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Mechanisms of Subtype-Specific Inhibition of N-methyl-D-aspartate Receptors by Ethanol

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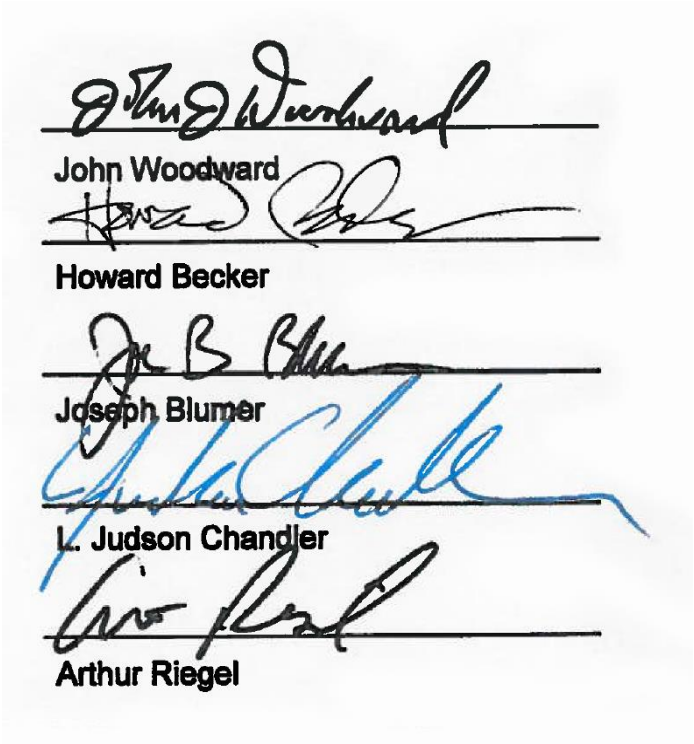


Table of Contents

List of Tables

List of Figures

Abstract

BENJAMIN ALEXANDER HUGHES. Mechanisms of Subtype-Specific Inhibition of N-methyl-D-aspartate Receptors by Ethanol. (Under the direction of JOHN J. WOODWARD).

Alcoholism is a debilitating and costly disease with rates of prevalence totaling nearly 6% worldwide. Treating this disease has proven expensive and in many cases ineffective due in large part to significant gaps in our knowledge of the molecular determinants that drive the pathogenesis of the disorder. Recently, the N-methyl-D-aspartate (NMDA) receptor has emerged as a candidate site for the primary inhibitory actions of ethanol on nervous system function. This receptor is an ion channel highly expressed in the nervous system that is activated by the excitatory neurotransmitter glutamate and has been implicated in the induction of a number of cellular phenomena including the molecular underpinnings of learning and memory. Understanding how ethanol affects the function of this channel both acutely and chronically is crucial to understanding the manifestation and progression of alcohol use disorders. By employing site-directed mutagenesis and patch-clamp electrophysiology with recombinant cell-expression of mutant and wild type NMDA receptor subunit cDNAs, my research has identified a number of novel interacting sites of ethanol with the NMDA receptor complex. Additionally, this work has highlighted essential differences in both ethanol sensitivity and gating mechanics of different NMDA receptor

subtypes, GluN2A- and GluN2B-containing in particular. Furthermore, by exploiting the phenomenon of bioluminescence resonance energy transfer (BRET) my work has also demonstrated that GluN2A- and GluN2B-containing NMDA receptor subtypes will co-assemble into novel receptor complexes. Further teasing the differential activity of ethanol on these NMDA receptor subtypes could lead not only to the development of novel and effective therapeutics for alcohol use disorders, but as well engender a better understanding of the molecular mechanisms that underlie learning and memory.

Chapter I. Background and Significance

I.1 Alcohol and the Human Condition

Alcohol enjoys an intimate relationship with nearly every human population on the planet and has in many ways shaped the trajectory of our collective development. Indeed, archaeological evidence suggests that the emergence of agrarian societies nearly 10,000 years ago (Johnson and Earle, 2000) may have been in part motivated by the desire to produce ethanol-containing beverages. Discovered by the molecular archaeologist Dr. Patrick McGovern amongst a number of pottery jars in a Neolithic North China village, the oldest example of a fermented beverage dates to nearly 9,000 years in age. Thanks to the efforts of an intrepid group of brewers at Dogfish Head Brewing Company (Milton, Delaware), this concoction is actually commercially available today. More contemporary examples of alcohol-driven social paradigm shifts abound as well, including the first demonstration by a nascent U.S. government that it could enact and enforce its own laws; evidenced by the suppression of violent protests in response to an unpopular whiskey tax, colloquially known as the Whiskey Rebellion of 1791.

As a rule, though, immoderate indulgence often begets misfortune, and alcohol use is no exception. Current estimates indicate that alcohol-related deaths constitute nearly 6% of all fatalities worldwide, with many indicators suggesting a rise in this trend (World Health Organization, 2014). An especially

prescient example of the more insidious nature of alcohol consumption occurred on the night of September 10th, 1897, when a heavily intoxicated London taxi driver named George Smith drove his cab into the side of a building, becoming the first known arrestee for operating a motor vehicle while inebriated (History.com, 2009). This infamous arrest has since faded from the public consciousness, though it heralded an enduring pattern of behavior that has resulted in the death or disability of hundreds of thousands of people in the United States alone. Harsh penalties including significant jail sentences and suspension of driving privileges have helped to curb the incidence of this phenomenon, but the behavior nevertheless persists with recent statistics pinning alcohol-related traffic fatalities at upwards of 10,000 annually (U.S. Department of Transportation, 2014). Drunk driving is emblematic of the profound effect alcohol intoxication has on an individual's capacity to appropriately weigh risk versus reward, and is perhaps the most potent reminder of the cost of alcohol over-indulgence. Even if one opts not to drive intoxicated, though, excessive alcohol consumption still elicits a number of pathological neurobiological and peripheral adaptations that, while far less apparent than a car accident, can be equally as debilitating for the imbiber.

In the coming sections, the body of literature probing the mechanisms of alcohol intoxication and abuse, from the earliest hypotheses on the molecular actions of ethanol to current models of circuit level adaptive changes that drive alcohol seeking, will be surveyed with special emphasis placed on gaps in

knowledge my work has sought to fill. Implicit in this review, though, will be a discussion of the novel molecular mechanisms of neuronal function discovered *a fortiori* in the pursuit of understanding the ever-prevalent scourge of alcoholism, revealing an existential duality often overlooked by the general public and researchers alike; that the uglier, pathological aspects of human behavior nevertheless offer a great deal of insight into the intrinsic nature of our nature.

I.2 Early Indications of Ethanol Action: Inhibition of Excitation

As discussed, the nature of ethanol's relationship with humanity is multifaceted, and indeed it is perhaps unsurprising that a functional gestalt for ethanol action on the central nervous system has proven equally elusive. The accumulation of motor impairment and other sedative-hypnotic behaviors observed with increasingly higher doses of alcohol led many to initially infer that alcohol primarily impaired the efficient transduction of excitatory neurotransmission (Wallgren and Barry, 1970). Though not necessarily a faulty assumption, this hypothesis lacked an adequately descriptive mechanism for ethanol-induced inhibition of excitation, leading researchers to probe the intrinsic processes governing neuronal excitability. Among these early reports it was observed that in the squid giant axon, 2-3% solutions of ethanol reliably inhibited action potentials. The doses employed by these studies (in the 320-480 mM range), however, are far above the lethal limit of mammals, casting doubt on their physiological relevance (Armstrong & Binstock, 1964; Moore, Ulbricht, & Takata, 1964). Nevertheless, some hypothesized that alcohol could impair the efficiency

of cation transport across the cell membrane, the elemental basis of action potentials, thereby blunting neuronal firing and reducing excitability. Indeed, experiments initially seemed to confirm that alcohol appeared to inhibit the efficiency of sodium/potassium exchange by the Na^+/K^+ ATPase both acutely and chronically (Israel & Salazar, 1967; Carmichael & Israel, 1975). While an attractive mechanism of action, this hypothesis would ultimately fall out of favor as further experiments demonstrated an apparent cell-type specificity for the actions of ethanol, thus excluding such a generalized feature of neurons as a primary mode of alcohol action (Wayner, Ono, & Nolley, 1975).

Incidentally, later efforts offered experimental evidence showing increased fluidity of plasma membranes following treatment with either anesthetics or ethanol suggesting a possible cause of blunted excitation (Chin & Goldstein, 1977; Goldstein, Chin, & Lyon, 1982). This work was largely an extension of the Meyer and Overton theory of anesthesia, which posits that volatile anesthetics elicit their effects by disrupting the plasma membrane. This theory was derived from observations showing a linear correlation between the lipid solubility of an agent and its potency as an anesthetic. This theory, and by extension the membrane-fluidity hypothesis of ethanol action, was called into question by experiments demonstrating that the activity of lipid-free, non-membrane bound soluble proteins such as luciferase exhibited the same Meyer-Overton sensitivity to anesthetics (Franks & Lieb, 1984). These observations demonstrated that anesthesia, and by extension alcohol intoxication, was not governed by

membrane dynamics alone, and further implied that proteins may harbor significant hydrophobic pockets that could accommodate anesthetic agents of various sizes. These findings, driven by the desire to understand the mechanisms of drug action, ultimately revealed an essential feature of protein structure that guides drug discovery to this day, and further underscores the reciprocal relationship between human pathology and scientific insight.

The inability of these proposed generalized features of alcohol action to sufficiently incorporate the diverse physiological effects of ethanol at behaviorally relevant doses implied that specific neuronal substrates must mediate intoxication. Phenomenologically, in brain homogenates, ethanol was consistently observed to elicit strong increases in concentration of the inhibitory neurotransmitter GABA that is primarily responsible for mediating inhibition in mammalian neurons. Subsequent radio-ligand binding studies observed that alcohol increased the binding of tritiated agonists to low-affinity GABA receptor sites, lending credence to this hypothesis (Hakkinen & Kulonen, 1959; Ticku, Burch, & Davis, 1983). While encouraging, these results could not reconcile observations intimating a direct effect of ethanol on processes mediating excitatory currents (Nicoll, 1972). Progress in resolving these substrates thus stalled, as the tools available to discriminate the myriad ion currents underlying excitatory neuronal activity remained coarse at best. With the development of high-affinity, high-potency agonists and antagonists, though, the means to isolate and define the receptors mediating excitatory currents became available. Initially,

however, work probing them as sites of alcohol action progressed slowly, as results from emerging reports made apparent that the receptors underlying excitatory, largely glutamatergic, neurotransmission were heterogeneous and exhibited highly idiosyncratic behavior that differentially participated in functional roles well beyond simple signal propagation. Indeed, the initial studies describing these myriad receptor populations added further complexity to a model of nervous system function that was itself nascent and incomplete.

I.3 Discrimination of Post-Synaptic Excitatory Receptor Populations

The earliest descriptions of the nervous system by Claudius Galen posited that the brain was the seat of nervous system function, sending hollow emanations to terminal points of action on muscle groups or elsewhere, and seemed to agree with the anatomical prominence of these structures amongst increasingly intelligent animals. The question of what transduced central nervous or sensory signals via these fibrils remained unknown, and, in lieu of better hypotheses, his assertion that they ferried spirits persisted well through the era of Descartes. Otto Dieters, a German anatomist working in the mid-nineteenth century, is credited with establishing the reticular hypothesis of neurotransmission, in which he asserted the nervous system was fully connected and uninterrupted, sending electrical signals to their targets nigh instantaneously through reticular branches (now known as axons). Among his staunchest supporters was Camillo Golgi, who discovered a process by which neurons could be efficiently stained and visualized under a microscope. Incidentally, a Spanish

contemporary of Golgi, Santiago Ramon y Cajal, used this very process, dubbed Golgi staining, to eventually visualize distinct gaps between neurons, refuting Golgi's firmly held belief in the reticular hypothesis.

The question of what purpose these gaps served, however, remained open, though many, including John Langley, a 20th century pharmacologist, inferred they formed some sort of chemical messaging system. Via the concerted efforts of John Langley, Otto Loewi, and others in describing the role of acetylcholine, an excitatory neurotransmitter, in signal transmission at the neuromuscular junction provided the first tangible evidence that signal transduction was likely receptor mediated. This key insight provided a framework for understanding the essential function of the central nervous system. While ligand/receptor dynamics are today understood to be the foundational basis of inter-neuronal communication, conclusive evidence of this paradigm did not emerge until 1952, when Alan Hodgkin and Andrew Huxley developed the voltage clamp method, widely considered a quantum leap in the study of cell to cell communication and for which they shared the Nobel Prize in 1963.

This method rests on the electronic principle of voltage, which essentially describes the "potential" of electrons, or in this case aqueously dissolved charged ions, to move across a resistor, specifically the cell membrane: the actual flow of electrons across a resistor is current. At its most basic, then, the setup consists of two electrodes, one placed extracellularly and the other intracellularly, that can, by measuring the differential electrical charges inside and

outside the cell, determine the membrane's potential difference. The intracellular electrode then injects current to establish and maintain an experimenter-defined voltage across the membrane. When a stimulus is applied that activates ion channels within the membrane, for example bath-application of an ion channel's cognate agonist, the amount of current the intracellular electrode must inject to proportionally offset the stimulus-induced change in voltage allows the experimenter to determine functional characteristics of these channels such as what types of ions these channels flux and how many ions they can pass (i.e. – current). Furthermore, this system allows experimenters to pharmacologically define and isolate what sort of ion channels underlie specific changes in membrane potential in an otherwise diverse cellular milieu of receptors. Refinements to this technique by Erwin Neher and Bert Sakmann during the 1970's and 80's, specifically introducing the concept of establishing a giga-Ohm seal between the recording electrode and the cell membrane, have subsequently allowed researchers to measure changes in current or voltage at the population, cellular, and single-channel level, and earned the pair a Nobel Prize in 1991.

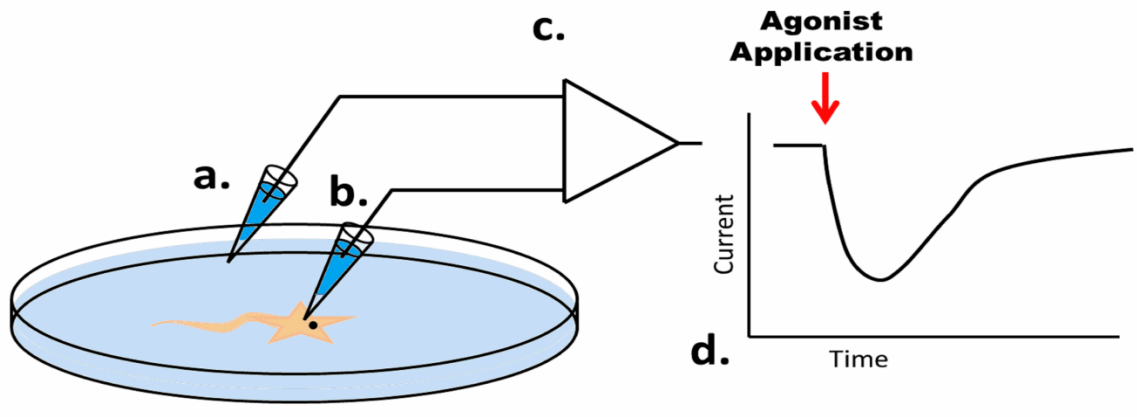


Figure I.1: Schematic depicts a voltage-clamp electrophysiological setup. The amount of current required to offset the voltage potential difference between the reference electrode (**a**) and the recording electrode (**b**) by the amplifier (**c**) in response to a stimulus, in this case agonist application, is read by the experimenter as current conducted by the receptor population being studied (**d**).

The electrophysiological techniques described above gave researchers the tight experimental control and fine temporal precision necessary to understand the elemental features of neurotransmission, and were essential to early reports that established both the ionic basis of neuron excitability and the receptor/channel-dependency of neural responses outlined by Langley, Loewi, and others. As it was largely unknown what types of receptors governed inter-neuronal signaling, much less their number and functional properties, the elucidation of these represented fertile ground for the application of this nascent experimental design. In tandem with continuing refinements to electrophysiological procedures, the development of potent, selective pharmacological agents proved invaluable in first discriminating the substrates of these neurotransmitters and their respective roles in neural activity. Among these early electrophysiological experiments were descriptions of amino acids evoking both neuronal excitation (*L*-glutamate) and inhibition (GABA) that seemed to reveal a binary mode of neuronal activity (Curtis & Watkins, 1960; Curtis & Watkins, 1963). By showing that differential activity of excitatory or inhibitory inputs on specific motor neurons in the squid could elicit either simple reflexes or complex motivated responses, Kupfermann and Kandel (1969) showed that this binary model of neurotransmission permitted the large scale computational

processing necessary to produce behavior. Additionally, the discovery of the mu-opioid receptor, and later additional peptidergic neurotransmitters such as endorphin and substance P, further amended this model by incorporating the concept of neuromodulation, in which certain classes of neurotransmitters do not themselves directly elicit neuronal activity but instead tune the degree of excitation or inhibition, thereby vastly increasing the computing power of neuronal communication (Pert & Snyder, 1973; Knoishi & Otsuka, 1974). Owing to the initial reports of robust glutamate-induced neuronal excitation, though, a considerable premium was placed on the characterization of potent and selective analogues of glutamate, and thus was it demonstrated that multiple receptor subtypes with diverse signaling properties likely mediate excitatory responses elicited by glutamate, weaving further nuance into the existing models of neurotransmission (Johnston, Curtis, Davies, & McCulloch, 1974; Biscoe et al., 1975).

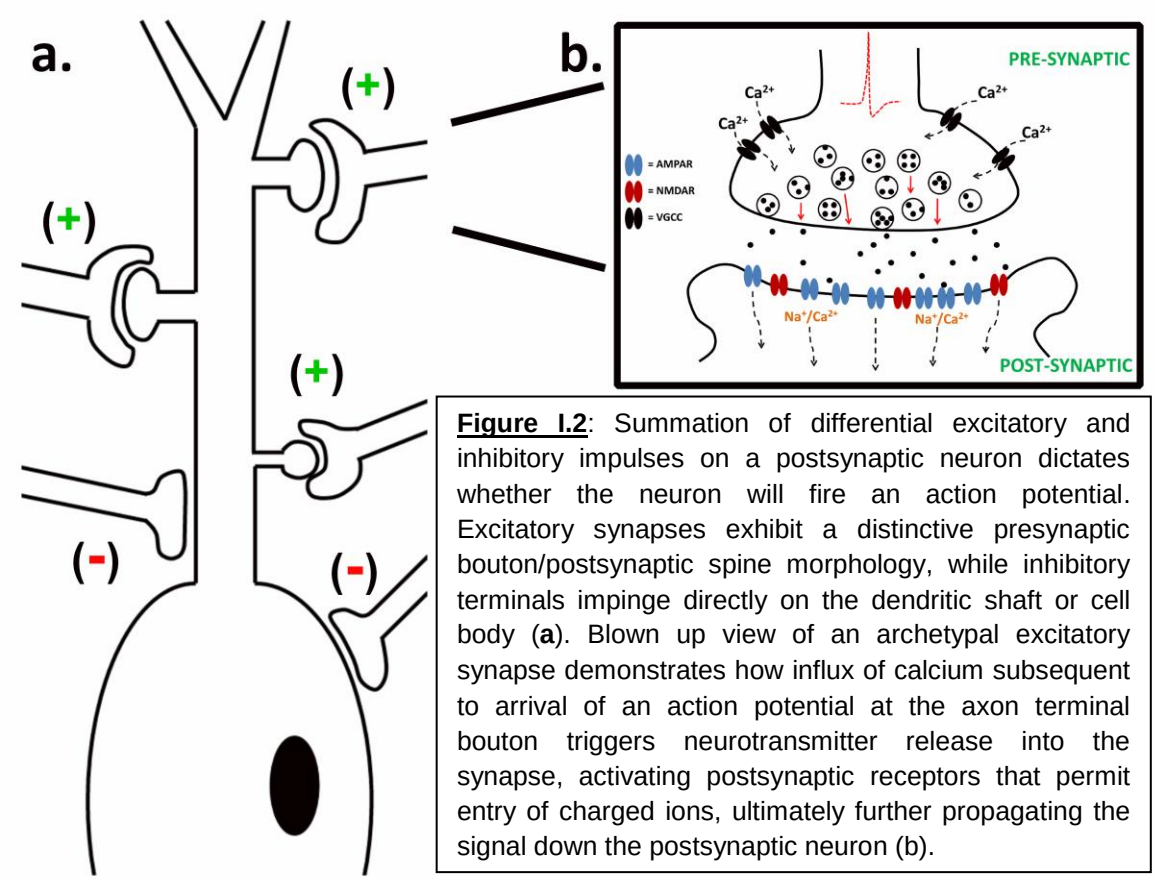


Figure I.2: Summation of differential excitatory and inhibitory impulses on a postsynaptic neuron dictates whether the neuron will fire an action potential. Excitatory synapses exhibit a distinctive presynaptic bouton/postsynaptic spine morphology, while inhibitory terminals impinge directly on the dendritic shaft or cell body (**a**). Blown up view of an archetypal excitatory synapse demonstrates how influx of calcium subsequent to arrival of an action potential at the axon terminal bouton triggers neurotransmitter release into the synapse, activating postsynaptic receptors that permit entry of charged ions, ultimately further propagating the signal down the postsynaptic neuron (b).

Interestingly enough, initial development of these glutamatergic analogues partly arose from the search for novel anti-parasitics and insecticides that were subsequently observed to elicit robust effects on neuronal excitation, echoing the guiding theme that pathological states drive our understanding of basic cellular function (Shinozaki & Konishi, 1970). Kainic acid was among these first analogues described and produced significant excitation with application, albeit to a much more modest degree than L-glutamate (Johnston, Curtis, Davies, & McCulloch, 1974). Structurally similar to kainic acid, quisqualate was similarly shown to elicit excitation, though considerably stronger than kainate and nearly two orders of magnitude more potent than L-glutamate (Shinozaki & Shibuya,

1974; Biscoe et al., 1975). When rank-ordered by potency, these analogues appeared to demonstrate two distinct receptor populations; these quisqualate- and kainate-sensitive receptors are now referred to as AMPA and Kainate respectively.

While kainate and quisqualate were shown to reliably evoke excitation, additional contemporaneous reports showed that structurally distinct aspartate analogues also elicited excitatory responses, albeit with slower kinetics, implying that a class of glutamatergic receptor existed apart from AMPA and Kainate receptors (Johnston, Curtis, Davies, & McCulloch, 1974; Biscoe et al., 1976). Discovery of the robust antagonistic properties of DAA, and later DL-AP5 with much higher potency, on these kainate-/quisqualate-insensitive currents finally resolved this orphan receptor, and it was subsequently named the N-methyl-D-aspartate receptor after its most potent aspartate-derived agonist (McLennan & Hall, 1978; Collingridge & Davies, 1979; Davies & Watkins, 1979; Evans et al., 1979; Davies & Watkins, 1982).

