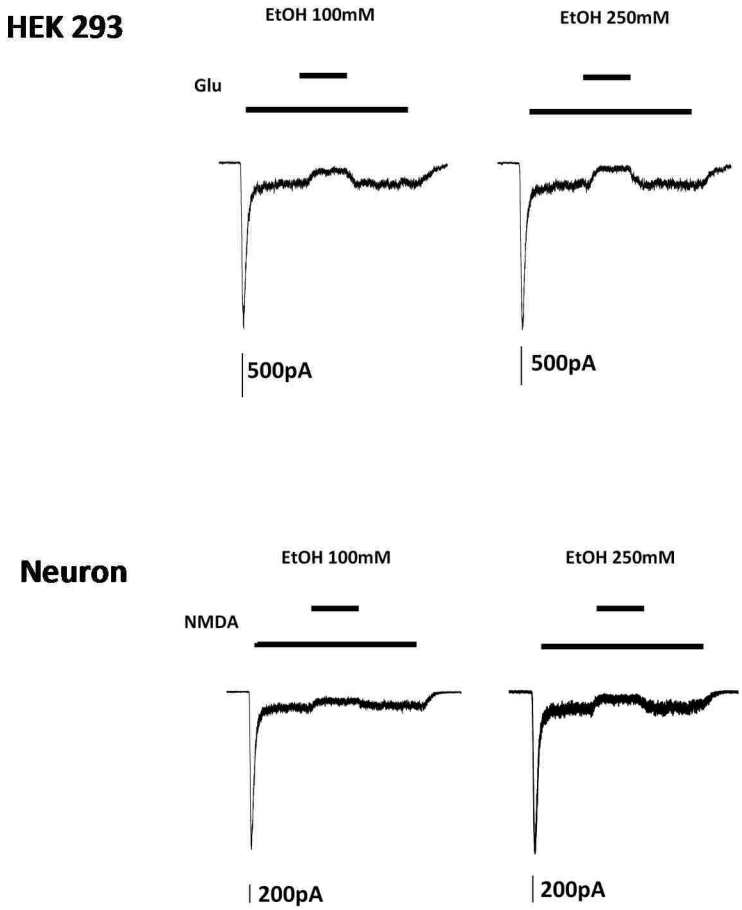

Figure 8.3. The characteristics of the GluN2A(M823W) mutant NMDA receptor observed in HEK 293 cells are also present in neurons

Records are currents activated by 300 μM glutamate and 50 μM glycine with 100 mM ethanol inhibition in either HEK293 cells (upper) or cortical neurons (lower) expressing GluN2A(M823W) NMDA receptors.

Ethanol sensitivity of GluN1/GluN2A(M823W) mutant NMDA receptors expressed in CNS neurons.

In order to characterize the ethanol sensitivity of GluN2A(M823W) mutant NMDA receptors expressed in rat cortical neurons, both 100 mM and 250 mM ethanol were used to inhibit NMDA-activated current since cortical neurons are more fragile

compared to tsA-201 cells and could not bear the whole panel of ethanol concentrations (Figure 8.3). Ethanol inhibition of GluN2A(M823W) mutant NMDA receptors was not significantly altered compared with wild-type receptors, which was consistent with the results in HEK 293 cells (100 mM: ~32 % inhibition; 250 mM: ~60% inhibition).

Ethanol sensitivity of GluN1/GluN2B(F637W) mutant NMDA receptors expressed in CNS neurons

Besides comparing the characteristics of GluN2A mutant subunits expressed in HEK293 cells and cortical neurons, I also tested whether the significantly decreased ethanol sensitivity of GluN2B(F637W) mutant receptors found in tsA-201 cells is a cell type-specific phenomenon. I expressed either wild-type GluN1/GluN2B NMDA receptors or GluN1/GluN2B(F637W) mutant NMDA receptors in rat cortical neurons and compared their ethanol sensitivity. However, no significant difference in ethanol sensitivity was noticed between wild-type receptors and mutant receptors (100 mM: ~35%; 250 mM: ~65%). Results are average percentage of inhibition in 4-6 cells.

Discussion

Because NMDA receptor activity in the neuronal environment is regulated by interactions with intracellular proteins such as scaffolding proteins and kinases, it is possible that NMDA receptor ion channel function and behavior could differ among different cell types (Dingledine *et al*., 1999; Yagi, 1999; Levitan, 1994; McBain and Mayer, 1994). It is difficult to utilize cortical neuronal cultures from NMDA receptor knockout mice since the NMDA receptor is vital for normal CNS function (Dingledine *et al*., 1999; Paoletti and Neyton, 2007). Previous studies have demonstrated that there are

developmental changes in localization and expression efficiency among different NMDA receptor subunit types, such that GluN1 and GluN2B subunits display high levels of expression within the first week, while the GluN2A subunit is barely detectable at the first week (Li *et al*., 1998). So it is reasonable to study the function of our recombinant GluN2A-containing NMDA receptor in neuronal cultures by transfecting them into P2/P3 rat neurons. However, it is impossible to utilize the same method to test the function of GluN2B-containing NMDA receptors. A possible explanation for this result may be that although GluN2A subunit-containing NMDA receptor expression level is very low in the P2/P3 period, GluN2B subunit-containing NMDA receptor expression level is constant from P2 to P21. So the cortical neurons used in this study may contain high levels of the native GluN2B subunit, which could obscure the current from activation of exogenous GluN2B mutant NMDA receptors. The only mutant GluN2A subunit tested in this chapter, M823W, showed the same desensitization pattern as results collected from HEK 293 cells (Ren *et al*., 2003a).