I.4 Unique Characteristics of the N-methyl-D-aspartate Receptor

Experiments immediately revealed that this novel receptor population exhibited a host of unique features distinct from AMPA- and kainate-sensitive receptor types. Chief among the first reports describing NMDA-sensitive receptors was a consistently noted innate sensitivity to magnesium not seen with kainate- and AMPA-type receptors as well as voltage-dependent regenerative currents similar to sodium channels in the axon that produce action potentials

(Ault et al., 1980; MacDonald, Porietis, & Wojtowicz, 1982). These observations have since been revealed to be governed by the same mechanism. Under normal hyperpolarized neuronally quiescent conditions extracellular magnesium infiltrates and blocks the central pore of the NMDA receptor. Upon increasing depolarization from AMPA/Kainate receptor activity, magnesium block of NMDARs is proportionally diminished, and with sufficient depolarization allows ion flux through the channel. Thus, the voltage sensitivity of the NMDA-receptor is a consequence of voltage-dependent magnesium blockade and not due to a receptor-intrinsic physical voltage gate in contrast to axonal sodium channels (Nowak et al., 1984; Mayer, Westbrook, & Guthrie, 1984). Depolarization-induced relief of NMDAR magnesium blockade is consistent with initial studies showing that NMDA application resulted in a slow accumulation of excitation (Johnston et al., 1974), though the significance of this feature would not be fully appreciated until later reports demonstrated that NMDA receptors are essential elements in the molecular mechanisms of learning and memory (discussed in greater detail in Section I.8).

While the innate Mg^{2+} sensitivity of the NMDAR was described, at least phenomenologically, soon after the discovery of the receptor itself, the underlying mechanism(s) that governed this feature remained unknown. Fortuitously, emerging reports on AMPA receptor function would guide researchers to an answer. Experiments conducted by the Seeburg laboratory in 1991 showed that some AMPA receptor subunits, most prominently GluA2, underwent RNA editing

that resulted in substitution of the coded glutamine (Q) codon at a critical channel-forming segment into a charged arginine (R) in certain AMPA subunits, resulting in a number of profound changes in channel activity (Sommer et al., 1991). Among these functional changes observed with mutation of GluA2 Q607 to an arginine was a significant attenuation of the calcium permeability of the receptor (Puchalski et al., 1994). The constitutive prominence of GluA2-containing AMPARs in the synapse under basal neurotransmission has subsequently been suggested to be a neuroprotective adaptation to guard against calcium-induced excitotoxicity. Specific inclusion of GluA2-lacking AMPARs after induction of LTP (discussed later in Section I.8) may represent a positive feedback loop to maintain calcium-dependent functional adaptations (Kim et al., 2001).

The presence of a similarly re-entrant pore-defining region in the NMDA receptor, coupled with reports of the high intrinsic calcium permeability of NMDARs, prompted researchers to speculate a central role for this region in governing ion selectivity. While the native cognate residue in NMDARs (N616) is an asparagine instead of the glutamine or arginine present in AMPARs, reports nevertheless observed bidirectional changes in the calcium selectivity of the mutant receptor contingent on N/Q or N/R mutation (Burnashev et al., 1992; Sakurada, Masu, & Nakanishi, 1993). Unexpectedly, though, these experiments also demonstrated significant attenuation of the magnesium sensitivity of the receptor with manipulation of this site. Ultimately, Retchless, Gao & Johnston

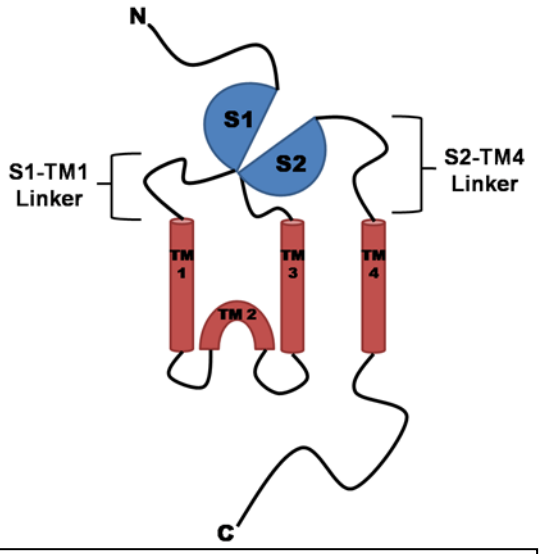


Figure I.3: General schematic depicting the broad topology of GluN subunits. As discussed, NMDARs are composed of 4 such subunits, two glycine-binding GluN1 and two glutamate-binding GluN2, in a complex. The S1 and S2 domains coordinate to define the extracellular ligand binding site, and the membrane-spanning domains, including the M2 reentrant loop, are depicted in red.

(2012) have shown that, while the sites probed by Burnashev et al. (1992) and Sakurada et al. (1993) participated in defining Mg^{2+} sensitivity, a single residue within the M3 domain of the GluN2 subunit (S632 in GluN2A) coordinates with residues (largely W608) in the M2 loop of GluN1 to primarily define the interacting site for Mg^{2+} action (for a schematic of basic GluN subunit topology see Figure 3). Additionally, comparison of residue homology between the four flavors of GluN2 subunits (A-D) revealed that innately different substitutions at the respective homologous GluN2 M3 residues correlated significantly with both the intrinsically lower Mg^{2+} sensitivities observed with GluN2C/D-containing receptors versus their A/B counterparts as well as their intrinsically different relative Ca^{2+} permeabilities.

Parsing the different glutamate-sensitive ion channels via pharmacological agents led many to wonder at the contributions of each receptor type in synaptic signaling. By recording currents in the presence and absence of the high potency NMDAR antagonist AP5, Jahr & Stevens (1987) dichotomized the receptor

populations based on response times to glutamate activation into a “fast” component group comprised of AMPA and Kainate receptors, and a “slow” component group consisting largely of NMDARs. Importantly, this AP5-sensitive, slow component population appeared to exclusively govern the induction of synaptic potentiation after high frequency stimulation, leading others to speculate that activation of NMDARs was the mechanism underlying the most common forms of synaptic potentiation. The aforementioned study by Jahr & Stevens (1987) showed a high apparent calcium permeability of NMDARs, and results from subsequent studies showed that indeed NMDARs exhibit an exceptional selectivity for Ca^{2+} flux that by some estimates mediates upwards of 50% of total charge transfer during excitation (Murphy, Thayer, & Miller, 1987; Forsythe & Westbrook, 1988). The significance of this feature is discussed in further detail in Section I.8, though in short it was observed in later studies that this high Ca^{2+} permeability acted as a link in an elegant signaling cascade between NMDAR-mediated activity and induction of activity of calcium-sensitive secondary effector proteins that drive synaptic strengthening. Interestingly, the studies discussed above demonstrating the dependency of Mg^{2+} sensitivity on individual residues in the reentrant M2 loop simultaneously revealed that these residues also govern the intrinsically high Ca^{2+} permeability of the NMDA receptor.

The interaction of multiple amino acids and domains in establishing basic functional parameters of ion channels has become a guiding theme in pharmacological research, and the studies outlined above provided the

foundation of comparative analysis in understanding the behavior of different receptor types. Indeed, until recently the AMPA receptor crystal structure served as the basis for hypothesized structures of the NMDA receptor (Sobolevsky, Rooney, & Wollmuth, 2002). Recently, however, the structure of the GluN2B-containing NMDAR was solved simultaneously by two independent groups (Karakas and Furukawa, 2014; Lee et al., 2014). Such homology comparisons have proven essential to our understanding of ethanol action, and their implementation along with single channel electrophysiological recordings have enabled researchers to parse the discrete domains and gating steps that elicit channel activation (discussed more thoroughly in Section II.1).

I.5 NMDARs Revealed to be Primary Sites of Ethanol Action

By this time, the broad strokes of innate NMDAR function and its role in synaptic transmission were rudimentally understood, but the question of how ethanol inhibited excitation remained open. As previously discussed in Section I.2, ethanol was shown, in addition to potentiating GABA-mediated inhibition, to directly affect excitatory post-synaptic activity (Nicoll, 1972). With the discrimination of three distinct excitatory, glutamate-sensitive receptor populations, divining substrate-specific actions of ethanol on neuronal excitation became possible. Seminal work by Lovinger, White, and Weight (1989) demonstrated that pharmacologically isolated NMDA currents were significantly inhibited by ethanol at concentrations associated with behavioral intoxication, while AMPA and Kainate receptor populations exhibited modest, if any,

attenuation at these concentrations. Given the emerging role of NMDARs in synaptic signaling, strengthening, and cognition, these results simultaneously aligned with behavioral manifestations of intoxication including depressed cognitive function and, with higher levels of intoxication, impairment of memory formation, as well as further intimated the elemental role of NMDARs in learning and memory.

I.6 Mechanisms of Direct Inhibition of NMDAR Function by Ethanol

While Lovinger, White, and Weight (1989) showed that the NMDA receptor constitutes a primary site of ethanol action, it nevertheless remained unclear precisely how ethanol blunts NMDAR-mediated current. Early reports hinted that ethanol may interfere with glycine binding to the GluN1 subunit, and indeed such findings proved contentious from the start. Initially, experiments appeared to show that ethanol inhibition of NMDAR-mediated calcium influx in cerebellar granule cells and NMDA-stimulated dopamine release in striatal slices could be blunted by the addition of increasing concentrations of glycine (Rabe & Tabakoff, 1990; Woodward & Gonzales, 1990). Later experiments examining NMDA currents in hippocampal neurons showed no ethanol-induced shift in the glycine EC_{50} as would be expected with a competitive antagonist (Peoples & Weight, 1992). The matter would not seem definitively settled, however, until work by Mirshahi and Woodward (1995) demonstrated no shift in glycine EC_{50} with ethanol application in recombinantly expressed GluN2A- and GluN2C-containing NMDARs. The disparity of results between these studies was likely

due to differing glycine sensitivities of fully complemented receptors in intact tissue: indeed, studies have shown that glycine sensitivity of the GluN1 subunit is affected by what concomitant GluN2 subunit is expressed, such that otherwise comparatively ethanol-resistant receptor populations could be recruited with increasing glycine concentrations in native tissue (Peoples & Weight, 1992; Ronald, Mirshahi, & Woodward, 2001; Chen et al., 2008). Consistent among all studies, though, was the observation that ethanol did not directly compete with glutamate or NMDA binding, nor did ethanol affect the magnesium sensitivity of the channel (Chu, Anantharam, & Treistman, 1995; Bhave et al., 1996). Thus, it was determined that ethanol inhibits NMDAR function via non-competitive, voltage-independent mechanisms that are distinct from any actions as a pore blocker.

Subsequent single-channel experiments by Wright, Peoples and Weight (1996) ultimately showed that ethanol inhibited NMDA receptor activity by reducing mean channel open time and channel open probability, consistent with its role as an allosteric modulator of channel gating. Further work with NMDARs found that, while truncation of C-termini of GluN1 or GluN2 could modify the ethanol sensitivity of the NMDA receptor, it was primarily extracellular, not intracellular, application of ethanol that resulted in NMDAR inhibition, suggesting that, while the C-termini could modulate the ethanol sensitivity of the channel, these effects were likely subsequent to ethanol interactions with core gating elements (Peoples & Stewart, 2000). Indeed, mutagenesis studies of the $GABA_A$

receptor revealed that when specific residues within the transmembrane 2 (TM2) or transmembrane 3 (TM3) domains were mutated the alcohol-induced potentiation in current was significantly attenuated **(**Mihic et al., 1997**)**. Based on this data, Ronald, Mirshahi and Woodward (2001) hypothesized that ethanol similarly acts as a modulator of NMDAR function by interactions with transmembrane elements. A homology scan of the TM domains in GluN1 and GluN2A-D subunits revealed a single residue in particular, F639 of GluN1 and the homologous sites in the modestly differentiated GluN2 subunits, of the TM3 domain was highly conserved among NMDA subunits. Mutation of this residue in GluN1 elicited robust reductions in ethanol sensitivity that occurred regardless of what GluN2 subunit is expressed. Interestingly, attenuation of ethanol sensitivity appeared positively correlated with the volume of the substituent (i.e. - the most robust reductions in ethanol sensitivity were observed with smaller amino acids) (Smothers & Woodward, 2006). Recent studies, though, seem to both challenge and confirm this observation, as substitution of GluN1 F639 with a large tryptophan elicited attenuations in ethanol sensitivity comparable to GluN1 (F639A) (Ren et al., 2012), while another study showed that GluN1 (F639C) restored wild type ethanol sensitivity following treatment with MTS reagents that covalently bind to cysteine residues, increasing their volume occupancy (Xu, Smothers, & Woodward, 2015). The discrepancy between these results remains an open question and will require further study. Functionally, substituent size also correlated positively (ie - smaller substituents displayed higher glycine sensitivity) with increases in glycine EC_{50}, and suggests that amino acid/ethanol co-

occupancy within this molecular space partially governs reductions in channel activity (Smothers & Woodward, 2006). Importantly, this residue does not face the pore, both confirming prior data ruling out any actions of ethanol as a pore blocker while also intimating possible interactions with residues in other gating elements including TM4 (Mirshahi & Woodward, 1995).

In Ronald, Mirshahi and Woodward (2001) the authors were unable to generate functional receptors in *X. laevis* oocytes expressing GluN1/GluN2A (F637A) mutants, however subsequent work by Ren et al. (2007) using human embryonic kidney (HEK) cells did obtain functional receptors and found that manipulation of the GluN2A (F637) site resulted in profound effects on intrinsic receptor function distinct from those observed with GluN1 (F639A)/GluN2A receptors. Perhaps most importantly though, changes in ethanol potency seen with GluN1/GluN2A (F637X) were negatively correlated with the volume of the substituent amino acid (i.e. - increasing substituent size increasingly blunted ethanol sensitivity), in contrast to GluN1 (F639X)/GluN2A mutants in which a robust positive correlation was observed. Furthermore, with GluN1/GluN2A (F637X) receptors, the volume occupied did not significantly correlate with changes in mean open time of the channel, suggesting that this site in GluN2 could instead act as a barrier to ethanol infiltration of adjacent sites (Ren et al., 2007). Indeed, substituent-specific effects on basic channel properties would be expected if ethanol interacted directly with this site. The disparity between these subunit-specific results and the highly homologous TM3 sequences between

GluN1 and GluN2 was initially attributed to a hypothesized structural shift of about 4 amino acids between the subunits (Sobolevsky, Rooney, & Wollmuth, 2002). However, results from recent crystallographic studies of the GluN1/GluN2B structure show that this is not the case (Sobolevsky, Rooney, & Wollmuth, 2002; Karakas & Furukawa, 2015; Science; Lee et al., 2014). In addition, a recent study recapitulated the aforementioned experiments with GluN2B-containing receptors and obtained results similar to those seen with GluN2A-containing NMDARs were observed with GluN1/GluN2B (F637X) (Zhao et al., 2015).

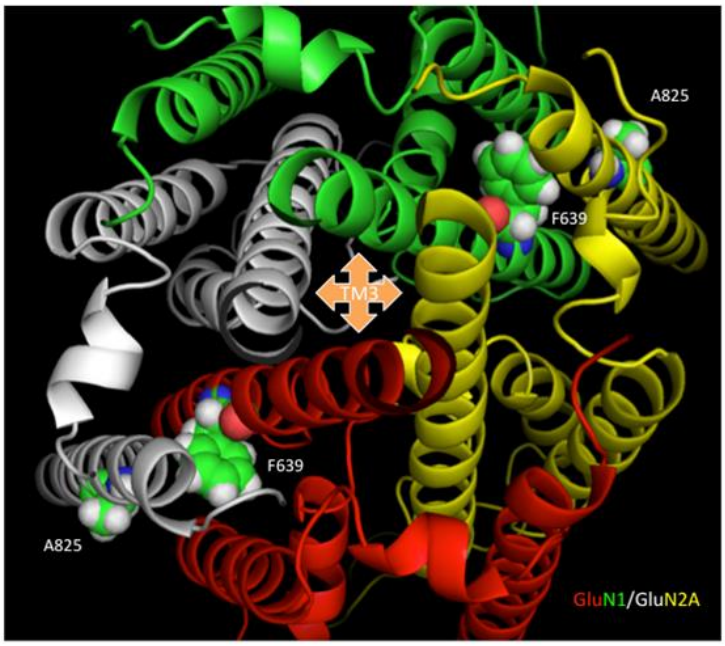


Figure I.4: Overhead view of the transmembrane domains that define the central ion-gating pore. Positions of GluN1 F639 residues are shown relative to A825 residues in the M4 domain of GluN2A. Note the size disparity between the two residues, as well as the seeming orientation of A825 toward the lipid interface instead of a more direct interaction with F639.

This disparity between GluN1 and GluN2 TM3 mutations with regard to their effects on ethanol inhibition may reflect an intrinsic asymmetrical contribution of the subunits and their respective gating domains to receptor

function, consistent with emerging reports (Kazi et al., 2013). Indeed, work by Xu et al. (2012) has further shown that when GluN2A- and GluN2B-containing NMDARs are rendered constitutively open, they display GluN2-subtype specific changes in ethanol sensitivity. Specifically, it was observed that GluN2A-containing receptors showed comparable decreases in ethanol sensitivity subsequent to either the GluN1 or GluN2 subunit being rendered open, with the larger effect observed with GluN2A. GluN2B-containing NMDARs on the other hand showed a robust decrease in ethanol sensitivity with GluN2 manipulation well above that observed with manipulation of GluN1, implying a higher proportional contribution of the GluN2B subunit to ethanol sensitivity in this receptor type. These data pose an interesting question; namely, whether the inherent difference in ethanol sensitivity observed between GluN2A- and GluN2B-containing NMDA receptor types is a product of intrinsically different gating mechanisms. This observation forms an essential part of the underlying hypothesis for the experiments discussed in Section II.

The central location of the F639 residue and the incomplete elimination of ethanol sensitivity of NMDARs following mutation at this site suggest that other sites may participate in channel inhibition. Further experiments discovered a residue, M823, in the TM4 domain of GluN2A that when mutated resulted in significantly attenuated ethanol sensitivity (Ren, Honse, & Peoples, 2003). The most robust attenuation of ethanol sensitivity was observed with mutation of M823 to a tryptophan (W), while the decrease in ethanol sensitivity observed with

other amino acid substitutions generally correlated negatively with spatial volume of the amino acid used. Importantly, though, manipulation of this site resulted in profound effects on basic channel properties including enhanced receptor desensitization, complicating an interpretation for M823 as a direct interacting site for ethanol. Thus, other residues proximal to the highly conserved M823 were evaluated for effects on ethanol inhibition, ultimately resulting in identification of other candidate targets of ethanol action by Honse et al. (2004). The essential finding of this report was that manipulation of residue A825 in the TM4 of GluN2A resulted in a reduction in ethanol sensitivity over and above that observed with similar manipulations of not only M823, but as well residues distal and proximal to A825. Furthermore, manipulation of A825 resulted in few changes in intrinsic receptor function, in contrast to M823. Later experiments showed that the analogous residue in GluN2B, G826, could elicit similar reductions in ethanol sensitivity of the channel. Importantly, though, both alanine and especially glycine residues lack appreciable side-chains for direct ethanol interactions, and in the case of glycine, may be required to impart essential tertiary structure to the helix, thus limiting interpretation of this data as evidence for a true site of ethanol action (Smothers & Woodward, abstract presented at the Annual Meeting of the Society for Neuroscience, 2005; Zhao et al., 2015).

Caveats aside, the lack of intrinsic alterations in receptor function with mutation of GluN2A (A825)/GluN2B (G826) similarly distinguishes this site as a potential gate-keeper for ethanol access into the central TM3 domains, not unlike

the effects observed with GluN1/GluN2 (F637) mutants. When both GluN1 (F639A) and GluN2A (A825W) were co-expressed, an appreciable increase in ethanol IC_{50} over and above single mutants resulted, intimating that these sites do not specifically interact to regulate ethanol sensitivity (Ren et al., 2012). Indeed, by definition if these residues were truly epistatic in defining an alcohol site one would expect a non-additive effect on ethanol sensitivity subsequent to tandem site mutation. Because of this, other pairs of residues between GluN1 TM3 and GluN2A TM4, as well as GluN1 TM4 and GluN2A TM3, were evaluated for potential interacting pairs that coordinate and define a site of ethanol action. The results indicated that residues adjacent to GluN1 (F639), G638 in particular, similarly resulted in robust increases in ethanol IC_{50} observed by Ronald, Mirshahi, and Woodward (2001) and that, along with F639, appeared to interact strongly with M823 and L824 in the TM4 of GluN2A. Neither residue, however, appeared to interact with A825 (see Figure I.4), consistent with structural models showing the side chain of A825 is pointed away from other proximal amino acids, and could be indicative of actions of ethanol on protein-lipid interactions (Sobolevsky, Rooney, & Wollmuth, 2002; Karakas & Furukawa, 2014; Lee et al., 2014). Surprisingly, though, when cognate GluN1 TM4 and GluN2A TM3 pairs were evaluated the authors observed significant attenuations in ethanol sensitivity with individual mutation of GluN2A TM3 residues, but no apparent shift in ethanol IC_{50} subsequent to mutation of GluN1 TM4 residues. Indeed, if interactions between the GluN1 TM4 and GluN2A TM3 domains were truly homologous to GluN1 TM3/GluN2A TM4 interactions one would expect robust

shifts with single mutation of cognate GluN1 TM4 residues. Thus it appears the fundamental interactions of GluN1 TM4 and GluN2 TM4 with other receptor domains are likely very different, consistent with observations that GluN1 and GluN2 do not contribute homologously to channel activation (Banke & Traynelis, 2003; Kazi et al., 2013; Cummings & Popescu, 2015). Residues L819 of GluN1 and F637 of GluN2A, though, did appear to coordinate robustly in defining the desensitization rate of the channel, reinforcing models that implicate the movement of TM3 directly in ion channel gating with TM4 modulating gating indirectly via its interactions with TM3 (Jones, VanDongen, & VanDongen, 2002; Ren, Honse, & Peoples, 2003). Thus, from these experiments it can be concluded that ethanol interacts both directly with TM3 residues to impede channel gating as well as disrupts the modulatory functions of TM4 in the gating cascade, and suggests TM4 residues are more than simple barriers to ethanol access.

The participation of a canonical modulatory domain such as TM4 in defining ethanol sensitivity of the NMDAR suggests the possibility that similarly modulatory regions may participate as well. Indeed, a number of extracellular and intracellular domains within the NMDA receptor are known to impact channel activity. Specifically, work by Gielen et al. (2009) showed that the amino terminal domain (ATD) interacts robustly with the ligand binding domain (LBD) of the GluN2 subunit, substantially affecting the spontaneous opening/closure of the ligand binding cleft. Based on these observations, Smothers, Jin, and Woodward

(2013) generated ATD-deleted GluN1 and GluN2 subunits and evaluated the ethanol sensitivity of these truncated mutants. Interestingly, the authors observed that deletion of the ATD of GluN2A subunit resulted in a significant increase in ethanol sensitivity of the receptor. While this effect could be attributable to relief of Zn^{2+} inhibition, this seems unlikely since prior reports by this laboratory showed that ethanol sensitivity was unchanged in receptors containing a point mutation that renders them zinc insensitive (Woodward & Smothers, 2003). A likelier explanation may instead rest on observations that ATD-deletion in GluN2A receptors results in reductions in channel open probability to similar levels seen with GluN2B receptors (Gielen et al., 2009). Therefore, in wild type GluN2A receptors, ethanol inhibition may be less substantial due to the intrinsically higher open probability as compared to GluN2B-containing receptors. ATD-deletion of GluN2A yields receptors with a lower open probability thus permitting an increase in the efficacy of ethanol (Smothers, Jin, & Woodward, 2013).

Several studies have shown that in brain, pharmacological antagonism or genetic elimination of GluN2B renders residual NMDAR currents ethanol insensitive, suggesting that GluN2B-containing NMDARs constitute the primary site of ethanol action on NMDAR currents (Roberto et al., 2004; Kash, Matthews, & Winder, 2008; Wills et al., 2012). As GluN2B selective antagonists such as ifenprodil exert their effects via interactions with the ATD, these results suggest that this domain is an important site of action for ethanol. In the Smothers, Jin,

and Woodward (2013) study, however, robust inhibition of GluN2B-containing NMDAR current by ethanol was still observed in mutants lacking the ATD that abolished the effects of ifenprodil. This observation raises the possibility that, while ethanol and GluN2B-selective antagonists operate at different sites on the receptor, their effects on channel function converge. Indeed, others have shown that ifenprodil elicits reductions in mean open time and channel open probability in a fashion similar to those observed with ethanol (Wright, Peoples, & Weight, 1996; Amico-Ruvio et al., 2012; Bhatt et al., 2013). Functionally, ATD-deletion of GluN2B results in no observable change in open probability, and further supports the conclusion that changes in open probability likely underlie the results seen with GluN2A ATD-deletion (Gielen et al.; 2009). In sum, it appears that the amino terminal domain, while a potent regulator of ligand-mediated channel gating, is not a primary site of ethanol action (Smothers, Jin, & Woodward, 2013). Nevertheless, probing the actions of the ATD on channel function has revealed that intrinsic receptor characteristics such as open probability partially dictate the differential ethanol sensitivities observed between GluN2 subtypes. These results are thus also important in informing the hypothetical basis for the experiments described in Section II.

I.7 Design-Specific Disparities in Ethanol Sensitivity Reveal Alternative Mechanisms of Alcohol Inhibition

It is important to note that while the aforementioned studies consistently show intramolecular actions of ethanol on NMDA receptor function, the effective

dose ranges appear to conflict with studies of NMDAR inhibition in neurons. Typical circulating blood levels of alcohol in an intoxicated person hover in the 0.08-0.30 g/dL range and roughly correspond to millimolar concentrations of 17-33. Most studies of ethanol action on recombinant NMDA receptors typically report ~30% inhibition of current with application of 60 mM ethanol, in stark contrast to the >60% inhibition observed by Lovinger et al. (1989) (although this figure has since been amended by other experiments to a more generally accepted value of ~50%) (Chandler, Sumners, & Crews, 1993). This discrepancy highlights a potentially important disparity between native and recombinantly-expressed NMDARs and suggests that additional factors likely participate in ethanol inhibition of NMDARs *in vivo*. Indeed, increasing evidence suggests the activity of kinases including Fyn tyrosine kinase potentiate NMDAR-mediated currents in neuronal tissue and blunt the ethanol sensitivity of this population. Further reports have modified this schema to include the activity of an NMDA-interacting phosphatase, Striatal-enriched Tyrosine Phosphatase (STEP), that when knocked out *in vivo* appears to abolish ethanol sensitivity of NMDAR-mediated current (Hicklin et al., 2011). As discussed more thoroughly in Section I.9, the action of these two secondary effectors on NMDAR-mediated currents appear to involve regulation of surface expression rather than direct actions on intrinsic receptor function. Nevertheless, while diametrically opposed, their mechanisms of action were both elucidated by experimental methods that critically hinged on both native and recombinant expression systems.

While the interplay of these mechanisms is discussed in greater detail later, it is prudent to further detail how the profound behavioral impairments observed with circulating levels of ethanol may differ from the relatively low level of NMDAR antagonism. This relationship between low efficacy and resulting high phenotypic response to NMDAR antagonism is not simply an idiosyncratic property of ethanol, as low doses of AP5 and resultant NMDA inhibition have similarly been shown to abolish NMDA-dependent up states in neuronal firing (Tu et al., 2007). This discrepancy implies not only a substantial role for NMDARs in cellular processes beyond simple signal propagation, but as well that in addition to direct actions on channel function these NMDA-dependent cascades may themselves be intrinsically sensitive to alcohol.

At the most basic level, the ability of drugs like ethanol to elicit substantial behavioral effects despite relatively modest inhibition of NMDARs may be related to the low levels of NMDAR expression at the synapse. Electron microscope studies of excitatory synapses have revealed that, unlike AMPA receptors, NMDA receptors exhibit a relatively intractable level of expression, averaging about 50 receptors per synapse regardless of size (Racca et al., 2000). AMPA receptors by comparison can vary widely in their expression, ranging from zero receptors in a synapse (a so-called “silent” synapse) to over 150 in large synapses (Nusser et al., 1998; Takumi et al., 1999). Thus the cumulative effect of 50% inhibition of NMDAR-mediated current results in the silencing of half the NMDA receptor population of nearly all excitatory synapses, and this may have

profound implications when one considers the paramount importance of NMDA receptor signaling in processes including cortical up-states, learning, and memory. In the next section, then, I will provide a brief primer on the role of NMDA receptors in the machinery that governs synaptic plasticity, the molecular basis of learning and memory, and attempt to reconcile the disparate levels of alcohol inhibition on NMDA receptor signaling observed between reductionist, recombinant experiments and those conducted in neuronal tissue.

I.8 NMDARs are Essential Regulators of Synaptic Plasticity

Excitatory glutamatergic synapses are morphologically defined by a feature called the post synaptic density (PSD) in which a host of scaffolding proteins assemble to form an extended matrix that incorporates a number of receptors and secondary effector proteins. Electron micrographs first revealed this structure as a large electron dense array embedded in spines along dendritic branches, and subsequent experiments established that PSD-containing synapses appear to be exclusively glutamatergic (Westrum & Blackstad, 1962; Allison et al., 1998). While successive reports continue to elucidate the finer points of PSD function, the central role of the PSD is to act as a molecular hub for the efficient transduction of trans-synaptic signaling between ion channels and secondary effector proteins that regulate receptor expression as well as basic cellular function (for review see Kennedy, 2000). Structurally, the PSD is a conglomerate of scaffolding proteins largely composed of membrane-associated guanylate kinases (MAGUKs) that are suspended within an actin cytoskeleton.

An essential feature of MAGUKs are the presence of multiple PDZ domains that allow the formation of peptide bonds with a number of different proteins simultaneously, ultimately permitting the assembly and organization of large, diverse signaling complexes at the cell membrane (Doyle et al., 1996; Hsueh, Kim, & Sheng, 1997). In tandem with cycling of the actin cytoskeleton between filamentous and globular forms, the integration and disintegration of MAGUK-protein interactions represent the most basic form of excitatory synaptic plasticity (Allison et al., 1998).

Interestingly, among the first MAGUK-interacting proteins discovered was the NMDA receptor, and indeed a large majority of neuronal NMDA receptors are found within this molecular milieu (Kornau et al., 1995; Niethammer, Kim, & Sheng, 1996). Many subtypes of NMDARs, though most notably GluN2A- and GluN2B-containing receptors, have extensive C-termini with myriad different consensus sequences that recognize not only PDZ domains but as well a host of different kinases and phosphatases (Kornau et al., 1995; Niethammer, Kim, & Sheng, 1996; Zhou et al., 2007; for review see Salter and Kalia, 2004). As previously discussed in Section I.4, NMDA receptors display unique channel characteristics including voltage dependence and a high calcium permeability relative to other glutamatergic ion channels. These properties in conjunction with an apparent affinity for C-terminal protein-protein interactions led many to hypothesize that the NMDA receptor itself may function as a fundamental link

between membrane depolarization and induction of cytoskeletal rearrangements associated with activity-dependent phenomena including long term potentiation.

Donald Hebb first postulated that learning was the aggregate result of distributed synaptic strengthenings, specifically positing that repeated pre-synaptic stimulations at a synapse would elicit enhanced post-synaptic responses over time. Owing to its veracity, this model has since been termed “Hebbian plasticity” (Hebb, 1949). It would not be until 1973, however, that this phenomenon would be experimentally described when Bliss and Lomo (1973) observed heightened synaptic responses in the dentate gyrus subsequent to high frequency stimulation of the perforant pathway stimulation. While the particulars of that phenomena, which they dubbed “long term potentiation” (hereafter referred to as LTP), vary, it is generally understood to constitute enhanced synaptic transmission after induction that persists for >30min and is often, though not always, post-synaptic and NMDA-dependent (for review see Nicoll & Roche, 2013). For the purposes of this discussion only NMDA-dependent LTP will be addressed.

The observation of synaptic LTP posed two important questions: namely, (1) what type(s) of receptors mediate this phenomenon, and (2) by what mechanism do these receptors induce synaptic potentiation? Using a variety of pharmacological agents Collingridge, Kehl, and McLennan (1983) demonstrated that kainate and AMPA receptors largely mediate fast excitatory synaptic transmission while NMDARs seem to contribute only modestly. Importantly and

somewhat counter-intuitively, the authors observed that application of an NMDAR-selective antagonist, DL-AP5, blocked the induction of synaptic potentiation, heavily implying a central role for the NMDA receptor in governing LTP. Additional experiments further showed that, while NMDARs are essential for the induction of LTP, AMPA-mediated currents appear to underlie the actual expression and maintenance of enhanced synaptic signaling, suggesting NMDARs are themselves not significantly potentiated (Muller, Joly, & Lynch, 1988; Davies et al., 1989).

As discussed previously, a number of idiosyncratic features make the NMDA receptor uniquely suited to fulfill a role as a central hub for coordinating synaptic signaling with the mechanics of synaptic strengthening. Among these features is the inherent voltage-dependent blockade of NMDARs by magnesium, whose relevance to long term potentiation became apparent subsequent to work published by Herron et al. (1986). In this study, the authors showed that induction of LTP was frequency-specific, requiring high levels of stimulation to relieve this magnesium block and allow ion flow through NMDARs. Thus, in concert with the NMDA receptor's intrinsically high calcium permeability, voltage-dependent magnesium blockade permits the NMDA receptor to act as a coincidence detector linking post-synaptic depolarization, via synaptic activation of AMPA/Kainate receptors, back-propagating depolarization from firing of an action potential, or both, with calcium-sensitive cellular mechanisms of synaptic potentiation.

Importantly, initial studies of LTP showed that expression of the phenomenon was not only sensitive to extracellular concentrations of Mg^{2+}, consistent with a central role for NMDARs in eliciting LTP, but to levels of free calcium as well (Dunwiddie & Lynch, 1979). Further experiments by this group revealed that high frequency stimulation of hippocampal tissue in low-calcium extracellular solution did not exhibit enhanced binding of tritiated glutamate observed with normal calcium levels, indicating calcium may be essential to post-synaptic mechanisms of potentiation (Lynch, Halpain, & Baudry, 1982). By intracellularly injecting EGTA, a calcium chelator, into the post-synaptic neuron, Lynch et al. (1983) determined that LTP is indeed a post-synaptic phenomenon, dependent on influx of extracellular Ca^{2+} into the post-synaptic neuron, later shown to occur specifically via influx through NMDA receptors (Frank et al., 1989).

Though at this point it was understood that the long-term cellular processes induced by NMDA activity relied significantly on calcium flux through NMDARs, the ultimate consequence of this calcium influx was not known. Davies et al. (1989) observed slow increases in the sensitivity of neurons to iontophoretically-applied AMPA receptor agonists after induction of LTP suggesting AMPARs mediate the expression of LTP. It remained unclear, however, if this was due to a functional change in the unitary conductance of the AMPA receptor or to increased AMPAR expression. More fundamentally, though, a link between NMDAR-mediated Ca^{2+} influx and this AMPA-mediated current

facilitation was lacking. Owing to the vast expression of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in the forebrain, by some estimates comprising nearly 2% of the total protein, CaMKII began to emerge as a possible intermediary in this cascade (Erondu & Kennedy, 1985). Experiments conducted by Malinow, Madison, and Tsien (1988) were among the first to pose and test the hypothesis that induction of protein kinase activity could link Ca^{2+} influx through NMDARs to enhancement of AMPA-mediated currents. Using intracellular injection of calmidazolium, a calmodulin antagonist, it was further shown that activation of CaMKII, a dodecameric kinase activated by calcium-bound calmodulin, is required for eliciting post-synaptic potentiation (Malenka et al., 1989). Importantly, experiments showed that the role of NMDA receptors in CaMKII activity was two-fold: namely, Ca^{2+} influx through NMDARs activated calmodulin binding to CaMKII, and the extensive C-terminal tails of GluN2 subunits (GluN2B in particular) act as molecular scaffolds for CaMKII to autophosphorylate its own regulatory domains thus further enhancing activity (Strack & Colbran, 1998; Leonard et al., 2002; Bayer et al., 2001).

While the observed effects of CaMKII antagonists on LTP suggested a necessary role for the protein, the final link between CaMKII activity and upregulation of AMPA current remained an open question. A tentative answer emerged when experiments by Tan, Wenthold, and Soderling (1994) showed that treatment of cultured hippocampal neurons with stimulators of CaMKII and PKA kinases resulted in a significant increase in AMPA receptor phosphorylation

that was blocked by pretreatment with the NMDA receptor antagonist AP5, or KN-62, a CaMKII inhibitor. Later studies ultimately showed that AMPA subunits, GluA1 in particular, displayed CaMKII consensus sequences that exhibited a strong positive correlation between their phosphorylation state and the magnitude of AMPA current potentiation (Barria et al., 1997; Mammen et al., 1997; Barria, Derkach, & Soderling, 1997). While contemporaneous evidence suggested that phosphorylation of AMPAR subunits was itself sufficient to increase AMPA-mediated current (Raymond, Blackstone, & Huganir, 1993), other mechanisms likely mediated upregulated AMPA current after LTP induction. Indeed, experiments performed by Ehlers (2000) demonstrated that elevated levels of synaptic activity could induce reinsertion of endosomally bound AMPARs into the plasma membrane. Further work similarly showed that induction of LTP critically relied on AMPA receptor insertion into the plasma membrane, definitively demonstrating that LTP-induced upregulation of AMPA current was the product of receptor phosphorylation that then signaled membrane targeting (Lu et al., 2001). Refinements to this model continued to emerge, ultimately revealing that differential subunit phosphorylation drove not only GluA1-containing AMPAR insertion into the membrane, but as well simultaneous GluA2-containing receptor internalization (Chung et al., 2000; Esteban et al., 2003).

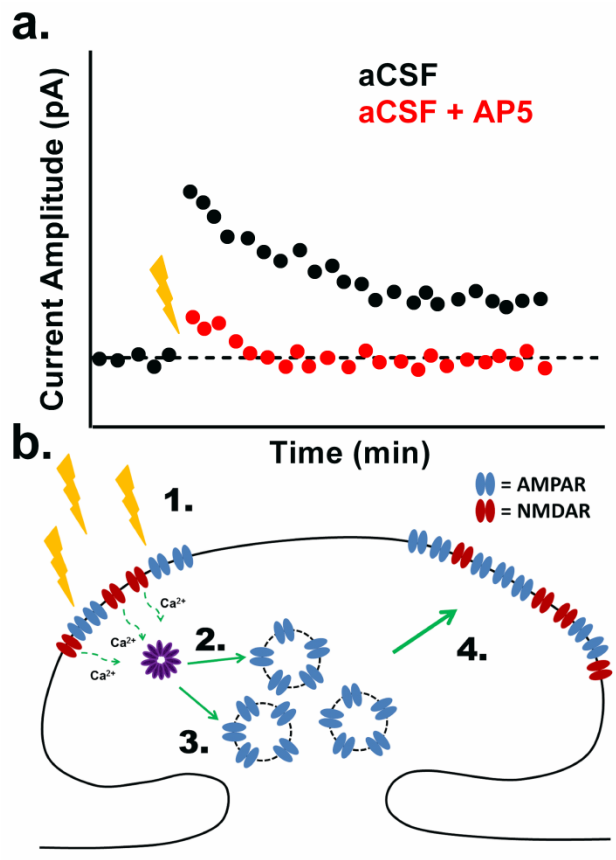


Figure I.5: Many forms of plasticity are NMDA dependent. Panel (a.) demonstrates that after high frequency stimulation (usually 3-4 1 sec bursts of 100 Hz stimulation) current amplitudes of the recorded neuron increase substantially, and remain elevated for hours after LTP induction. When an NMDA antagonist (AP5) is present during stimulation, though, LTP is not induced. Panel (b.) illustrates the molecular mechanisms underlying observed increases in current amplitude following LTP induction. 1.) High frequency stimulation causes AMPARs to open, relieving Mg^{2+} blockade of NMDARs and permitting subsequent Ca^{2+} influx. 2.) Ca^{2+} then binds Calmodulin, activating CaMKII (purple), that then 3.) phosphorylates endosomally-bound AMPA receptors, 4.) signaling their insertion into the plasma membrane. The net result is thus a "potentiated" synapse.

While the mechanism of LTP thus described represents only one of a number of NMDA-dependent forms of plasticity, it nevertheless highlights the multiple interactions and events that NMDA receptor activity governs that guide and shape homeostasis. That homeostasis is itself such a dynamic process has led many to question whether drugs of abuse impair or co-opt this innate machinery to drive drug-seeking behavior. Indeed emerging evidence suggests dysfunction in NMDA receptor homeostasis is, if not a primary etiology, a secondary pathology of extended alcohol exposure that seems to promote maladaptive seeking behaviors. The dichotomy of alcohol action between acute

inhibition of NMDAR function and secondary disruptions of NMDA-dependent plasticity and homeostasis is an interesting phenomenon, and indeed it remains unclear as to whether the two events are mutually exclusive. This suggestion forms the hypothetical basis for experiments detailed in Section III. In any case, the alcohol-induced changes in NMDA receptor function and expression with chronic exposure appear to be driven by mechanisms distinct from those described that drive LTP, and constitute a form of “meta-plasticity”, in which the regulators of plasticity themselves become the targets of modulation, and serves to further underscore the critical importance of NMDARs in pathology of alcoholism.

I.9 Homeostatic Changes in NMDA Receptor Expression After Extended Alcohol Exposure

Given the pivotal role of the NMDA receptor in eliciting plasticity, inhibition of its activity should have profound effects on normal synaptic function even with low levels of antagonism, echoing the point made in Section I.7. Furthermore, the previously detailed two-fold role of NMDARs in eliciting plasticity, specifically their high innate calcium permeability and predisposition to act as a molecular scaffold, explains the high phenotypic response to relatively modest levels of NMDA antagonism by alcohol. In fact, as will be detailed, substantial evidence has emerged suggesting that alcohol inhibits not only NMDAR channel gating, but additionally alters the regulation of receptor surface expression in a highly dynamic fashion.

Although results from initial experiments by Peoples and Stewart (2000) discounted a role of intracellular domains in mediating the direct actions of alcohol on NMDA receptor function, the prevalence of studies suggesting that secondary effector proteins known to associate with NMDARs could influence ethanol sensitivity in neurons nevertheless accumulated. Among the first reports describing this phenomena were observations by Miyakawa et al. (1997). In this study, knockout of Fyn, a neuronally enriched tyrosine kinase and intimate associate of NMDA receptors, in mice resulted in the elimination of acute tolerance to ethanol inhibition typically seen with extended alcohol application. This finding fostered other studies to determine what role, if any, kinases had in governing the sensitivity of NMDARs to acute ethanol exposure.

Chief among these was a report by Anders et. al. (1999) that showed a robust reduction in ethanol sensitivity, and concomitant increase in tyrosine phosphorylation, of recombinantly expressed GluN2A-containing NMDARs when co-expressed with Fyn. Oddly, though, no effect was seen with co-expression of Fyn and GluN2B-containing NMDARs, that displayed similar increases in tyrosine phosphorylation as GluN2A and that is known to intimately associate with Fyn (Salter and Kalia, 2004). Later work by this laboratory ultimately demonstrated that the effect of Fyn co-expression on GluN2A ethanol sensitivity was due to Fyn-mediated relief of tonic Zn^{2+} inhibition, consistent with observations that the phosphorylation state of C-terminal tyrosines on GluN2A can affect Zn^{2+} sensitivity of the channel (Zheng et al., 1998; Woodward, 2004).

Nevertheless, the kinase-mediated reductions in ethanol sensitivity reported by these authors, though statistically significant, appeared modest at best and such receptors retained significant amounts of inhibition. Indeed, evaluation of co-expression of other kinases or mutation of C-terminal consensus sites of phosphorylation revealed only slight or no changes in ethanol sensitivity of recombinantly expressed NMDARs (Anders et al., 1999; Xu, Chandler, & Woodward 2008; Xu, Smothers, & Woodward, 2011). Furthermore, single channel studies of NMDARs have shown that phosphorylation state of C-terminal residues appear to impact channel function, underscoring this disparity and ultimately becoming an essential justification for the experiments detailed in Section III (Murphy et al.; 2014 : Aman et al.; 2014).

Irrespective of these aforementioned conflicts in data, substantial evidence simultaneously emerged that the phosphorylation state of NMDAR C-terminal residues correlated significantly with receptor surface expression. While association of kinases and phosphatases with NMDARs was already documented, a report by Hall and Soderling (1997) was among the first showing that under basal circumstances, neuronal NMDARs exhibit extensive GluN2 subunit phosphorylation. This increases following cell stimulation with glutamate application and GluN2 phosphorylation correlates significantly with receptor inclusion in the plasma membrane (Wang & Salter, 1994). Strangely, the authors reported that primarily serine, not tyrosine, residues accounted for this increase in phosphorylation, in stark contrast to earlier studies showing that GluN2

subunits display extensive tyrosine phosphorylation after induction of LTP (Lau & Huganir, 1995; Rostas et al., 1996). This discrepancy may have been due to the use of dispersed cultured neurons by Hall and Soderling, as opposed to use of intact tissue employed by others, since these cells are not fully developed and exhibit lower levels of tyrosine kinase expression, including Fyn (Yagi et al., 1994). Nevertheless, this spurred others to investigate the role of post-translational modifications of GluN2 C-termini in the regulation of receptor surface expression, culminating in the elucidation of a highly dynamic and subunit-selective process that is inherently alcohol-sensitive.

While Miyakawa et al. (1997) demonstrated an essential association of Fyn and GluN2B-containing NMDARs in mediating acute ethanol tolerance, further revelations by Yaka et al. (2002) and others showed that extensive interactions between a host of secondary effector molecules coordinated during basal and post-stimulation conditions effectively govern the relative surface expression of predominantly GluN2B-containing NMDARs (Roche et al., 2001; Lavezzari et al., 2003; Prybylowski et al., 2005). This GluN2B-dependency specifically seems to be due to the presence of the high-consensus YEKL (tyrosine-glutamate-lysine-leucine) domain at the extreme C-terminus that promotes binding of adapter protein 2 (AP-2) (Roche et al., 2001; Lavezzari et al.; 2003). As its name implies, the function of this protein largely consists of acting as an adapter between the substrate, in this case the GluN2B subunit of NMDA receptors, and the recruitment and binding of clathrin, a protein that self

assembles into cage-like structures forming endosomes that ultimately internalize the AP-2-bound substrate. Importantly, the binding of AP-2 to the YEKL site on GluN2B is intimately determined by the phosphorylation state. Specifically, AP-2 will only bind when Y1472 of this consensus site is dephosphorylated. Thus, the Fyn-dependent acute tolerance to ethanol observed by Miyakawa et al. (1997) could have been a product of blunted AP-2 association subsequent to heightened phosphorylation of GluN2B, and indeed later work by Yaka et al. (2002), Yaka, Phamluong, and Ron (2003), and Wang et al. (2007) has since borne out this mechanism. In addition, work by these authors and others has modified this schema to include the essential ethanol-sensitive element RACK1 that, following alcohol exposure, dissociates from the complex allowing Fyn to phosphorylate GluN2B.