It was surprising that I did not observe significant effects on alcohol sensitivity of the mutant receptors expressed in neurons or in GluN2A(M823W) subunits expressed in HEK 293 cells. In addition to the possibility of background expression of native receptors in cultured neurons, there may be other explanations for this. It should first be noted that while the GluN2A(M823W) mutant dramatically alters macroscopic desensitization (Ren *et al.*, 2003a), its effect on alcohol sensitivity is more subtle (Ren *et al.*, 2003b), especially compared to other mutants (Ren *et al.*, 2012). Thus it may be difficult to obtain significant differences using only two ethanol concentrations in a small number of cells. Furthermore, a lesser ability to control the conditions in distal processes

of neurons may render receptors in these regions more susceptible to modulation by processes such as phosphorylation.

Although the sample number in this study is limited, and only one mutant type expressed in cortical neurons was characterized, this mutant did show a similar ion channel gating pattern as well as pattern of ethanol action compared to the same construct expressed in HEK-293 cells. It is important to notice that studies performed in our laboratory using neurons cultured from GluN2A subunit knockout mice have demonstrated little or no influence of cell type on NMDA receptor ion channel gating or ethanol sensitivity. Because it is difficult to limit the effect of endogenous GluN2B subunits in cells expressing transfected GluN2B constructs, future studies could address whether GluN1 subunit mutants show similar alterations on ion channel gating and ethanol action when co-transfected with the GluN2A subunit into neurons.

GENERAL CONCLUSIONS

Because alcohol addiction is an enormous problem in U.S. society, it is very important to generate novel therapeutic methods. There are currently a small number of available drugs designed to reduce cravings for alcohol, such as naltrexone (Ganellin and Triggle, 1996). However, these drugs may help to reduce heavy drinking, but not to maintain abstinence (Spagnolo *et al*., 2014). Previous studies have pointed out a role of the GluN2B subunit in alcohol action (Miyakawa *et al*., 1997; Kash *et al*., 2009; Wills *et al*., 2011), and the ability to selectively regulate alcohol sensitivity of the GluN2B subunit could be a powerful tool in the treatment of alcohol addiction. However, there is still very little information about the molecular mechanisms of alcohol action on the GluN2B subunit. Studies in this thesis provide key information about the interaction between the GluN2B subunit and the ethanol molecule by identifying ethanol-sensitive positions in the GluN2B subunit, which may in turn help to identify novel therapeutic treatments for alcohol addiction. In addition to characterization of ethanol-sensitive positions in the GluN2B subunit, this study also aided in developing a better structural model of the M3 and M4 domains of the GluN2B subunit, understanding the relationship between structure and function of these domains, and determining the effects of alcohol on these domains.

Studies in the present dissertation initially demonstrated that mutations at a phenylalanine (Phe^{637}) in the third transmembrane domain of the GluN2B subunit could alter NMDA receptor ethanol sensitivity, apparent glutamate potency, apparent agonist affinity, and apparent desensitization. These results indicate that this position is important for both ethanol action and ion channel function. In addition to studying the main

ethanol-sensitive position in the GluN2B subunit, this dissertation also compared the GluN2A and GluN2B subunits with respect to ethanol-sensitive positions and their functions on ion channel gating. Although the GluN2A and GluN2B subunit M3 and M4 domains are highly homologous, there are both important similarities and differences in the action of ethanol on these subunits.

Subsequent experiments showed that individual tryptophan substitution at a number of positions in the M3 and M4 domains of GluN1/GluN2B NMDA receptor could alter ethanol sensitivity. Moreover, upon introduction of dual tryptophan substitutions into adjacent positions in the GluN1 and GluN2B subunits, functional interactions with respect to ethanol sensitivity between pairs of residues in the M3 domain of one subunit type and the M4 domain of the other subunit type were characterized by using two-way ANOVA and mutant cycle analysis. In addition, these pairs of residues could also interactively regulate ion channel function (agonist affinity and apparent desensitization).

To exclude the possibility that the M domain mutations we have identified alter NMDA receptor function in a cell-specific manner that may not be relevant to the function of the CNS, I recorded from cultured P2-P3 rat cortical neurons. One GluN2A mutant, M823W, did show a similar ion channel gating pattern as well as ethanol action pattern compared to the same construct expressed in HEK-293 cells. Since it is difficult to limit the effect of endogenous GluN2B subunits in cells expressing transfected GluN2B mutant constructs, future studies will address whether GluN1 subunit mutants show similar alterations on ion channel gating and ethanol action when co-transfected with the GluN2A subunit into CNS cortical neurons.

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