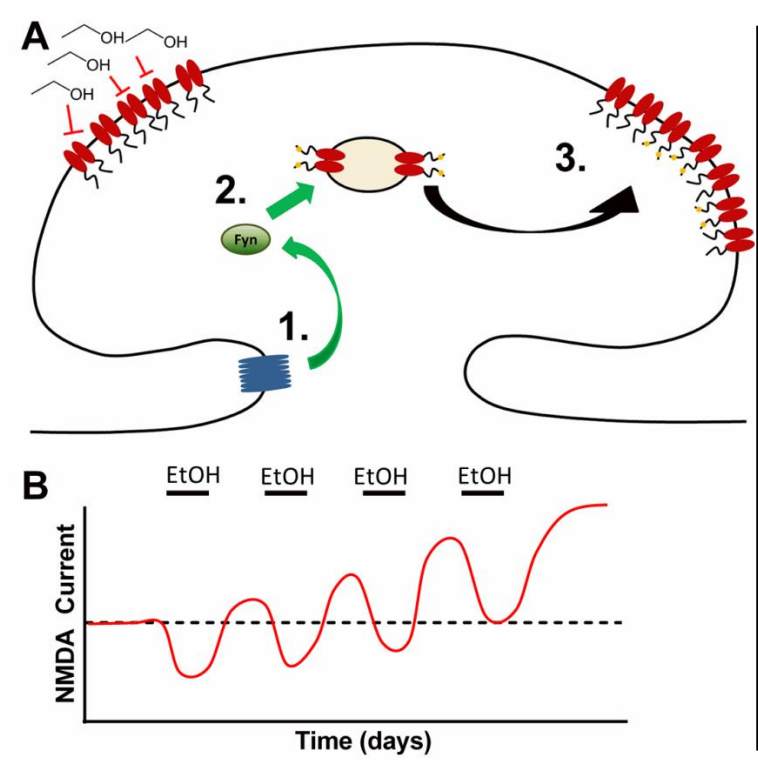


Figure I.6: Diagram of repeated ethanol exposure on NMDA receptor expression. Panel (a.) shows how Fyn-mediated phosphorylation promotes the insertion of endosomal pools of NMDARs into the plasma membrane. Panel (b.), adapted from Wang et al. (2010), shows the cumulative effect on NMDAR-mediated current of ethanol-induced Fyn activity. Ethanol treatment enhances Dopamine D1 receptor signaling (1.), inducing Fyn activity (2.), that promotes NMDAR insertion into the plasma membrane (3.), overcoming ethanol inhibition.

Thus, the induction of Fyn activity, potentially via enhanced Dopamine D1 receptor signaling, following ethanol administration appears to be a homeostatic mechanism that restores NMDAR-mediated current blunted by ethanol (Wang et al., 2010; Xu et al., 2015). This effect is observed functionally as a rebound in NMDAR current over and above baseline levels that is apparent following alcohol washout. The effector cascade described above likely underlies this phenomenon specifically by heightening Y1472 phosphorylation, thereby blocking AP-2 binding to GluN2B and ultimately both stabilizing NMDAR surface expression and facilitating the insertion of endosomal pools of NMDA receptors into membrane. This increase in surface-bound receptors is opposed to functional up-regulation via enhanced unitary conductance, etc. based on the

previously detailed work of Anders et al. (1999), Xu et al. (2008), and Xu et al. (2011)**.** Interestingly, repeated induction of this phenomenon in dorsal striatum by cycles of ethanol administration appears to result in persistently heightened NMDAR signaling well above baseline, and has been given the moniker “long-term facilitation” by Wang et al. (2007). Long-term facilitation appears essential to the pathogenesis of maladaptive behaviors with chronic alcohol exposure, and its expression in dorsomedial striatum appears sufficient to drive elevated alcohol drinking behaviors (Wang et al., 2010).

While an attractive mechanism for acute tolerance observed with ethanol exposure, the converse seemed equally plausible for governing acute inhibition by ethanol: specifically, that ethanol-induced dephosphorylation of GluN2B, subsequently provoking internalization of the receptor complex by AP-2, may partially underlie ethanol inhibition and resolve the disparate observations in ethanol sensitivity noted between recombinant and native tissue experiments. Indeed, building on an accumulating body of evidence showing high interacting specificity of striatal-enriched protein tyrosine phosphatase (STEP) with GluN2B, Wu et. al. (2010) showed that not only did ethanol exposed neuronal tissue exhibit significant dephosphorylation of GluN2B C-terminal tyrosine residues (e.g. – Y1252, Y1336, and Y1472), but that such ethanol treatment also correlated with increased STEP33, a soluble form of STEP, expression (Snyder et al., 2005; Kurub et al., 2010). Further work by this group ultimately showed that in brain slices treated with a dominant-negative form of STEP, STEP C/S, no ethanol

inhibition was observed. Interestingly though, and consistent with the canonical functions of Fyn suggested by Miyakawa et al. (1997) and Wang et al. (2007), compensatory facilitation of NMDAR-mediated current was still observed following ethanol washout.

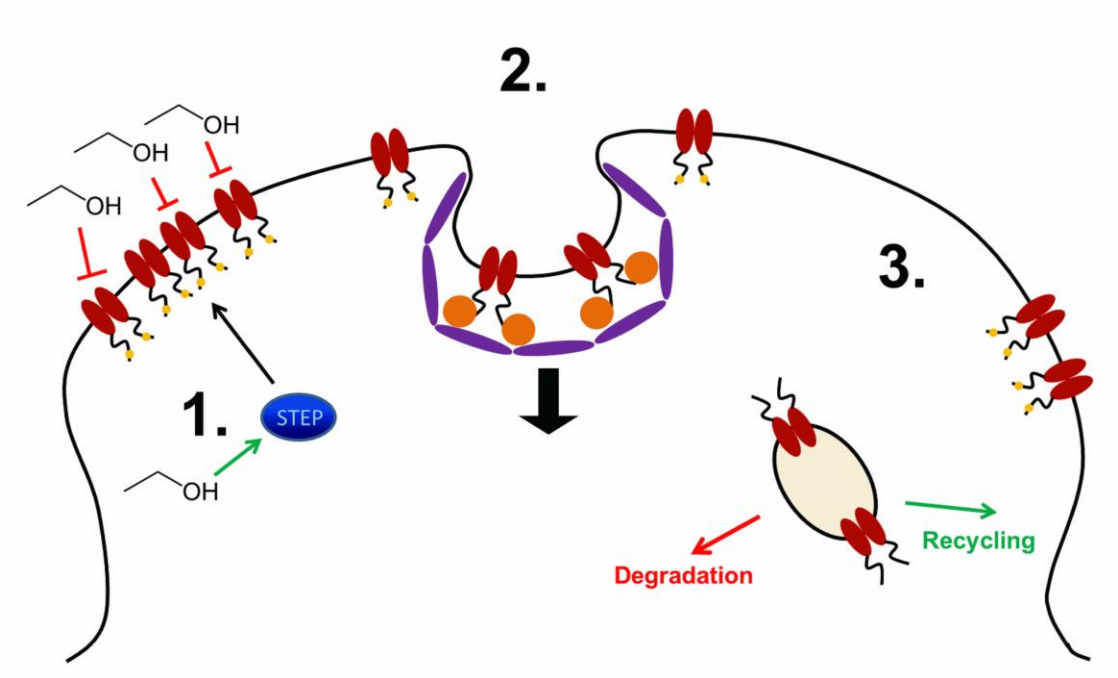


Figure I.7: Diagram showing ethanol-induced STEP activity resulting in diminished NMDAR-mediated current. 1.) Ethanol initially blocks NMDAR current via direct interactions with the receptor, but with extended application induced the activity of STEP. 2.) STEP dephosphorylates C-terminal tyrosine residues on GluN2B, signaling the binding of AP-2 (orange) to GluN2B, ultimately recruiting clathrin (purple) resulting in endocytosis of affected NMDAR complexes. 3.) The net result is diminished NMDAR current. After washout of ethanol, the endosomally-bound NMDARs can be targeted either for proteolytic degradation, or, as work by Wang et al. (2010) has shown, reinserted into the plasma membrane.

Still unclear, however, is in what manner these opposing cascades are induced, and indeed whether or not they are in fact mutually exclusive. Emerging reports suggest that a central factor temporally distinguishing STEP-mediated

NMDAR down-regulation and Fyn-mediated compensatory up-regulation is ethanol-induced facilitation of activity of the dopamine D1 receptor (Xu et al., 2015). Specifically, induction of PKA activity by ethanol/D1 results in STEP inhibition, resulting in PTPα dis-inhibition (a regulator of Fyn activity) that ultimately dephosphorylates regulatory sites on Fyn permitting up-regulation of NMDAR-mediated current. This model is consistent with previous work showing persistent up-regulation and synaptic targeting of GluN2B-containing NMDARs that was PKA dependent and that may represent a sensitization of the system to ethanol-induced synaptic plasticity (Carpenter-Hyland, Woodward, & Chandler, 2004; Wang et al., 2007; Clapp et al., 2010). That this paradigm does not include a basis for ethanol-induced STEP activity is curious and seems to argue that STEP up-regulation is not necessarily a primary feature of alcohol inhibition of NMDA receptors. Indeed, while others have shown that enhanced phosphorylation of GluN2B by Fyn does not result in any appreciable increase in unitary enhancement of receptor function, the converse experiment, whether dephosphorylation of STEP-interacting residues on GluN2B directly affects channel function and ethanol sensitivity, is as yet untested and represents a gap in the literature that studies outline in Section III has sought to fill.

I.10 Differential Ethanol Sensitivity of NMDAR Subtypes: Implications for Compensatory Plasticity

As discussed, the NMDA receptor is a critical link in a number of cellular processes that form the basis of synaptic plasticity. Significant evidence shows

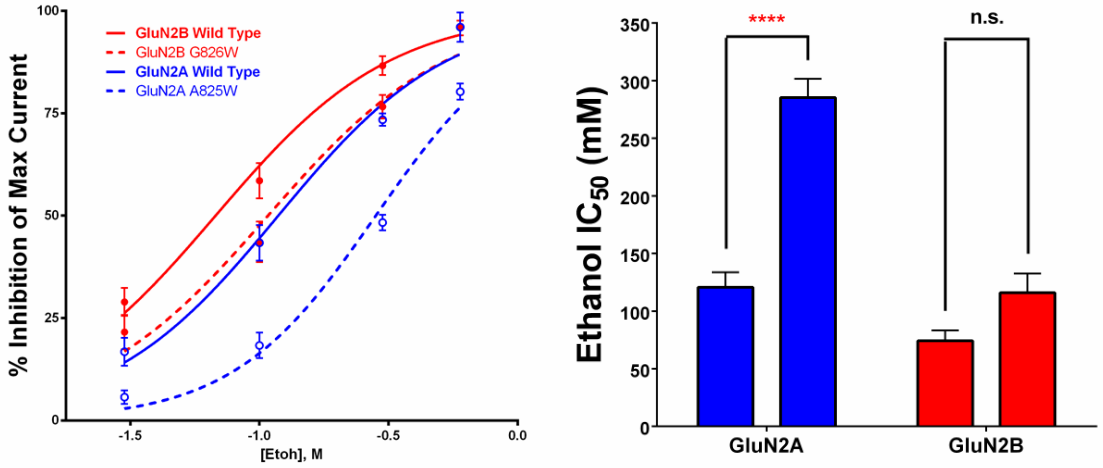
that in addition to the acute effects of ethanol on receptor function, extended application can result in the recruitment of adaptive mechanisms that up-regulate receptor function and restore homeostasis. Such maladaptive changes in the activity of these metaplastic elements are not only persistent but have been shown to drive pathological behaviors. Though these late phase mechanisms of NMDAR regulation display an apparent preference for GluN2B-containing NMDARs, it is important to remember that NMDAR populations exist as a rich milieu of subtypes at most synapses and that accumulating evidence shows exhibit differential sensitivities to ethanol depending on GluN2 subtype. The significance of this becomes apparent when considering work by Foster et al. (2010) and Kim et al. (2005) demonstrating vastly different roles for GluN2A- and GluN2B-containing NMDARs in eliciting specific types and magnitudes of synaptic plasticity. Thus a fundamental understanding of how ethanol preferentially inhibits these receptor subtypes could reveal the relative contribution of these receptor types in the pathogenesis of alcoholism. This question, in concert with the possible extraneous intramolecular sites of alcohol action on the NMDA receptor outlined in Section I.6, thus represents the driving hypothesis of experiments discussed in the following section.

Chapter II. Differential Gating of GluN2A and GluN2B Receptor Types Govern Ethanol Sensitivity

II.1 Introduction

As discussed extensively in Section I, the NMDA receptor is an essential mediator of excitatory neurotransmission, and in addition acts as a central link that transduces levels of excitation into long term cellular adaptations. NMDARs are large, tetrameric complexes composed of two obligate, glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits and each display their own unique intramolecular interacting sites, as well as vastly divergent sites of intermolecular interaction such as their extensive intracellular C-termini. Owing to its prominence in forebrain signaling, NMDA receptor dysfunction has been implicated in a number of pathological states including addiction and schizophrenia (Cull-Candy et al., 2001). In addition to their innate gross complexity is the potential inclusion of 8 different splice variants of GluN1 as well as four different isoforms of GluN2 subunits that themselves can vastly affect the functional properties and intermolecular interactions of the fully complemented receptor. It is perhaps unsurprising, then, that a precise understanding of how ethanol inhibits these channels remains elusive. Nevertheless, work by our laboratory and others have identified a number of key sites within the core membrane-spanning (M) elements that, when mutated, can substantially alter the ethanol sensitivity of the channel.

Two sites in particular, F639 in the M3 domain of GluN1 and A825 in the M4 domain of GluN2A, robustly diminish ethanol sensitivity when mutated to a smaller alanine (A) residue or larger tryptophan (W) residue respectively (Ronald et al., 2001; Ren et al., 2003; Smothers and Woodard, 2006). When the analogous M3 residue of GluN2A, F637, was mutated, however, no apparent change in ethanol sensitivity was observed, suggesting non-homologous roles for GluN1 and GluN2 transmembrane elements in dictating ethanol sensitivity (Ren et al.; 2008). Furthermore, studies by our laboratory and others have shown that not only does NMDAR sensitivity to ethanol vary in a GluN2 subtype-dependent fashion (e.g. between GluN2A and GluN2B subtypes), but that mutating homologous M4 sites of alcohol activity between GluN2A and GluN2B (e.g. -A825W and G825W) also elicits unequal alterations in ethanol sensitivity similarly described above between GluN1/GluN2 M3 residues (Xu et al., 2012; Zhao et al., 2015; Hughes and Woodward; unpublished observations).



(Hughes and Woodward; Unpublished Observations)

Figure II.1: The left panel shows concentration-response relationships between wild type and mutant GluN2A- and GluN2B-containing NMDA receptors expressed in HEK cells with acute application (10 sec) of increasing concentrations of ethanol. The right panel shows the quantification of the effects of M4 residue mutation on ethanol IC_{50} values (left panel), revealing non-homologous changes in alcohol sensitivity between GluN2A and GluN2B receptor types.

Kinetic modeling of NMDA receptor gating developed by Popescu and Auerbach (2004) has shown that, while the transmembrane elements are core determinants of channel gating, other extracellular sites also govern channel activity. Specifically, work by Kazi et al. (2013) has shown that the linker regions between the S1/M1 and S2/M4 domains participate strongly in unequally assigning GluN subunit participation in the gating cascade, with the GluN2 subunit in particular predominantly determining the final closed to open transition state. Their results also revealed that conformationally restricting the movement of these linkers by cysteine-crosslinking elicited reductions in open probability and mean open time of the channel that strongly resembled those observed in wild type receptors exposed to ethanol (Wright et al., 1996; Kazi et al., 2013). Indeed, Smothers and Woodward (2006) showed that receptors containing both the GluN1 F639A and GluN2A A825W mutations still displayed residual sensitivity to alcohol, suggesting other sites of alcohol action.

Thus, via conformationally restricting or enhancing mobility of these linker regions, we sought to evaluate a three-fold hypothesis: 1.) Changes in linker domain mobility will significantly alter ethanol sensitivity, 2.) Altering linker domain mobility will reveal fundamentally different gating profiles between

GluN2A- and GluN2B-containing NMDA receptors, and 3.) The essential gating differences between GluN2A- and GluN2B-containing receptors will partially explain the intrinsic differences in ethanol sensitivity between the receptor subtypes. Experimentally, we have chosen to exploit the phenomenon of cysteine substitution and disulfide crosslinking described by Kazi et al. (2013) to restrict linker mobility, in which intra-domain proximal residue pairs are mutated to cysteines and allowed to spontaneously form disulfide bridges, restricting the movement of that region. Increasing the mobility of the linker regions was accomplished by substituting select intra-domain residues to a rotationally active glycine residue.

II.2 Materials and Methods

Cell culture and mutagenesis

Human embryonic kidney (HEK) 293 cells were obtained from ATCC (Manassas, VA) and maintained in 10 cm culture dishes containing serum-supplemented DMEM in a humidified incubator with 5% CO_2. For recordings, cells were split and plated on poly-ornithine coated 35 mm dishes and 24 hrs later transfected with cDNA plasmids using Lipofectamine 2000 (Invitrogen Inc, Carlsbad, CA). Plasmids containing rat GluN1, wild-type or mutant rat GluN2A or GluN2B, and an enhanced green fluorescent protein for cell selection were transfected at a 3:3:1 ratio unless otherwise noted. All mutant receptor subunits were generated using the Quik Change II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and were subsequently verified via sequencing

(Genewiz, South Plainfield, NJ). Residues selected for cysteine substitution were specifically derived from Kazi et al. (2013) and are as follows (residue numbering schema used by Wollmuth laboratory indicated in parentheses): Intra-GluN1 M1:M4 = S549 (531):F810 (792), Intra-GluN2A M1:M4 = V544 (525):D815 (796), Intra-GluN2B M1:M4 = V545 (526):D816 (797), Inter GluN1 M4:GuN2A M1 = P805 (787):S554 (535), Inter GluN1 M4:GluN2B M1 = P805 (787):S555 (536). These residues were previously shown in Kazi et al. (2013) to only exhibit crosslinking when expressed in tandem, with no change in receptor function observed in single cysteine mutants. Prior to transfection, cell media was exchanged for fresh media containing 5 mM $MgCl_2$ and 0.5 mM AP5 (Abcam, Cambridge, MA) to prevent excitotoxic cell death. Experiments were conducted 24 hrs post-transfection and after extensive washing to remove NMDA antagonists.

Electrophysiological recordings

Dishes containing transfected cells were mounted on an Olympus IX50 inverted microscope (Waltham, MA) and were perfused with an extracellular solution at a rate of 1-2 mL/min at room temperature. Extracellular recording solution contained the following (in mM); NaCl (135), KCl (5.4), $CaCl_2$ (1.8), HEPES (5), glucose (10), (pH adjusted to 7.4 with 1 M NaOH, and osmolarity adjusted to 315-325 mOsm with sucrose). Patch pipettes (2-5 MOhms) were pulled from standard wall borosilicate glass (1.5 x 0.85 mm) and filled with internal solution containing the following (in mM); CsCl (140), $MgCl_2$ (2), EGTA

(5), HEPES (10), NaATP (2), NaGTP (0.3), (pH adjusted to 7.2 with 2 M CsOH, and osmolarity adjusted to 290-295 mOsm with sucrose). Transfected cells were identified by eGFP fluorescence and whole-cell voltage clamp recordings were performed using an Axon Instruments 200B microamplifier (Molecular Devices, Union City, CA). Cells were held at -70 mV to monitor breakthrough and maintained at this potential unless otherwise noted. Access resistance was monitored throughout the experiment and cells demonstrating unstable holding currents or significant changes in series resistance were excluded from analysis. NMDA currents were evoked using a Warner FastStep multi-barrel perfusion system (Hamden, CT) programmed to switch between extracellular recording solution and solution containing agonist (10 µM glutamate and glycine) or agonist plus ethanol (30-300 mM). For cysteine crosslinking experiments, once all concentrations of ethanol were tested, cells were treated with 10 mM DTT for 20 seconds immediately followed by three consecutive 2-sec pulses of 10 µM Glu/Gly. These responses were used to determine percent DTT-potentiation by comparing them to the mean of currents obtained in the same cell during the ethanol experiments. Glutamate concentration-response curves were acquired by increasing the concentration of glutamate from 0.1-30 µM for GluN2A experiments, and 0.03-10 µM for GluN2B experiments. The order of solutions was interleaved to account for any time-related effects. For MK-801 decay experiments, 5 µM MK-801 (Sigma-Aldrich, St. Louis, MO) was applied in the continuous presence of 10 µM glutamate/glycine for 7.5 seconds, then the cell was returned to glutamate/glycine-only solution. All data were filtered at 1-2 kHz

and acquired at 5 kHz using an Instrutech ITC-16 digital interface (HEKA Instruments, Bellmore, NY) and analyzed offline by Axograph X software (Axograph Scientific, Sydney, NSW, Australia). Ethanol was purchased from Pharmco-Aaper (Brookfield, CT) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Data analysis

For glutamate concentration-response experiments, Prizm 6.0 software (GraphPad Software, San Diego, CA) was used to calculate EC_{50} and Hill coefficient via the equation $y = E_{max} / (1 + 10^{((Log\{IC_{50} \text{ or } EC_{50}\} - Log[x])* n_H)})$, where E_{max} is the maximum current evoked, n_H is the Hill coefficient, y is the measured response amplitude, and $[x]$ is the concentration of glutamate. Curves were fit to data obtained from individual cells with the minima and maxima constrained to zero and 100 respectively, and derived log EC_{50} values were statistically compared using one-way analysis of variance (ANOVA) and Dunnett's post test. All values are reported as mean ± S.E.M. Note that in Figure 6 all mutants were analyzed simultaneously with those in Figure 5F and only divided into separate graphs for clarity. For ethanol experiments, a two-way ANOVA with Dunnett's post test was used to statistically compare mutation-dependent changes in ethanol sensitivity across doses versus wild type. In DTT experiments, responses from individual cells were analyzed using a one-sample t-test (control set to 100%) followed by inter-group comparison using one-way ANOVA and Dunnett's post-hoc test. In MK-801 decay experiments, a single

exponential function was fit to the MK-801-induced current decay for each cell using the curve fitting routine in AxographX (Axograph Scientific, Sydney, NSW, Australia), and the time constant of inhibition was then used to calculate the rate of inhibition ($1/\tau_{MK\text{-}801}$).

II.3 Results

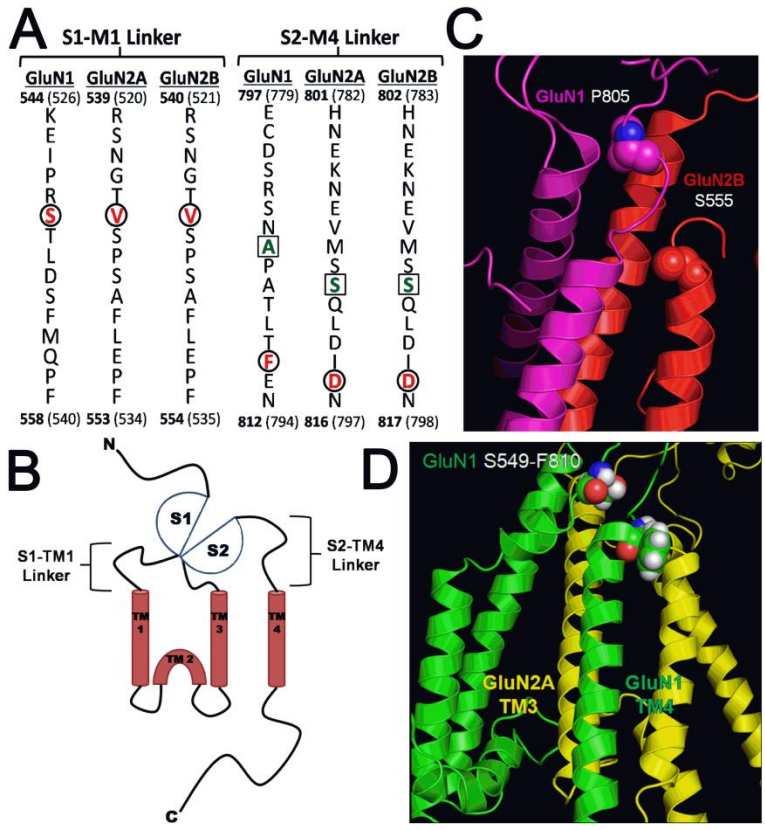


Figure II.2: Sequence of extracellular linkers and structural topology of GluN subunits. *A*, Amino acid sequence of S1-M1 and S2-M4 linker domains of GluN1 and GluN2 subunits. *B*, Cartoon depicts the broad structural topology of GluN subunits, highlighting the locations of the ligand binding domain-forming S1/S2 domains, transmembrane (TM) domains, and the S1-M1/S2-M4 linker domains. *C-D*, Structural models showing the relative locations of cysteine-substituted residues of inter- and intra-subunit crosslinked GluN2B- (*C*) and GluN2A-containing (*D*) receptors. MacPymol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC) was used to render structures of GluN1/GluN2B (PDB ID: 4PE5; Karakas and Furukawa, 2014) and a homology model of the GluN1/GluN2A receptor described in a previous study (Xu et al., 2012) that was based on the GluA2 structure (PDB ID: 3KG2; Sobolevsky et al., 2009).

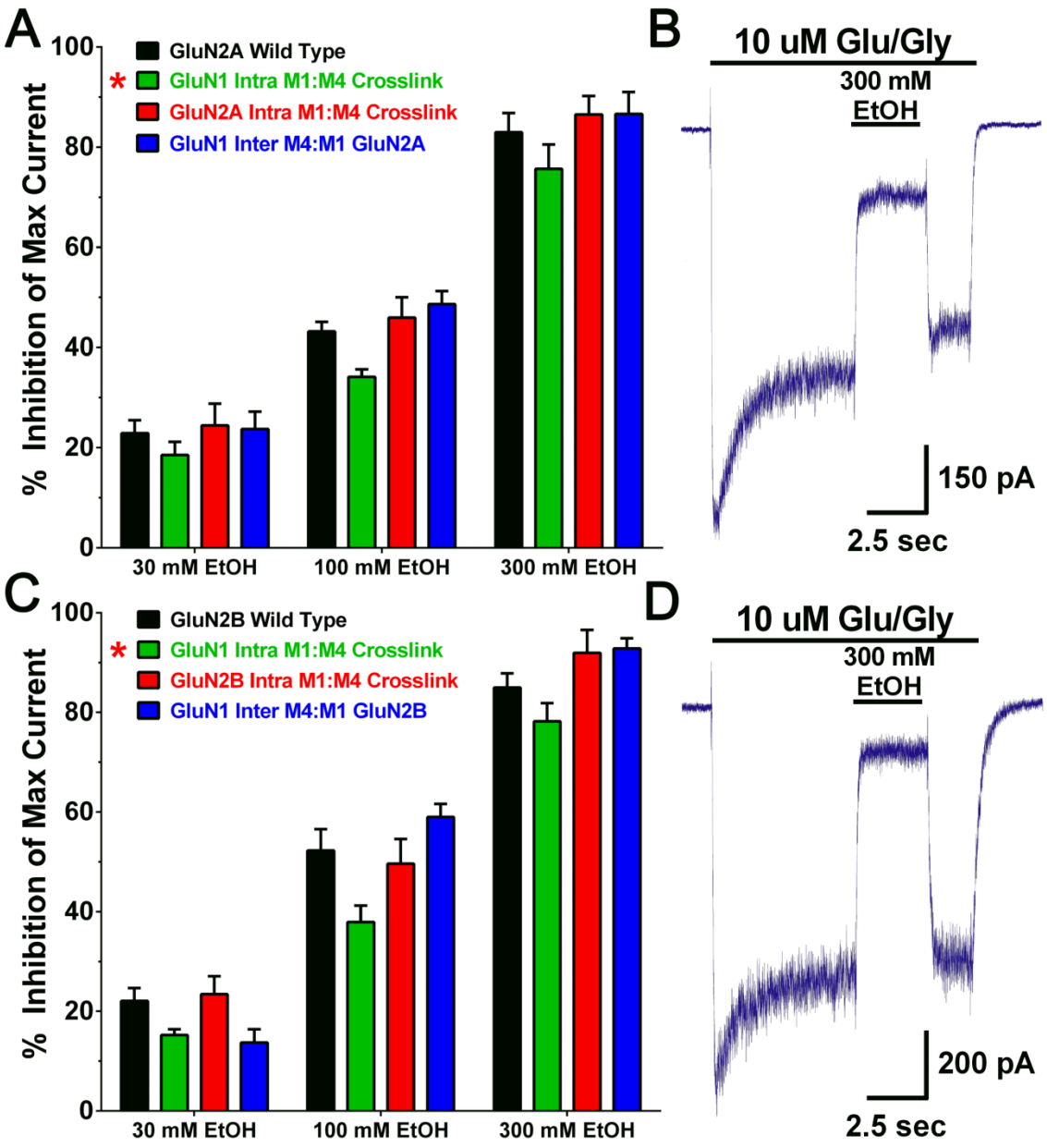

A
B
C
D
GluN2A Wild Type
GluN1 Intra M1:M4 Crosslink
GluN2A Intra M1:M4 Crosslink
GluN1 Inter M4:M1 GluN2A
% Inhibition of Max Current
30 mM EtOH
100 mM EtOH
300 mM EtOH
10 uM Glu/Gly
300 mM EtOH
150 pA
2.5 sec
GluN2B Wild Type
GluN1 Intra M1:M4 Crosslink
GluN2B Intra M1:M4 Crosslink
GluN1 Inter M4:M1 GluN2B
200 pA

Figure II.3: Concentration-dependent inhibition of crosslinked receptors by ethanol. *A*, Intra-GluN1 crosslinked GluN2A receptors showed a significant reduction in ethanol sensitivity compared to GluN1/GluN2A wild type receptors. Bar graph shows mean ± S.E.M. inhibition of agonist-evoked currents by 30, 100, and 300 mM doses of ethanol (***** $p < 0.05$; two-way ANOVA with Dunnett's test; effect of mutation, $F_{3,87} = 5.002$; N = 6-10 cells). *B*, Representative trace showing current inhibition by 300 mM ethanol of a GluN1/GluN2A wild type NMDAR. *C*, Similar to results observed in GluN1/GluN2A receptors, only intra-GluN1 crosslinked GluN2B receptors showed a significant reduction in ethanol sensitivity compared to GluN1/GluN2B wild type receptors. Bar graph shows mean ± S.E.M. inhibition of agonist-evoked currents by 30, 100, and 300 mM doses of ethanol (***** $p < 0.05$; two-way ANOVA with Dunnett's test; effect of mutation, $F_{3,93} = 7.232$; N = 7-10 cells). *D*, Representative trace showing current inhibition by 300 mM ethanol of a GluN1/GluN2B wild type NMDAR.

Intra-subunit GluN1 S1-M1/S2-M4 linker crosslinking decreases ethanol sensitivity of GluN2A- and GluN2B-containing NMDA receptors

Figure II.2.A shows the amino acid sequence of S1-M1 and S2-M4 linkers of GluN1, GluN2A and GluN2B subunits and schematic (Fig. II.2.B) and molecular (Fig. II.2.C/D) models of these regions. Results from previous studies show that NMDA receptors crosslinked via S1-M1/S2-M4 substituted cysteines show marked reductions in open probability and mean open time of GluN1/GluN2A NMDA receptors (Kazi et al., 2013) that resemble effects in wild type receptors treated with ethanol (Wright, Peoples, & Weight, 1996). On this basis, we hypothesized that linker crosslinking would occlude ethanol inhibition of NMDARs. As shown in Figure II.3.A, intra-subunit crosslinking of GluN1 peripheral linkers caused a modest but significant decrease in ethanol sensitivity compared to GluN1/GluN2A wild type that was not observed with inter- or intra-subunit GluN2A linker crosslinking (two-way ANOVA with Dunnett's test; effect of mutation, $F_{3,87} = 5.002$, $p = 0.003$; effect of ethanol treatment, $F_{2,87} = 297.6$, $p < 0.0001$; interaction, $F_{6,87} = 0.282$, $p = 0.944$; N = 6-10 cells). A similar subunit-

specific effect was observed with GluN2B-containing NMDARs (Figure II.3.C), as only intra-subunit GluN1 linker crosslinked GluN1/GluN2B receptors showed a significant reduction in ethanol sensitivity (two-way ANOVA with Dunnett's test; effect of mutation, $F_{3,93} = 7.232$, $p = 0.0002$; effect of ethanol treatment, $F_{2,93} = 381.6$, $p < 0.0001$; interaction, $F_{6,93} = 2.918$, $p = 0.0499$; N = 7-10 cells). As a control, the sensitivity of single intra-subunit GluN1 cysteine substitutions at S549 or F810 to 100 mM ethanol was tested, and no change was observed in either GluN2A- or GluN2B-containing receptors (GluN1/GluN2A wild type = 45.8%, GluN1 S549C/GluN2A = 42.8%, GluN1 F810C/GluN2A = 51.4%; ANOVA; $F_{2,25} = 3.066$, $p > 0.05$; N = 6-10 cells: GluN1/GluN2B wild type = 55.9%, GluN1 S549C/GluN2B = 52.1%, GluN1 F810C/GluN2B = 51.5%; ANOVA; $F_{2,25} = 0.377$, $p > 0.05$; N = 6-10 cells: values reflect percent inhibition). Note that in the present study, the wild-type GluN1/GluN2A receptors show a modest but reliable potentiation by DTT not observed with GluN1/GluN2B receptors. This is similar to that reported by others and is likely due to relief of tonic zinc inhibition as DTT is an effective chelator of zinc (Kohr et al., 1994). The subunit-dependent effect of DTT on wild-type receptor currents is consistent with the finding that GluN2A-containing, but not GluN2B, receptors are highly sensitive to low nanomolar concentrations of zinc found in most experimental recording solutions.

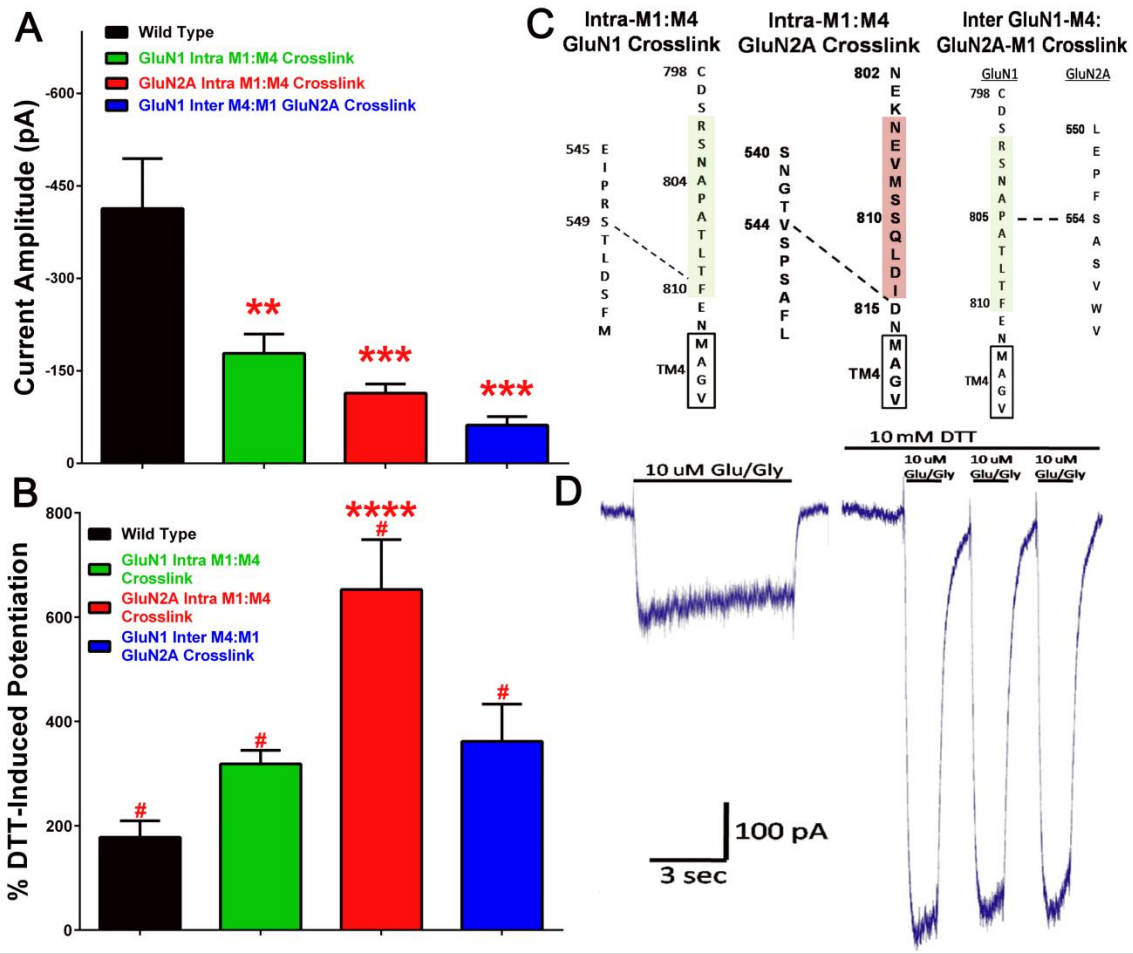


Figure II.4: Crosslinking M1 and M4 linker domains of GluN1/2A receptors alters receptor function. *A,* Mean current amplitude of GluN1/GluN2A wild type, intra-, and inter-subunit crosslinked receptors in response to 10 uM glutamate/glycine. Bar graph shows mean amplitude ± S.E.M. of WT and mutant receptors from 6-10 cells(****** $p < 0.001$, ******* $p < 0.0005$; one-way ANOVA with Dunnett's test). *B,* DTT treatment enhances steady state current amplitude of GluN1/GluN2A crosslinked receptors. Bar graph shows mean percent potentiation of current amplitude by DTT (10 mM; 20 s) ± S.E.M. from 6-10 cells (# $p < 0.05$; one-sample t-test: ******** $p < 0.0001$; one-way ANOVA with Dunnett's test). *C,* Schematic diagram showing location of cysteine-substituted residues in intra- and inter-subunit M1:M4 linker crosslinked GluN1/GluN2A receptors. *D,* Representative trace demonstrating potentiation of intra-GluN2A crosslinked receptors by DTT treatment.

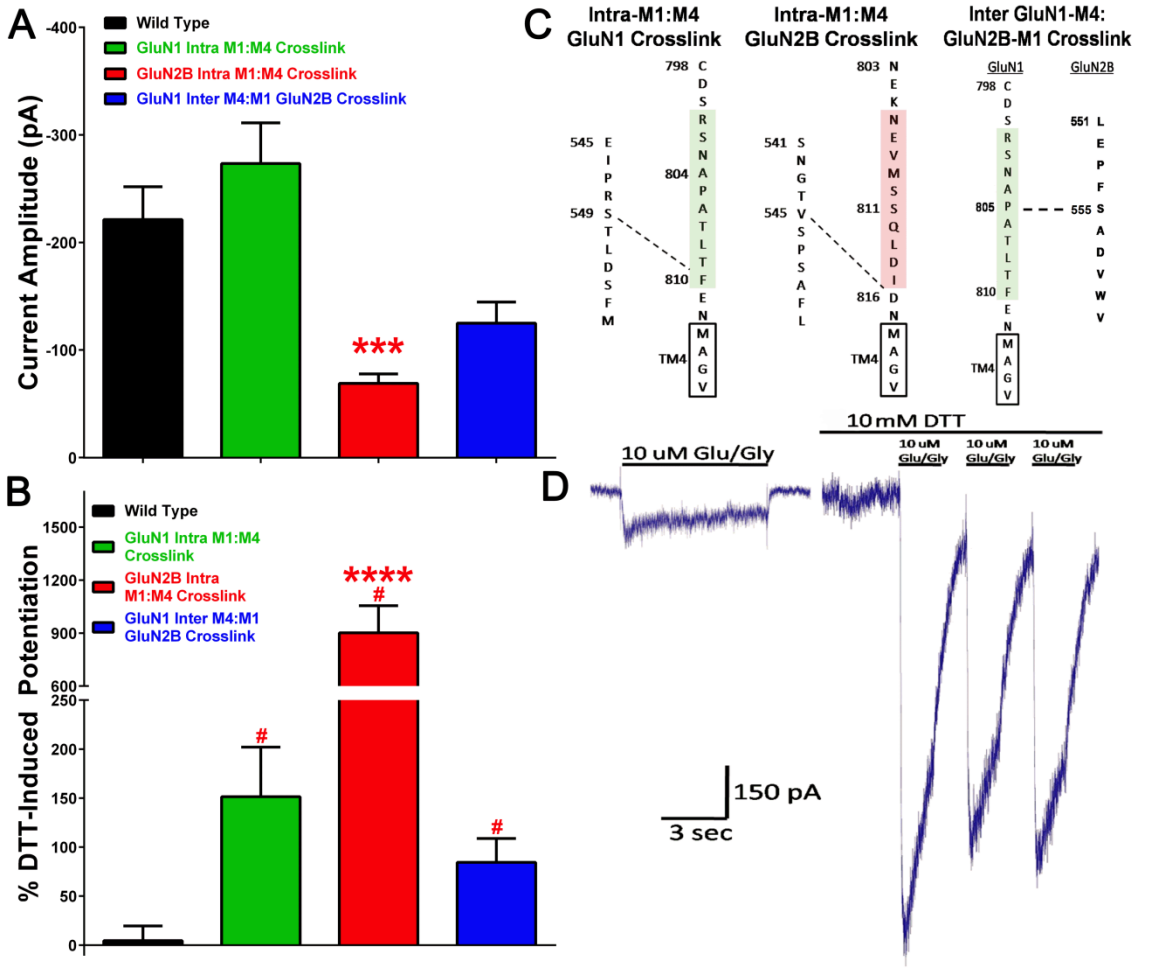


Figure II.5: Crosslinking M1 and M4 linker domains of GluN1/2B receptors alters receptor function. A, Bar graph shows mean amplitude ± S.E.M. of wild type and mutant GluN1/GluN2B receptors from 7-10 cells (*** $p < 0.0005$; one-way ANOVA with Dunnett's test). B, Effects of DTT treatment on intra- and inter-subunit crosslinked GluN1/GluN2B receptors. Bar graph shows mean ± S.E.M. percent potentiation of current amplitude by DTT (10 mM; 20 s) from 7-10 cells (# $p < 0.05$; one-sample t-test: **** $p < 0.0001$; one-way ANOVA with Dunnett's test). C, Schematic diagram showing location of cysteine-substituted residues in intra- and inter-subunit M1:M4 linker crosslinked GluN1/GluN2B receptors. D, Representative trace demonstrating potentiation of intra-GluN2B crosslinked receptors by DTT.

Subunit-dependent attenuation of NMDA receptor function via inhibition of S1-M1/S2-TM4 linker mobility

Structural modeling and experimental evidence from the Wollmuth laboratory shows that the S1-M1 linker of one subunit lies proximal to the

corresponding S2-M4 linker of the other in space, and using this information, we selected residues in GluN subunits for cysteine mutation (Sobolevsky, Rosconi, & Gouaux, 2009; Karakas & Furukawa, 2014; Kazi et al., 2013). As shown in Figure II.4.A, both intra- and inter-subunit crosslinking in GluN2A-containing NMDARs produced robust decreases in current amplitude, that was rescued by a 20 second application of DTT (10 mM) indicating crosslinking of substituted cysteines (Figure II.4.B; one-sample t-test; $p < 0.05$). Interestingly, the degree of DTT potentiation of cysteine-substituted GluN2A receptors was greater for intra-subunit crosslinked receptors (~650%) than for inter-subunit receptors (~375%). For GluN2B-containing NMDARs, however, only intra-GluN2B crosslinked receptors and inter-subunit GluN1 M4:GluN2B M1 receptors showed an appreciable decrease in current amplitude under non-reducing conditions (Figure II.5.A). DTT treatment enhanced these currents with the largest DTT-induced current potentiation, over 900%, observed for intra-subunit GluN2B receptors (Figure II.5.B; one-sample t-test; $p < 0.05$).

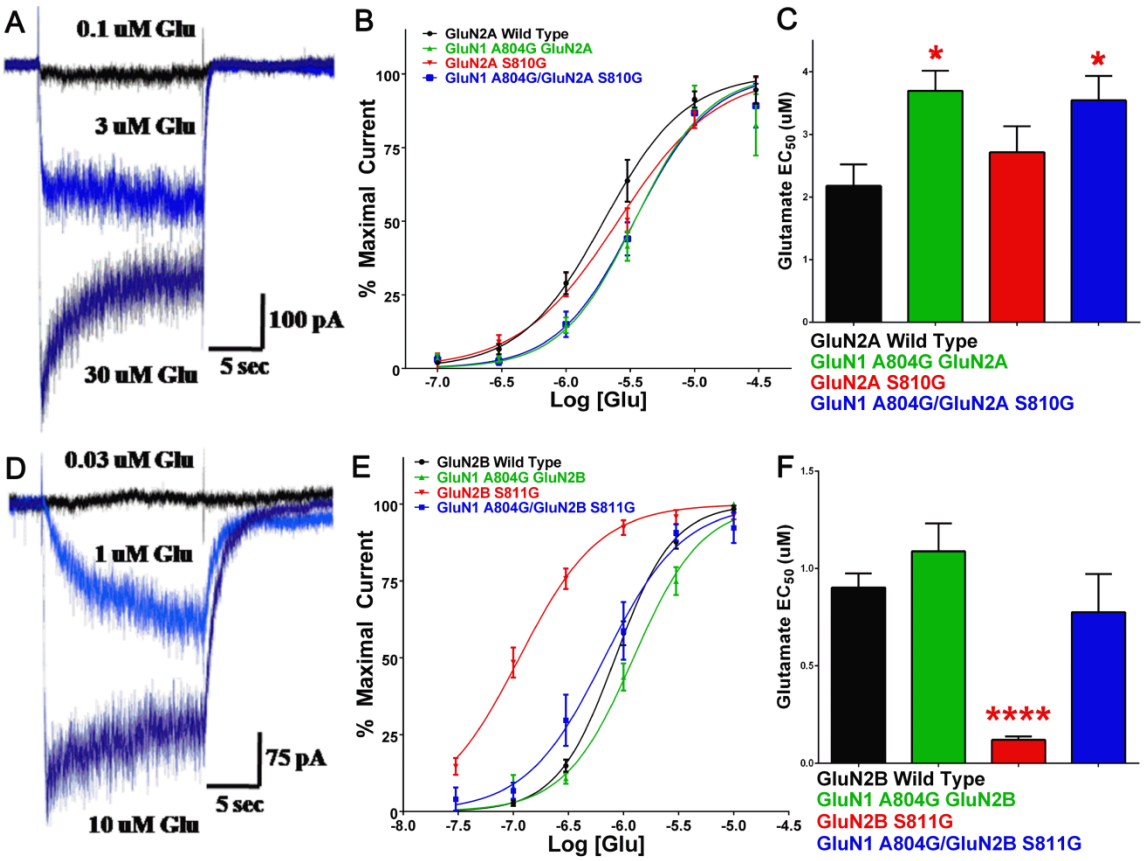


Figure II.6: Pre-TM4 glycine mutations in GluN1/GluN2A and GluN1/GluN2B receptors alter glutamate potency. *A,* Representative traces from a GluN1/GluN2A-expressing HEK cell showing response to 0.1 µM, 3 µM, and 30 µM concentrations of glutamate. *B/C,* Concentration-response curves (B) and summary graph (C) of glutamate EC_{50} values for GluN1/GluN2A wild type and glycine-substituted mutants. Curves shown are best fits to the equation given in *Materials and methods.* Bar graph shows mean ± S.E.M. EC_{50} values for glutamate-activated currents in wild type and mutant GluN1/GluN2A receptors. Data for GluN2A receptors are from 7-9 cells (***** $p < 0.05$; one-way ANOVA with Dunnett's test). *D,* Representative traces from a GluN1/GluN2B-expressing cell showing response to 0.03 µM, 1 µM, and 10 µM concentrations of glutamate. *E/F,* Concentration-response curves (E) and summary graph (F) of glutamate EC_{50} values for GluN1/GluN2B wild type and glycine-substituted mutants. Curves shown are best fits to the equation given in *Materials and methods.* Bar graph shows mean ± S.E.M. EC_{50} values for glutamate-activated currents in wild type and mutant GluN1/GluN2B receptors. Data for GluN2B receptors are from 7-9 cells (* $p < 0.05$, **** $p < 0.0001$; one-way ANOVA with Dunnett's test).

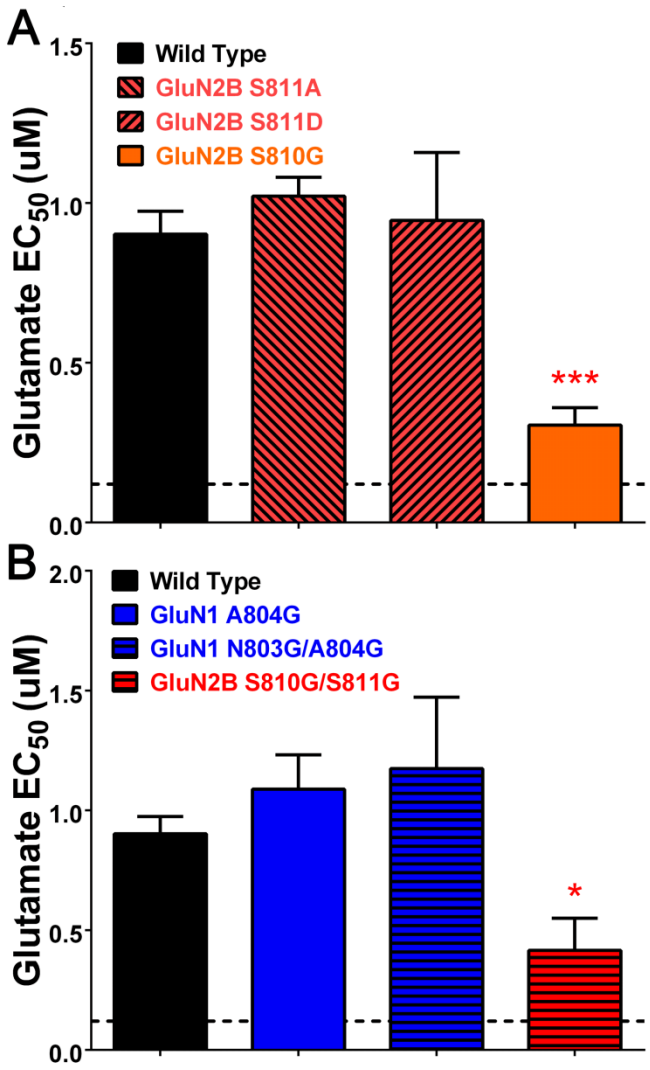


Figure II.7: Changes in glutamate potency in response to mutation of Pre-TM4 GluN2B residues. *A,* Substitution of serine (S) 811 of GluN2B with an alanine (A) or aspartate (D) did not significantly change glutamate potency, while mutation of the preceding S810 residue to a glycine produced a significant leftward shift in glutamate potency. For comparison, the effect of GluN2B (S811G) on glutamate potency (data from Figure 5) is represented by the dashed line. Data shown are mean EC_{50} values ± S.E.M. derived from individual curve fits of 5-10 cells using the equation given in *Materials and Methods* (******* $p < 0.001$; one-way ANOVA and Dunnett's test). *B,* Substitution of glycine at analogous positions in GluN1 did not alter glutamate potency, while glutamate EC_{50} of GluN2B (S810G) mutant was not affected by also substituting a glycine at position 811. Data shown are mean ± S.E.M. EC_{50} values derived from individual curve fits of 5-10 cells using the equation given in *Materials and Methods* (***** $p < 0.05$; one-way ANOVA and Dunnett's test).

Pre-TM4 residues of GluN1 and GluN2 regulate receptor function in a subunit-dependent manner

The results shown in Figures II.4 and II.5 and work by others show that restricting S2-M4 extracellular linker domain movement by disulfide crosslinking significantly attenuates channel gating and function in GluN2A-containing NMDARs (Kazi et al., 2013). To determine what effect increasing the flexibility of this region has on channel activity, select residues in the Pre-TM4 region, the terminal end of the S2-M4 linker before TM4, were mutated to the rotationally

active glycine residue. As seen in Figure II.6, robust changes in glutamate potency were observed in glycine-substituted mutants and these effects were subunit-dependent. Specifically, substitution of alanine 804 to glycine (A804G) in the Pre-TM4 of GluN1 produced a small but significant rightward shift in glutamate potency in GluN2A-containing NMDARs that was not observed with a mutation at the analogous serine 810 (S810G) in GluN2A (Figure II.6.B/C). This effect persisted when GluN1 (A804G) was co-expressed with GluN2A (S810G) (EC_{50} values: GluN1/GluN2A wild type = 2.18 µM, GluN1 A804G/GluN2A = 3.69 µM, GluN1/GluN2A S810G = 2.72 µM, GluN1 A804G/GluN2A S810G = 3.54 µM; ANOVA and Dunnett's post test; $F_{3,25} = 4.007$, $p < 0.05$; N = 7-8 cells). For GluN2B-containing NMDARs, however, mutation of serine 811 to glycine (S811G) produced a profound leftward shift in glutamate potency (Figure II.6.E/F) that was not observed in GluN1 (A804G)/GluN2B receptors. This change in glutamate potency was eliminated by co-expression of GluN1 (A804G) (EC_{50} values: GluN1/GluN2B wild type = 0.90 µM, GluN1 A804G/GluN2B = 1.09 µM, GluN1/GluN2B S811G = 0.12 µM, GluN1 A804G/GluN2B S811G = 0.78 µM; ANOVA and Dunnett's post test; $F_{8,67} = 9.019$, $p < 0.0001$; N = 7-10 cells). These effects were specific for glycine substitutions, as mutation of GluN2B S811 to either an alanine (A) or aspartate (D) did not significantly change glutamate potency (Figure II.7.A). Furthermore, substitution at adjacent residues in GluN1 (A803, A804) did not significantly affect the glutamate EC_{50} value and adding a second glycine residue at S810 in GluN2B did not further enhance glutamate potency (Figure II.7.B). As a further examination of the effects of glycine

substitutions on channel function, we used the rate of MK-801 inhibition as an index of channel open probability (Chen et al., 1999; Hansen et al., 2013). As shown in Table II.2, there were no significant differences in the rate of MK801 block for any of the glycine mutants tested. Table II.1 summarizes the functional characteristics of GluN2A- and GluN2B-containing wild type and glycine-substituted NMDARs and shows values for mean peak and steady state current amplitude as well as a measure of macroscopic desensitization (steady state to peak ratio).

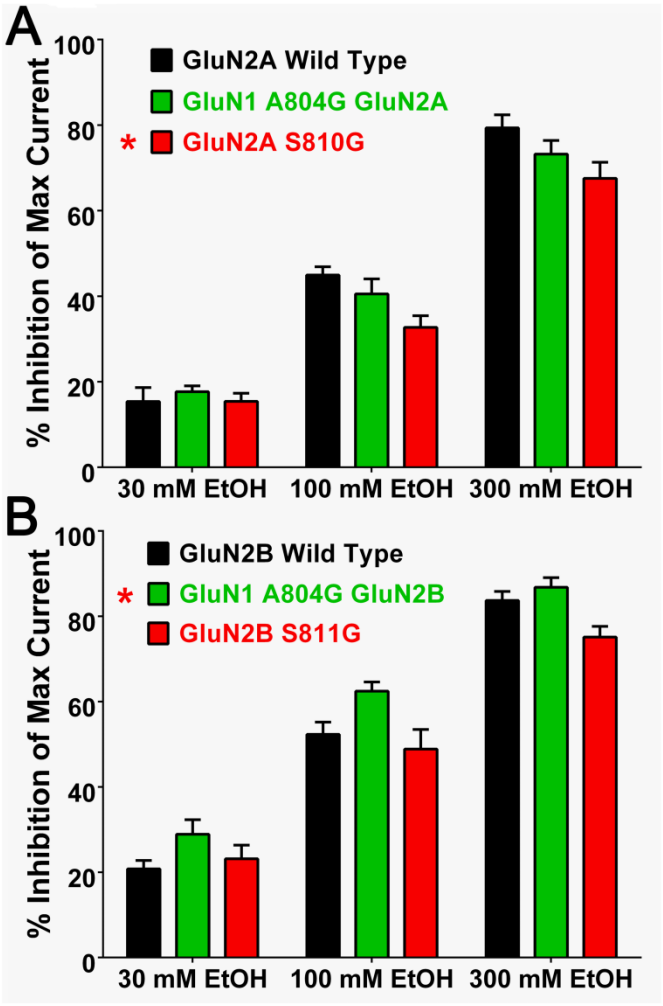


Figure II.8: Concentration-dependent inhibition of glycine-substituted receptors by ethanol. A, GluN2A (S810G) mutant showed a significant reduction in ethanol sensitivity compared to wild type GluN2A. Data shown are mean ± S.E.M. inhibition of agonist-evoked currents across three ethanol doses (* $p < 0.05$; two-way ANOVA with Dunnett's test, effect of mutation, $F_{2,63} = 5.91$, N = 7-10 per group). B, In contrast to GluN1/GluN2A receptors, GluN1 (A804G)/GluN2B significantly increased ethanol sensitivity, with no change observed in GluN2B (S811G) mutants. Data shown are mean ± S.E.M. inhibition of agonist-evoked currents across three ethanol doses (* $p < 0.05$; two-way ANOVA with Dunnett's test, effect of mutation, $F_{2,90} = 7.517$, N = 7-18 per group).

Subunit-dependent changes in ethanol sensitivity of Pre-TM4 glycine-substituents

Results from studies carried out by our laboratory and others have shown that a number of sites within the TM3 and TM4 domains of GluN subunits influence the ethanol sensitivity of NMDARs (Ronald, Mirshahi, & Woodward, 2001; Ren, Honse, & Peoples, 2003; Xu, Smothers & Woodward, 2012). To examine whether glycine mutations in nearby Pre-TM domains also affect ethanol inhibition, we determined the ethanol sensitivity of wild type and glycine-substituted receptors. As shown in Figure II.8.A/B, while all wild type and glycine-

substituted GluN2A- and GluN2B-containing NMDARs displayed concentration-dependent inhibition by ethanol, there were subunit-dependent differences in ethanol potency. For example, the GluN1/GluN2A (S810G) mutant showed a significant reduction in ethanol sensitivity compared to GluN1/GluN2A wild type NMDARs while this effect was not observed in GluN1 (A804G)/GluN2A receptors (two-way ANOVA with Dunnett's post test; effect of mutation, $F_{2,63} = 5.91$, $p = 0.0044$; effect of ethanol treatment, $F_{2,63} = 288.0$, $p < 0.0001$; interaction, $F_{4,63} = 0.1857$, $p = 0.1857$; N = 7-10 cells). In contrast, GluN1 (A804G)/GluN2B receptors were significantly more sensitive to ethanol, while inhibition of GluN1/GluN2B (S811G) receptors was indistinguishable from that of GluN1/GluN2B wild type (two-way ANOVA with Dunnett's post test; effect of mutation, $F_{2,90} = 7.517$, $p = 0.001$; effect of ethanol treatment, $F_{2,90} = 262.8$, $p < 0.0001$; interaction, $F_{4,90} = 1.131$, $p = 0.347$; N = 7-8 cells).

Table II.1: Functional characteristics of GluN2A- and GluN2B-containing mutant NMDA receptors.

Subunit/Mutant Expressed	**I_{Peak} pA (n)**	**$I_{Steady\ State}$ pA (n)**	**SS:Peak Ratio (n)**	**Rate of MK-801 Block s^{-1} (n)**
GluN1-1a/GluN2A WT	-927.63 ± 273.8 (8)	-462.66 ± 80.4 (8)	0.627 ± 0.073 (8)	2.99 ± 0.73 (9)
GluN1 A804G	-1064.97 ± 322.1 (7)	-453.26 ± 108 (7)	0.515 ± 0.07 (7)	3.62 ± 0.42 (8)
GluN2A S810G	-621.22 ± 193.2 (7)	-412.1 ± 114.1 (7)	0.814 ± 0.118 (7)	2.57 ± 0.58 (8)
N1 A804G/2A S810G	-767.37 ± 231.5 (7)	-455.97 ± 159.4 (7)	0.638 ± 0.096 (7)	N.D.
GluN1-1a/GluN2B WT	-375.01 ± 52.8 (22)	-285.65 ± 44.6 (22)	0.749 ± 0.048 (22)	1.21 ± 0.33 (9)
GluN1 A804G	-677.16 ± 207.1 (7)	-384.27 ± 113.1 (7)	0.651 ± 0.108 (7)	1.23 ± 0.26 (8)
GluN2B S811G	-186.82 ± 47.5 (10)	-119.1 ± 17.7 (10)	0.813 ± 0.102 (10)	1.60 ± 0.14 (8)
N1 A804G/2B S811G	-724.94 ± 147.4 (7)	-328.48 ± 113 (7)	0.391 ± 0.082* (7)	N.D.

(* denotes statistically significant deviation from wild type; one-way ANOVA with Dunnett's post-hoc test; * = $p < 0.05$; N.D. not determined)

Table II.2: Summary of effects of selected mutations on NMDA receptor function and ethanol sensitivity.

Crosslinked Receptors	**GluN1/ GluN2A WT**	**Intra-GluN1/ GluN2A**	**GluN1/ Intra-GluN2A**	**GluN1/ GluN2B WT**	**Intra-GluN1/ GluN2B**	**GluN1/ Intra-GluN2B**
Mean Amplitude (Steady State)	**-413.3 pA**	**-178.6 pA** **	**-113.9 pA** ***	**-221.4 pA**	**-273.5 pA**	**-69.1 pA** **
DTT *Potentiation*	**178% ↑**	**318.6% ↑**	**653% ↑** ****	**4.5% ↑**	**151% ↑**	**902% ↑** ****
Δ Ethanol Sensitivity	-	↓	-	-	↓	-
Glycine Mutant Receptors	**GluN1/ GluN2A WT**	**GluN1 (A804G)/ GluN2A**	**GluN1/ GluN2A (S810G)**	**GluN1/ GluN2B WT**	**GluN1 (A804G)/ GluN2B**	**GluN1/ GluN2B (S811G)**
Glutamate EC_{50}	**2.2 μM**	**3.7 μM** *	**2.7 μM**	**0.9 μM**	**1.09 μM**	**0.12 μM** ****
Δ Ethanol Sensitivity	-	-	↓	-	↑	-
Rate of MK-801 Block (s^{-1})	**2.99**	**3.62**	**2.57**	**1.21**	**1.23**	**1.6**

NOTE: Underlined values indicate statistically significant deviations from wild type. (one-way ANOVA with Dunnett's post-hoc test; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$)

II.4 Discussion

In the present study we tested whether manipulating the mobility of extracellular linker domains involved in regulating NMDA receptor gating and function would significantly affect the ethanol sensitivity of the receptor. The results show that mutations within these domains cause significant changes in receptor gating, glutamate potency, and ethanol sensitivity that are subunit-dependent. These findings highlight a disparity between the structural homology conserved between GluN2A and GluN2B subunits and the vastly divergent functional and pharmacological characteristics that each subunit imparts to receptor activity. Furthermore, the data suggest that such fundamentally different subunit-specific contributions to gating may underlie the intrinsic difference in ethanol sensitivity previously observed between GluN2A- and GluN2B-containing receptors (Masood et al., 1994; Mirshahi & Woodward, 1995).

Cysteine substitutions at select sites can elicit spontaneous crosslinking and subsequent conformational locking of protein regions and this approach has been used to probe the functional role of various domains within NMDARs (Talukder & Wollmuth, 2011; Kazi et al., 2013). Work by the Wollmuth laboratory used cysteine-substituted receptors that conformationally impeded S1-M1 and S2-M4 linker movements, and these studies revealed differential contributions of GluN1 and GluN2 linkers to receptor gating (Kazi et al., 2013). Specifically, when kinetic data from these receptors were fit to models of NMDA receptor gating developed by Kussius & Popescu (2009), C3-C2 and C2-C1 gating transitions

were predominantly dictated by the GluN2A subunit (Kazi et al., 2013), while GluN1 and GluN2 subunits made equal contributions to the final C1-O1 transition step (Talukder & Wollmuth, 2011). The results of the DTT experiments in the present study are consistent with this conclusion as both GluN2A- and GluN2B-cysteine-substituted receptors showed enhanced current amplitudes following thiol reduction with the highest potentiation observed for intra-GluN2 crosslinked receptors. While the modest potentiation observed with intra-GluN1 crosslinked receptors may simply be due to an incomplete interaction of the cysteines, this seems unlikely based on structural models demonstrating a high degree of proximity of the mutated residues (Karakas & Furukawa, 2014; Xu et al., 2012).

Notably, robust whole-cell currents were obtained from intra-GluN1 crosslinked GluN2B-containing receptors that showed only slight DTT-potentiation, while intra-GluN1 crosslinking of GluN2A-containing receptors showed significant mean current amplitude reduction with robust recovery by DTT treatment. This finding as well as the largely homologous results seen with inter-subunit crosslinking between receptor subtypes supports the conclusion that GluN2-specific, not GluN1, differences likely account for subunit-dependent disparities in DTT potentiation. This conclusion is further supported by the change in ethanol inhibition with intra-GluN1 crosslinked receptors that occurred regardless of the GluN2 subunit expressed. While studies have demonstrated that GluN2A- and GluN2B-containing receptors (Erreger et al., 2005; Amico-Ruvio & Popescu, 2010) exhibit substantially different channel characteristics

including open probability, mean open time, and glutamate dissociation rate, work by the Wollmuth laboratory (Kazi et al., 2013) first discriminated unequal contributions of the GluN1 and GluN2 subunits within GluN2A-containing NMDARs to receptor gating. Our results thus raise the intriguing possibility that intrinsic proportional contributions of GluN1 and GluN2 to gating are themselves fundamentally different between GluN2A- and GluN2B-containing NMDARs, and could underlie the divergent gating profiles observed between the receptor types.

As a counterpoint to the crosslinking experiments, we generated S2-M4 glycine mutants to increase the intrinsic flexibility of this region. Substitution or addition of glycine residues within discrete regions of ion channels including the NMDAR has been shown to enhance movement and function of these channels (Kellenberger et al., 1997; Kazi et al., 2014). When the changes in glutamate potency of glycine mutants are compared between receptor subtypes, it becomes apparent that glycine substitution reveals fundamental differences in the contribution of S2-M4 linkers to channel function that are not fully resolved with cysteine crosslinking. As observed with GluN2A-containing receptors, glycine substitution in the S2-M4 of GluN2A results in no demonstrable change in glutamate potency, while glycine substitution in the S2-M4 of GluN1 produces a significant decrease in glutamate potency that persists upon co-expression of a glycine-substituted GluN2A. In contrast, glycine substitution in the S2-M4 of GluN2B produces a profound increase in glutamate potency that is nullified by co-expression of the GluN1 (A804G) subunit, while GluN1 (A804G)/GluN2B

receptors show no discernable change. At first blush it seems curious that changing the intrinsic flexibility of the glycine-binding GluN1 subunit would alter the potency of the receptor for glutamate, or in the case of GluN2B (S811G) receptors, restore glutamate potency to wild type levels. Others, however, have shown that the GluN2 subunit can similarly impact the glycine potency of NMDARs in a subunit-dependent manner (Chen et al., 2008) suggesting a reciprocal interaction between subunits.

The observed disparity between the effects of glycine substitution on glutamate potency in GluN2A- and GluN2B-containing receptors, when considered in light of results from crosslinking experiments, implies non-homologous, subunit-specific contributions to receptor function. Indeed, studies have shown that GluN2A-containing NMDARs exhibit a much higher open probability compared to GluN2B-containing receptors (Erreger et al., 2005; Gielen et al., 2009; Hansen et al., 2013) and the results of the MK-801 blocking experiments in the present study reflect these intrinsic differences. These subunit-dependent differences in open probability appear to be attributable, at least in part, to interactions between the amino terminal domain (ATD) and ligand binding domains (LBD) that affect spontaneous opening and closure of the ligand binding cleft in GluN2 subunits (Gielen et al., 2009). The high sequence homology of core transmembrane gating elements between the GluN2 subunits further supports this ATD-LBD interaction model. Based on these findings, we posit that the lack of effect on glutamate potency observed for GluN2A S2-M4

glycine mutants is due to a ceiling effect on gating, in which actions of the ATD on activity of the ligand binding domain precludes any mutation-induced facilitation of the steps between agonist binding and channel gating. However, due to the much lower opening probability of GluN2B NMDARs, possibly reflective of subunit differences in ATD-LBD interactions, facilitating agonist activity by increasing the flexibility of the S2-M4 linker profoundly increases glutamate potency. In addition, this change is likely not simply due to increased spontaneous movement of core gating elements, as there was no change in the rate of MK-801 block in glycine-substituted mutants. Indeed, results from the DTT experiments support this conclusion as intra-GluN2B crosslinked receptors showed a significantly larger potentiation of current over intra-GluN1 crosslinked GluN2B receptors compared to GluN2A-containing receptors. Thus, we conclude that mechanistically, the S2-M4 region acts as a significant element in transduction of agonist binding to channel gating in GluN2A- and GluN2B-containing NMDARs and that manipulation of the flexibility of this region reveals a substantially higher contribution to channel gating of the GluN2 subunit in GluN2B-containing NMDARs compared to GluN2A-containing NMDARs.

The primary goal of the present study was to determine if manipulation of S2-M4 linker flexibility would elucidate mechanisms of ethanol action on NMDAR function distinct from core transmembrane sites. While others have shown that GluN1 M3/GluN2 M4 residue interactions are non-homologous to GluN1 M4/GluN2 M3 interactions in defining ethanol sensitivity (Ren et al., 2012), the

hypothesis underlying this study nevertheless initially rested on the assumption that the mechanisms of gating between GluN2A- and GluN2B-containing receptors were largely homologous. The functional data presented, though, argue against this assumption and indeed results from the ethanol experiments further support this conclusion. Specifically, a significant decrease in ethanol sensitivity was observed in GluN2A (S810G) receptors but not GluN1 (A804G)/GluN2A receptors. The opposite was observed with GluN2B-containing receptors, though, as a significant increase in ethanol sensitivity was observed only with GluN1 (A804G)/GluN2B receptors. As the amino acid sequence of the S2-M4 is identical between GluN2A and GluN2B subunits, it was predicted that either constraining (via cysteine-substitution) or enhancing (via glycine-substitution) S2-M4 linker mobility would elicit similar effects on ethanol sensitivity of GluN2A- and GluN2B-containing NMDARs. The subunit-dependent effects of glycine-substitution on ethanol sensitivity observed in the present study agree with emerging findings (Zhao et al., 2015) and suggest that ethanol does not impede receptor gating of GluN2A- and GluN2B-containing receptors in a homologous manner, perhaps as a consequence of the fundamentally different contributions of individual GluN2 subunits to channel activity. In sum, we demonstrate, in agreement with others, that extracellular linker domains of GluN subunits are significant elements in the transduction cascade between agonist binding and pore opening and that differential modulation of the mobility of these regions reveal fundamental differences in GluN2 subunit contributions to receptor gating. Furthermore, the subunit-specific effects on ethanol sensitivity, though

modest, reported herein argue that intrinsic differences in ethanol sensitivity between GluN2A- and GluN2B-containing receptors may largely be a consequence of essential differences in gating between the receptor types instead of different structural sites of action.

Chapter III. Secondary Mechanisms of NMDAR Inhibition by Ethanol

III.1 Introduction

As discussed in Section I, the NMDA receptor is a critical link between neuronal excitation and the adaptive processes that drive synaptic strengthening and depression. The direction and intensity of NMDAR-mediated synaptic alterations is largely a product of what GluN2 subunit-containing NMDARs are predominantly activated, with extensive evidence suggesting GluN2B-containing receptors exhibit the most robust predilection for inducing neuroadaptive responses. Importantly, the NMDA receptor is a primary site of action for the inhibitory effects of ethanol, and such inhibition correlates strongly with behaviorally relevant doses of alcohol impairing known NMDAR-mediated phenomena including learning and memory. While our laboratory and others have identified a number of sites within the core membrane-spanning elements of the receptor as sites of alcohol action, emerging evidence suggests that inter-molecular interactions between the receptor and secondary effector proteins could also mediate ethanol-induced reductions in NMDAR current.

Among these studies are reports of physiologically relevant doses of ethanol inducing activity of striatal-enriched protein phosphatase (STEP) that appears to interact specifically with GluN2B-containing NMDARs and promotes internalization of the receptor complex (Alvestad et al., 2003; Wu et al., 2010; Hicklin et al., 2011). This schema is based on work previously showing that extreme C-terminal tyrosine (Y) residues on GluN2B, e.g. Y1472, can signal

clathrin-mediated internalization of the receptor complex based on phosphorylation state (Roche et al., 2001; Prybylowski et al., 2005). In Hicklin et al. (2011) the authors observed a consistent reduction in phosphorylation of residue Y1472 on GluN2B with ethanol treatment that correlated with receptor internalization and STEP activity. Importantly, the authors showed that with knockdown of STEP protein, cultured neurons displayed ethanol resistance as well as curtailed GluN2B-containing NMDAR internalization. Indeed, when considered with data correlating heightened GluN2B-containing NMDAR surface expression with ethanol resistance, such a mechanism of sustained ethanol inhibition of NMDAR-mediated currents becomes viable (Wang et al., 2007).

While attractive, it nevertheless remains unclear as to whether such differential phosphorylation of C-terminal tyrosine residues on GluN2B contribute to ethanol sensitivity strictly via receptor internalization or if the phospho-state of these residues can directly impact receptor function. Initial studies seeking to understand the effect of phosphorylation on AMPAR currents seemed to show that post translational modification of C-terminal amino acids could regulate receptor function directly (Raymond et al., 1993). Indeed, later work with the NMDA receptor appeared to show that differential phosphorylation of single residues within the C-termini of GluN2 subunits was sufficient to alter ion permeability and receptor desensitization at the single channel level (Murphy et al., 2014; Aman et al., 2014). Thus, in the present study we sought to determine if the phosphorylation state of three C-terminal tyrosine residues on GluN2B

previously shown to be robust sites of STEP activity could alter the intrinsic ethanol sensitivity and receptor function of recombinantly expressed NMDARs. If dephosphorylation of these tyrosines represents a key step in the inhibition of channel currents by alcohol, then rendering these sites permanently dephosphorylated should mimic ethanol inhibition and occlude any further action of ethanol.

III.2 Methods

Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells were obtained from ATCC (Manassas, VA) and maintained in 10 cm culture dishes containing serum-supplemented DMEM in a humidified incubator with 5% CO2. Cells were split every 48 hours. For recordings, cells were split on poly-ornithine coated 35 mm dishes and 24 hours later transfected using Lipofectamine 2000 reagent (Invitrogen Inc, Carlsbad, CA). Plasmids containing rat GluN1, wild-type or mutant rat GluN2B, and an enhanced green fluorescent protein for cell selection were transfected at a 2:2:1 ratio unless otherwise noted. Mutant receptors were generated using the Quik Change II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and nucleotide substitutions were verified via sequencing (Genewiz, South Plainfield, NJ). Prior to transfection, media was exchanged with fresh serum-supplemented DMEM containing 0.5 mM AP5 to prevent glutamate-mediated excitotoxicity (Cik et al., 1994). AP5 was removed by extensive washing prior to recording.

Electrophysiology

Dishes containing transfected cells were mounted on an Olympus IX50 inverted microscope and perfused with an extracellular recording solution at a rate of 1-2mL/min. Extracellular recording solution contained the following (in mM); NaCl (135), KCl (5.4), $CaCl_2$ (1.8), HEPES (5), glucose (10), (pH adjusted to 7.4 with 1M NaOH, and osmolarity adjusted to 315-325 mOsm with sucrose). Patch pipettes (2-4 MOhms) were pulled from standard wall borosilicate glass (1.5 x 0.85 mm) and filled with internal solution containing the following (in mM); CsCl (140), $MgCl_2$ (2), EGTA (5), HEPES (10), NaATP (2), NaGTP (0.3), (pH adjusted to 7.2 with 2M CsOH, and osmolarity adjusted to 290-295 mOsm with sucrose). Transfected cells were identified by eGFP fluorescence and whole-cell voltage clamp recordings were performed at room temperature using an Axon Instruments 200B microamplifier (Molecular Devices, Union City, CA). Cells were held at -70 mV to monitor breakthrough and maintained at this potential unless otherwise noted. Whole-cell capacitance and series resistance were compensated for and access resistance was monitored throughout the experiment. Cells demonstrating unstable holding currents or significant changes in series resistance were excluded from analysis. NMDA currents were evoked using a Warner FastStep multi-barrel perfusion system programmed to switch between extracellular recording solution and solution containing agonist (10 µM glutamate and glycine) or agonist plus ethanol (10-600 mM). Glutamate dose-response curves were established using a similar method by increasing the

concentration of glutamate from 0.03-10 μM. The order of solutions was interleaved to monitor current rundown and changes >15% were excluded from analysis. For current-voltage relationship experiments, stable whole-cell configurations were first established at -70 mV, after which cells were held at 0 mV. Extracellular solution containing agonist and $MgCl_2$ was then washed on and the cell was subjected to a ramp protocol consisting of a jump to -80 mV, a 1.3 sec ramp to +80 mV, and a return to 0 mV holding potential. This protocol was repeated three times and I-V curves were obtained from the average of the three traces. All data were filtered at 1-2 kHz and acquired at 5 kHz using an Instrutech ITC-16 digital interface (HEKA Instruments, Bellmore, NY) and analyzed offline by Axograph X software (Axograph Scientific, Sydney, NSW, Australia).

Data Analysis

Data are expressed as mean ± SEM and were analyzed by analysis of variance using Prism 5.0 software (Graphpad Software, San Diego, CA) where indicated. Concentration-response curves for ethanol and glutamate were analyzed using non-linear regression. Not all ethanol concentrations could be tested on each cell thus preventing using Prism to calculate individual IC_{50}s for each cell. Instead for these data, a two-parameter (slope and EC_{50}) logistic function was estimated for all curves using SPSS (IBM Corporation, Armonk, NY) and individual curve IC_{50}s were then analyzed for group differences via ANOVA. Preliminary analysis indicated that slopes were not different and all curves for

each group were estimated simultaneously with shared logistic slopes. For all analyses, statistical significance was set at $p<0.05$.

III.3 Results

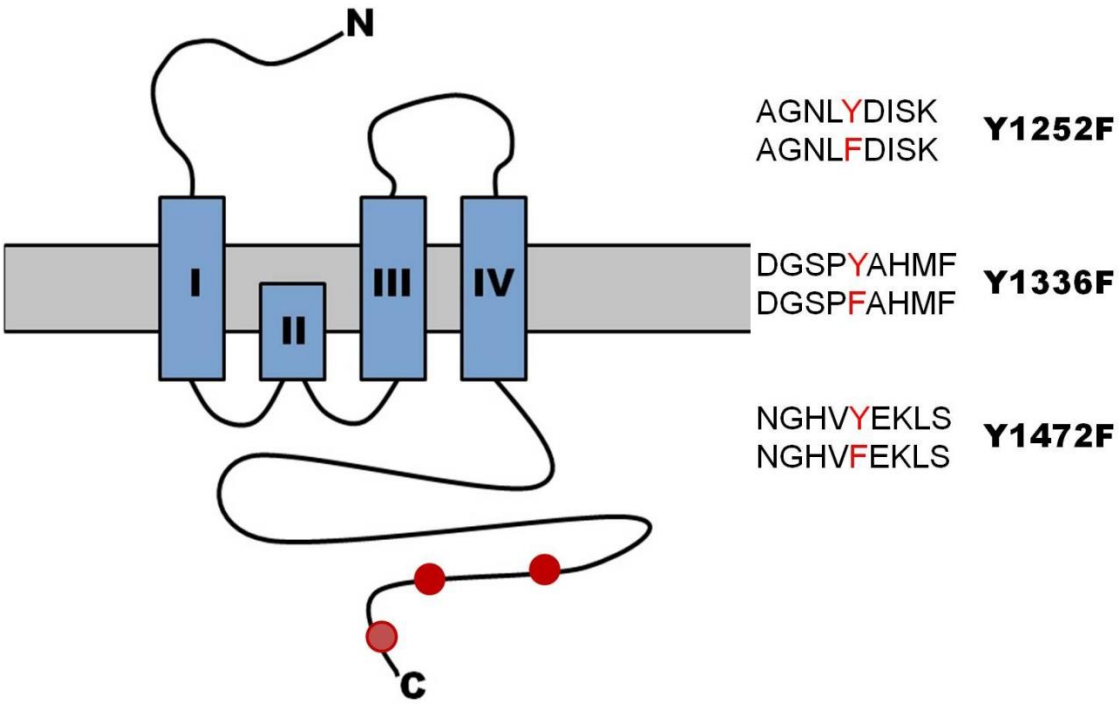


Figure III.1: Schematic cartoon of the NMDA GluN2B subunit. The extracellular N-terminus, four transmembrane domains denoted by the four blue boxes, and the intracellular C-terminus are depicted in the diagram. Red circles on the intracellular C-terminus indicate the approximate location of tyrosine residues 1252, 1336, and 1472. Also depicted are comparisons of wild-type (top) and mutant (bottom) amino acid sequences for each mutation studied.

Figure III.1 shows a schematic of the GluN2B subunit, highlighting the N-terminal domain, the glutamate binding site, transmembrane domains, and the intracellular C-terminal region of the GluN2B subunit. Residues that were mutated from tyrosine to phenylalanine were at positions 1252, 1336 and 1472 of

the full length rat GluN2B subunit. Wild-type and mutant GluN2B constructs were co-expressed with GluN1-1a subunits in HEK293 cells and whole-cell patch clamp electrophysiology was used to determine the sensitivity of ethanol.

	NR1-1a		
Mutant Expressed	**Peak Current, pA**	**Steady-state Current, pA**	**SS:Peak Ratio**
NR2B-WT	-261.6 ± 19.6 (41)	-182.4 ± 14.0 (41)	0.70 ± 0.02 (41)
Y1472F	-226.1 ± 26.3 (22)	-152.4 ± 18.9 (22)	0.67 ± 0.02 (22)
Y1336F	-205.6 ± 23.6 (25)	-134.7 ± 15.8 (25)	0.65 ± 0.02 (25)
Y1252F	-280.6 ± 17.6 (17)	-215.1 ± 14.2 (17)	0.78 ± 0.04 (17)
Triple Mutant	-305.8 ± 59.4 (20)	-190.4 ± 27.9 (20)	0.68 ± 0.04 (20)

Table III.1 summarizes the functional characteristics of wild-type and mutant receptors in terms of peak and steady-state amplitude and the steady-state to peak ratio, a marker of macroscopic receptor desensitization. While no significant differences were observed between wild-type and mutant receptors for peak current amplitude, one-way ANOVA revealed an overall significant effect between groups for steady-state current amplitude and steady-state to peak ratio ($f(4,119)=2.2694$, $p=0.034$; $f(4,119)=2.729$, $p=0.032$ respectively). Post-hoc analysis using Bonferroni and Dunnett's tests, however, demonstrated no

significant differences between wild-type and mutant receptors for either parameter.

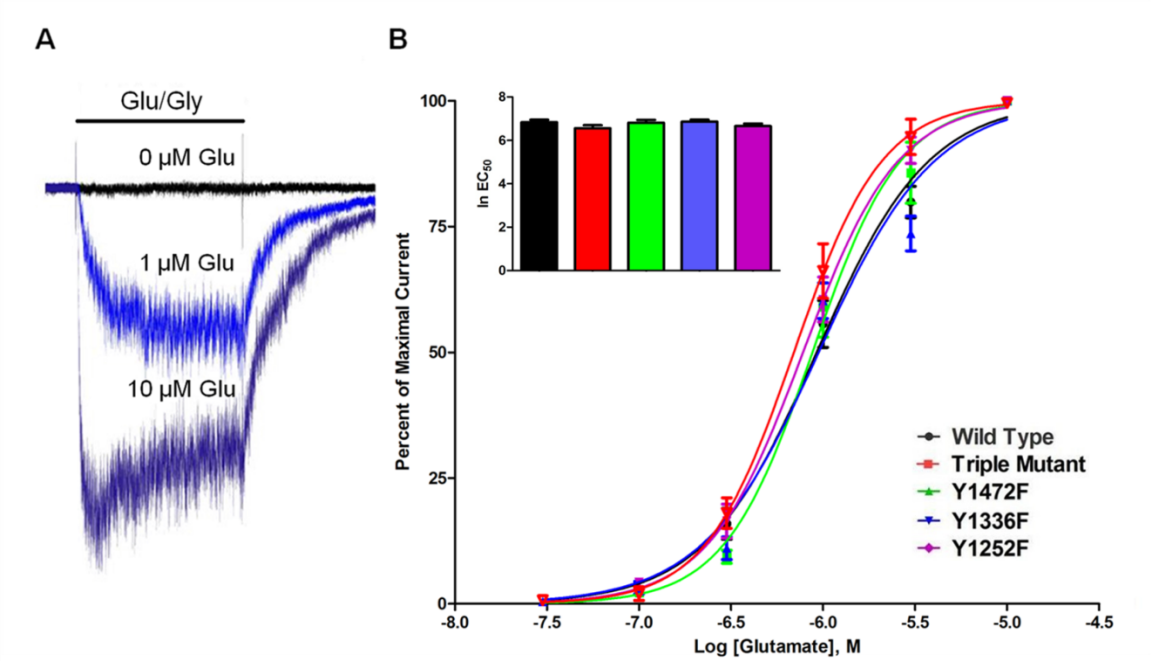


Figure III.2: Concentration-response relationship for glutamate activation of mutant and wild-type GluN2B-containing NMDARs. (A) Example traces showing currents from wild-type NMDAR transfected cell during exposure to to 0.03 μM (black), 1 μM (light blue), and 10 μM glutamate (dark blue). (N = 6 - 8 cells). All recordings performed in the presence of 10 μM glycine. (B) Currents are expressed as percent of maximal response to various concentrations of glutamate (0.03-10 μM). Non-linear regression yielded EC50 values of 0.92 μM (wild-type), 0.88 μM (Y1472F), 0.95 μM (Y1336F), 0.78 μM (Y1252F), and 0.69 μM (triple mutant). Inset bar graph shows a comparison of ln EC50 values expressed as mean ± SEM.

To determine whether manipulation of the three tyrosine residues altered the receptor's affinity for agonist, concentration-response curves for glutamate were generated. As shown in Figure III.2, increasing concentrations of glutamate evoked increases in whole-cell current in both wild-type and mutant receptors. Nonlinear regression analysis revealed no significant difference in glutamate affinity for any of the mutants tested. Calculated EC_{50} values for glutamate were

0.92 μM (wild-type), 0.88 μM (Y1472F), 0.95 μM (Y1336F), 0.78 μM (Y1252F), and 0.69 μM (triple mutant). ANOVA revealed no significant differences between groups for glutamate EC_{50} values ($f(4,27)=0.822$, $p=0.523$).

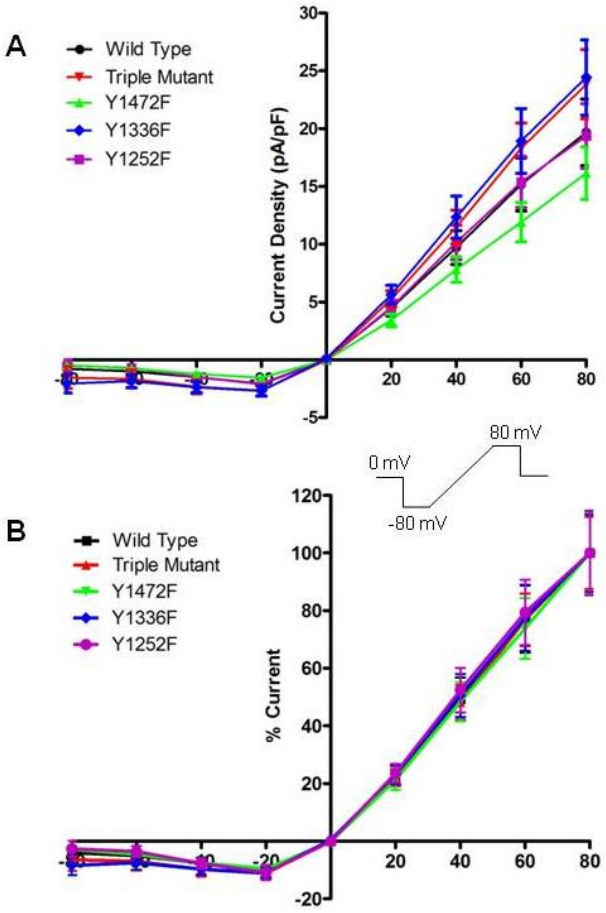


Figure III.3: Current-voltage relationship of wild-type and phospho-mutant NMDARs. (A) Cells were voltage clamped at 0 mV and then stepped to -80 mV for 200 ms followed by a 1.3s ramp to +80 mV for another 200 ms before returning to 0 mV. All recordings were performed in the presence of 2 mM Mg++. (N = 7 – 9 cells) (B) Current-voltage relationship of wild-type and mutant receptors after normalizing current to that obtained at +80 mV.

As a follow-up to the agonist dose-response experiments, the effect of tyrosine mutations on the voltage-dependent magnesium block of NMDA receptors was also determined. Figure III.3.A shows glutamate-evoked currents

in the presence of 2 mM Mg++ in cells held at different membrane potentials. In these studies, the concentrations of glutamate and glycine were held at 10 µM and current amplitude was expressed as a function of cell capacitance. Analysis of the current-voltage relationship revealed no significant differences between wild-type and mutants receptors although overall amplitudes did tend to vary. To account for this variability, I/V curves generated in each cell were normalized to the amplitude measured at +80 mV where Mg block is minimal. Figure III.3.B shows these normalized data and confirms there were no differences in voltage-dependent block between wild-type and mutant receptors.

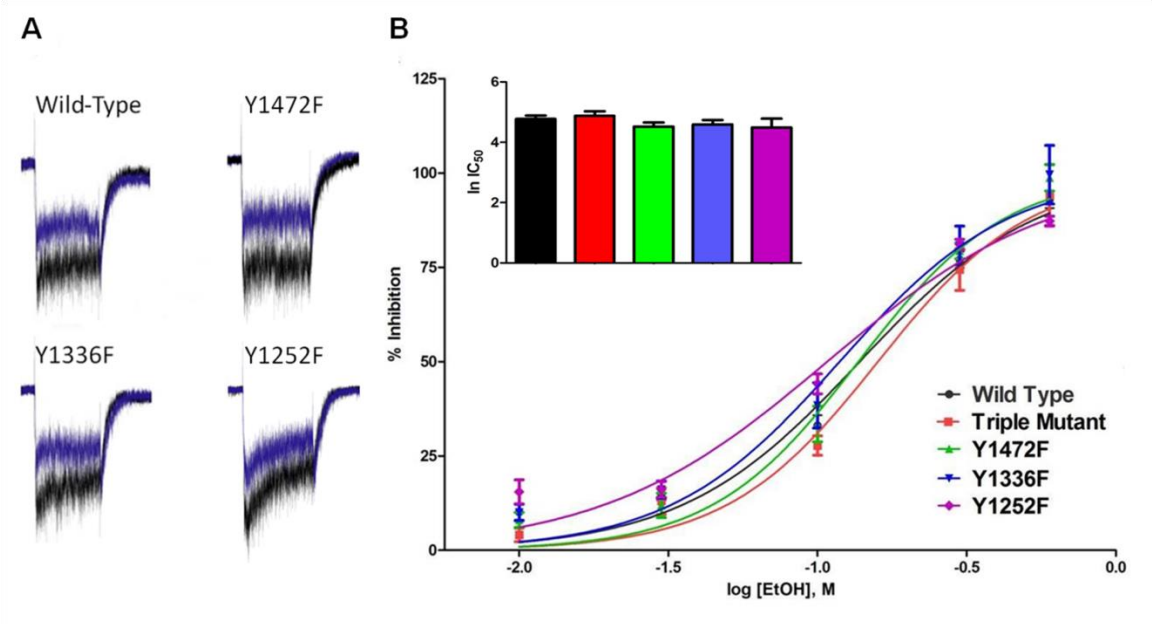


Figure III.4: Concentration-response effect of ethanol on wild-type and mutant GluN2B-containing NMDARs. (A) Example traces from a cell expressing wild-type and mutant GluN2B receptors. Agonist-only (blue) current; agonist + 100 mM EtOH (black). (B) Data are expressed as percent inhibition by ethanol (10-600 mM) of glutamate-evoked currents. (N = 6 – 8 cells per concentration). Non-linear regression yielded estimated IC_{50} values for ethanol of 136 mM (wild-type), 135 mM (Y1472F), 118 mM (Y1336F), 104 mM (Y1252F), and 156 mM (triple mutant). Inset bar graph shows a comparison of ln IC_{50} values expressed as mean ± SEM.

Finally, the effect of a series of ethanol concentrations on glutamate-activated currents was determined for wild-type and mutant receptors. In these studies, currents were evoked with 10 μM glutamate/glycine in the absence or presence of ethanol (10-600 mM). As shown in Figure III.4, ethanol caused a concentration-dependent inhibition of wild-type and mutant receptors. At the lowest concentration tested (10 mM), currents were inhibited by approximately 5-15% while 600 mM ethanol completely eliminated NMDA receptor currents. In fact, in all receptors tested, 600 mM ethanol resulted in slightly greater than 100% inhibition due to the presence of outward currents at this concentration. These currents may have resulted from ethanol's inhibition of leak current or from the slight change in ionic strength (~4%) produced when preparing the 600 mM ethanol solution. To examine these possibilities, non-transfected cells were voltage-clamped at -70 mV and holding currents were monitored before and during administration of ethanol. At a concentration of 600 mM, ethanol produced outward currents in non-transfected cells with an average amplitude of 11.63 pA ± 5.25 pA (mean ± SEM). No such effect was observed when ethanol was replaced with water suggesting that ethanol's effect on holding current was due to inhibition of a leak current and not a change in ionic strength. To account for this effect, the mean amplitude of the outward current measured in non-transfected cells was subtracted from the currents obtained in transfected cells during exposure to 600 mM ethanol. Regression analysis of the ethanol concentration-response curves yielded estimated IC_{50} values of 136 mM for wild-type, 135 mM for Y1472F, 118 mM for Y1336F, 104 mM for Y1252F, and 156

mM for the triple mutant. There were no significant differences between groups for ethanol IC_{50} ($f(4,86)=1.48$, $p=0.215$).

III.4 Discussion

In the present study, site-directed mutagenesis and whole-cell patch-clamp electrophysiology were used to examine the ethanol sensitivity of recombinant NMDARs with mutations that mimic permanently dephosphorylated tyrosine residues implicated in the inhibitory actions of ethanol. All mutant receptors tested in HEK293 cells showed robust glutamate-activated currents and all mutants retained wild-type like sensitivity to ethanol. The results of this study strongly suggest that dephosphorylation of these C-terminal tyrosine residues does not mediate the acute inhibitory actions of ethanol on recombinant NMDARs. They also suggest that ethanol inhibition of NMDA receptors expressed in native neurons is not solely due to dephosphorylation at these sites as has been suggested from studies in brain slices (Wu et al., 2010; Hicklin et al., 2011). Indeed, it is more likely that the phosphorylation state of these residues may represent intermediate sites of regulation in a larger cascade of events that are induced during long exposures to ethanol.

NMDA receptors are subject to tyrosine phosphorylation and the Src-family kinase Fyn is highly enriched in neurons where it associates with GluN2B-containing NMDARs. Over-expression of a constitutively active form of Fyn kinase in cerebellar granule cells resulted in robust increases in NMDA mini-EPSCs with little change in overall kinetics, suggesting increased membrane

insertion of NMDARs (Prybylowski et al., 2005). Co-expression of Fyn kinase with recombinant NMDA receptors to enhance tyrosine phosphorylation had no effect on the ethanol sensitivity of GluN1/GluN2B currents, supporting the hypothesis that changes in NMDAR currents observed in neuronal tissue by Fyn is due to altered trafficking (Kohr and Seeburg, 1996; Anders et al., 1999). Work conducted by Nakazawa et al. (2001) further showed that Fyn preferentially phosphorylates residues Y1252, Y1336, and Y1472 on GluN2B.

The interaction of Fyn kinase with GluN2B receptors is highly brain region dependent. Work by Yaka et al. (2003) showed that compartmentalization of Fyn with NMDARs occurs prominently in hippocampus and dorsal striatum but not in cerebral cortex. This interaction requires the scaffolding protein RACK1 and enhanced scaffolding of Fyn and GluN2B via RACK1 during ethanol exposure allows for increased membrane insertion of GluN2B-containing NMDARs, resulting in enhanced currents and the development of a functional form of acute ethanol tolerance (Wang et al., 2007; Wang et al., 2010). This mechanism is consistent with the lack of acute ethanol tolerance at the electrophysiological and behavioral level observed in Fyn-deficient mice (Miyakawa et al., 1997).

While activation of Fyn and enhanced NMDAR membrane trafficking may compensate for reduced NMDA signaling during acute ethanol inhibition, recent data from other laboratories suggests that dephosphorylation of key GluN2B tyrosine residues by striatal enriched protein phosphatase (STEP) may directly underlie the inhibitory actions of alcohol. STEP is abundantly expressed in the

striatum and hippocampus, and associates with GluN2B-containing NMDARs (Pelkey et al., 2002). It has been hypothesized that STEP mediates the inhibitory actions of ethanol on NMDARs by dephosphorylating Y1472 on the C-terminus of GluN2B. Using a phospho-Y1472 GluN2B antibody, Wu et al. (2010) demonstrated a significant reduction in immunoreactivity in hippocampal slices following acute administration of 80 mM ethanol. Further work showed that mice lacking STEP showed no change in the phosphorylation of Y1472 following ethanol administration (Hicklin et al., 2011). Inhibiting STEP with dominant-negative peptide eliminated ethanol inhibition of NMDAR-mediated EPSCs in hippocampal slices. Interestingly, however, a compensatory rebound in NMDAR current was observed after washout of ethanol in these experiments, likely due to the activity of Fyn (Yaka et al., 2003).

In the present study, mutating Y1472 to phenylalanine in order to mimic a permanently dephosphorylated state had no effect on ethanol sensitivity or channel function of recombinant GluN2B receptors. These results are consistent with prior work by our laboratory that found co-expression of various kinases including c-Src and Fyn also had no effect on the ethanol sensitivity of recombinant GluN2B-containing NMDARs, despite significant enhancement of tyrosine phosphorylation of these subunits (Anders et al., 1998; Anders et al., 1999). Results from recombinant and brain slice studies instead suggest that acute administration of ethanol likely induces multiple effects. First, ethanol directly inhibits channel function on a sub-second time scale, perhaps by

interacting with regions of GluN1 and GluN2 subunits that are involved in receptor gating (Ren et al., 2012; Ronald et al., 2001; Smothers and Woodward, 2006; Xu et al., 2011). Next, via an unknown mechanism, STEP is activated and preferentially recruited to GluN2B subunits where it dephosphorylates Y1472, promoting their removal from the post-synaptic density and internalization (Hicklin et al., 2011; Prybylowksi et al., 2005). Finally, ethanol-induced activity of Fyn via Rack1 scaffolding compensates for STEP-induced internalization, producing enhanced surface expression of NMDARs that is revealed following ethanol washout (Wang et al., 2007). Such a scenario may exist in brain regions such as hippocampus and striatum where STEP and Fyn prominently associate with GluN2B-containing NMDARs. Other brain areas such as cortex that do not show robust interactions with STEP and Fyn show significant inhibition of NMDA-mediated EPSCs without evidence of a rebound in NMDA EPSCs following cessation of ethanol exposure.

In summary, the results of this study show that the phospho-state of key C-terminal tyrosine residues of the GluN2B subunit does not directly regulate ethanol sensitivity. Rather, these residues likely influence apparent ethanol sensitivity via alterations in the trafficking and surface expression of GluN2B-containing NMDARs. Such data demonstrate that the actions of ethanol are myriad and complex and may modulate NMDAR activity through a number of different mechanisms.

Chapter IV: Mechanisms of Triheteromeric GluN1/GluN2A/GluN2B NMDA Receptor Regulation

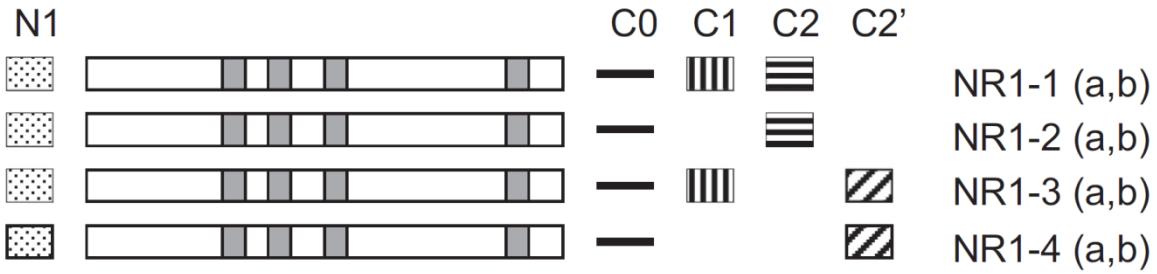
IV.1 Introduction

As discussed in previous sections, NMDA receptors can exhibit vastly different kinetic profiles as well as highly idiosyncratic intermolecular signaling cascades depending on the GluN2 subunit subtype being studied. Due largely to the high fidelity of many subtype-specific pharmacological agents such as ifendprodil and TCN-201, much of the work teasing out the role of different NMDA receptor subtypes in neuronal function has necessarily probed “pure” diheteromeric populations. In these studies, receptors are composed of two obligate GluN1 subunits and two copies of the same GluN2 subtype (e.g. 2A) arranged in a 1:2:1:2 orientation. However, evidence including that from early studies of NMDA receptor expression has suggested that triheteromeric NMDA receptors containing two different GluN2 subunits exist and may even predominate in some brain areas (Wafford et al., 1993; Dunah et al., 1996). Indeed, by exploiting intrinsic receptor properties such as differential magnesium sensitivity between GluN2A- and GluN2B-containing NMDARs in tandem with sub-maximal concentrations of subtype specific antagonists, researchers have parsed out unique pharmacological and kinetic profiles of triheteromeric NMDARs that appear to better model the NMDA-dependent signaling dynamics observed at synapses (Rauner & Kohr, 2011; Tovar, McGinley, & Westbrook, 2013).

While the method described above has proven effective at generally probing the idiosyncratic nature of triheteromeric NMDARs, “cleanly” isolating a population of triheteromers for study, even in recombinant expression systems, has remained a challenge. Recently, however, two research groups working independently established a method that took advantage of the complementary retention motifs on $GABA_{B1}$ and $GABA_{B2}$ subunits that drive dimerization of native GABA-B receptors. These motifs were cloned onto the ends of cDNAs encoding GluN2A and GluN2B in order to bias expression of a triheteromeric GluN1/GluN2A/GluN2B receptor conformation in oocytes (Terunuma, Pangalos, & Moss, 2010; Hansen et al., 2014; Stroebel et al., 2014). Exploitation of this phenomenon has not only confirmed the unique signaling characteristics of triheteromeric NMDARs observed by Rauner and Kohr (2011), but has enabled researchers to now study the effects of a vast array of pharmacological agents, on this heretofore neglected receptor population. As outlined in Section II, assumptions of congruent action of alcohol on different GluN2-containing receptors have proven untenable, suggesting the intriguing possibility that ethanol could exhibit substantially different actions on triheteromeric NMDARs both at the level of channel function as well as the novel signaling cascades these receptors may govern.

At the most basic level, though, significant questions remain regarding the intrinsic regulation of these novel receptors. Specifically, it remains unknown exactly what factors drive the preferential expression of N1/2A/2B triheteromeric

receptors at the synapse. While the process of diheteromer vs. triheteromer sorting could be stochastic and simply driven by cell-specific differences in subunit expression, experiments have shown that the magnitude of the surface expression of GluN3-containing NMDARs is critically dependent on what GluN1 splice variant is present (Smothers & Woodward, 2009). Indeed, GluN1 splice variants are determined primarily by alternating inclusion/exclusion of C-terminal cassettes averages ~20 amino acids in length that contain differential retention motifs and can affect intrinsic channel properties such as receptor desensitization and calcium permeability (Traynelis et al., 2010). GluN1 subunits are further discriminated as “A” or “B” type variants depending on the absence or presence of an N-terminal stretch of amino acids, known as Exon 5, and can affect receptor complex assembly within the ER (Meddows et al., 2001; Qiu et al., 2009).



From: Smothers & Woodward (2009)

In addition, Clapp et al. (2010) observed a significant upregulation of C2’ cassette-containing GluN1 splice variants following chronic ethanol exposure. Considering that GluN1 splice variants are defined by the presence or absence of differential endoplasmic reticulum retention motifs, this phenomenon implies that NMDA receptors undergo directed, GluN1-subtype specific sorting prior to

insertion into the plasma membrane. Furthermore, data has shown that at the level of the endoplasmic reticulum, GluN1 and GluN2 subunits exhibit directed assembly, to the extent that only fully complemented tetramers are allowed to exit and traffic to the plasma membrane (Riou et al., 2012). Taken together, this data and the enriched presence of triheteromers at the synapse suggests that triheteromeric NMDA receptors likely exhibit some form of directed sorting prior to insertion into the plasma membrane, and that such sorting may be critically dependent on the expression of GluN1 splice variants. The profound GluN1 and GluN2 subtype-specific expression changes of NMDA receptors by ethanol exposure outlined above and in Section I thus suggest triheteromeric NMDA receptor populations may be similarly affected. Thus, we hypothesize that altered expression of GluN1 splice variants after ethanol exposure represents a mechanism for altered triheteromeric NMDAR expression. To test this hypothesis, HEK 293 cells were tranfected with cDNAs expressing GluN2A-YFP and GluN2B-R.Luciferase (Rluc) along with one of the eight different GluN1 splice variants. We then used bioluminescence resonance energy transfer (BRET) to evaluate the intrinsic association of GluN2A and GluN2B subunits within the same receptor complex.

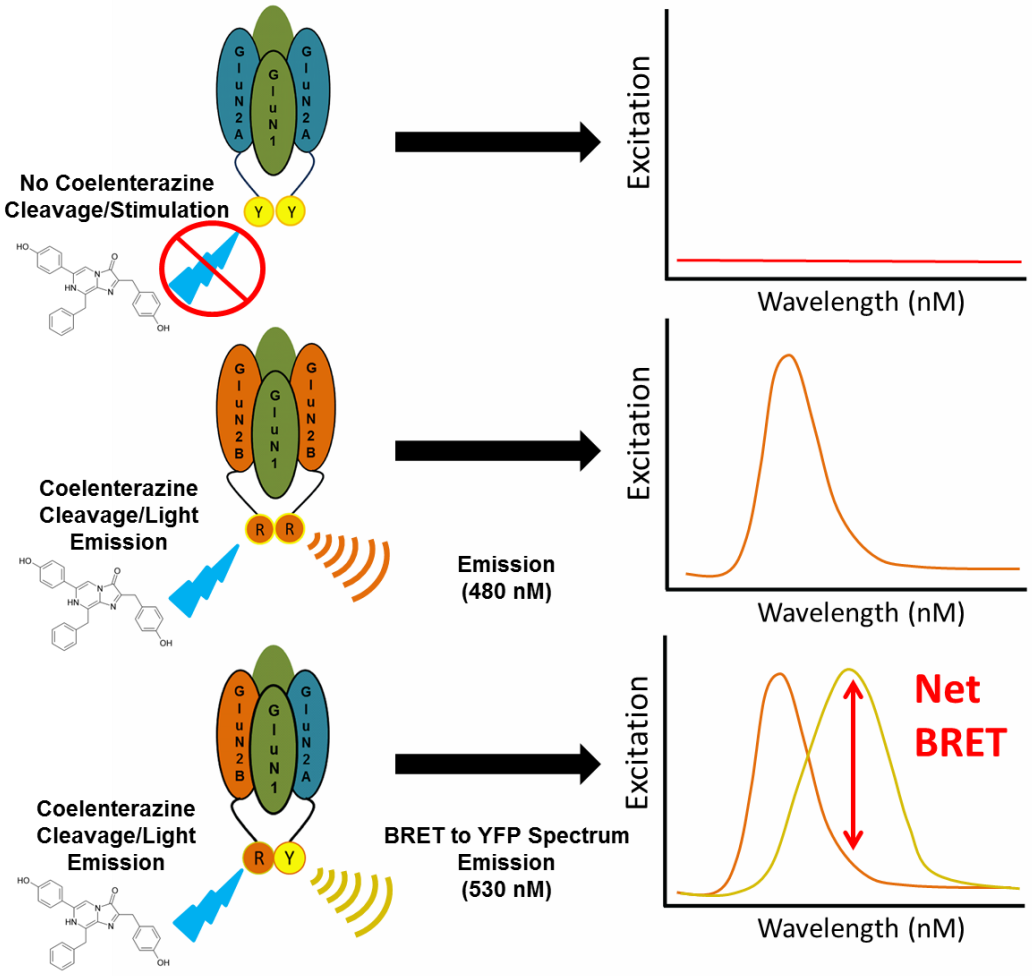


Figure IV.1: Figure depicts the experimental basis of BRET. Luciferase from *Renilla reniformis* (Rluc; R) decarboxylates coelenterazine and as a by-product releases light at the 480 nm wavelength. If Rluc comes into proximity (e.g. within 100 Angstroms) with a fluorescent protein like yellow fluorescent protein (YFP; Y), such as in an NMDA receptor complex, the photon released by coelenterazine digestion will instead undergo resonance energy transfer and be emitted in the longer wavelength YFP emission spectrum. This BRET-mediated YFP emission can then be quantified and compared between GluN1 splice variant expression groups, allowing the experimenter to determine the relative rates of triheteromerization between these groups.

IV.2 Methods

Cell culture and BRET

In brief, HEK-293 cells were maintained in 10 cm culture dishes with 10% serum-supplemented DMEM in a humidified incubator supplied with 5% CO2 and split every other day. Fusion proteins were generated by cloning an enhanced yellow fluorescent protein (YFP) cDNA onto the C-terminus of GluN2A and a bioluminescent luciferase from *Renilla reniformis* onto the C-terminus of GluN2B. For experiments, cultures were plated onto 6-well plates and transfected with GluN1, GluN2A-YFP, and GluN2B-Rluc at a 2:1:1 ratio using Lipofectamine 3000 (Invitrogen; Carlsbad, CA) reagent. The culture medium was supplemented with 0.5 mM AP5 (Abcam; Cambridge, MA) to prevent excitotoxic cell death, and experiments were performed 24 hours after transfection. Bioluminescence resonance energy transfer (BRET) experiments were conducted according to procedures outlined in Oner et al. (2010). Briefly, cells were washed once with 1x phosphate-buffered saline and harvested with Tyrode's solution containing (in mM): NaCl 140, KCl 5, CaCl2 2, NaH2PO4 0.37, NaHCO3 24, HEPES 10, and 0.1% glucose (w/v) (pH adjusted to 7.4 with NaOH). Cells were distributed in triplicate into gray 96-well optiplates and all fluorescence/luminescence signals measured using a TriStar LB 941 plate reader (Berthold Technologies, Oak Ridge, TN). The luciferase substrate Coelenterazine H (3 uM final concentration) was added for 2 min after which luminescence was measured (donor, 480 ± 20; acceptor, 530 ± 20 nm). BRET was defined as the ratio of 530 ± 20/480 ± 20,

and net BRET values were obtained by subtracting the background signal detected from expression of cells expressing the Rluc-tagged construct alone.

Data analysis

Statistical comparisons including one-way and two-way Analysis of Variance were conducted using Prism 6.0 (Graphpad Software, Inc., San Diego, CA).

IV.3 Results

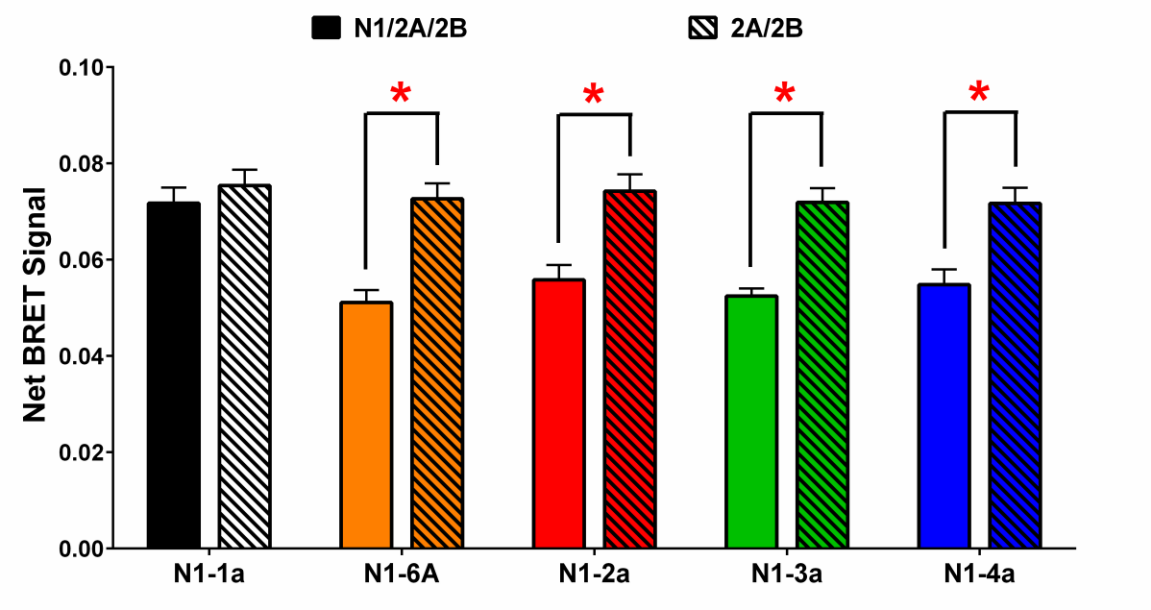


Figure IV.2: Analysis of Net BRET values between GluN2A-YFP and GluN2B-Rluc reveals that the presence of GluN1 results in a significant reduction in BRET signal for all variants except GluN1-1a. (* = $p < 0.001$, two-way ANOVA with Bonferroni's post hot test; interaction $p < 0.05$, $F_{4,100} = 2.725$; experimental condition $p < 0.001$, $F_{4,100} = 5.102$; splice variant $p < 0.0001$, $F_{1,100} = 69.19$)

BRET Reveals GluN1 Splice Variant-Independent Triheteromeric Assembly

In Figure IV.2, BRET values were compared between GluN1+ and GluN1- experimental groups among the different splice variants. With the exception of GluN1-1a, all other splice variants including the mutant GluN1-6A exhibited a significant decrease in net BRET signal compared to net BRET values of observed in the absence of GluN1 expression. As discussed in Stroebel et al. (2014), the GluN1-6A mutant has six key C-terminal residues that were mutated to alanines, thus nullifying the high fidelity ER retention motifs normally present in the wild-type GluN1-1a variant. The reduction in BRET with GluN1-6A and lack of such an effect with the wild-type variant thus likely reflects alterations in the degree of ER retention between these two variants. The inclusion of a GluN1-lacking experimental condition (hashed bars in Figure IV.2) stems from work by Qiu et al. (2005) showing that GluN2 subunits will spontaneously co-assemble in the absence of GluN1 within the ER, and thus provides both an estimate of BRET signal generated by GluN1-independent assembly and a control condition that different GluN1 subtype expressing groups can be normalized against. From this data it is inferred that, while a significant population of triheteromers are likely being assembled, diheteromeric populations are assembled as well although to a lesser degree. While these results cannot definitively rule out the possibility that the decrease in BRET is simply an artifact of higher surface area of the plasma membrane relative to the ER resulting in reduced random BRET signal, this is unlikely as there is no correlation between signal intensity and expression levels of GluN2A-YFP and GluN2B-Rluc (Figure IV.5) and in fact such data argues instead for specificity of the observed signal. Additionally, changes in BRET

signal may be due to presence of GluN1 driving YFP and Rluc apart, leading to an interpretation in which reduction in BRET signal instead implies enhanced triheteromerization. This possibility cannot be ruled out with the data presented, although co-immunoprecipitation of GluN2A and Western blotting for GluN2B can verify that the BRET signal genuinely reflects complexed GluN2A/GluN2B subunits.

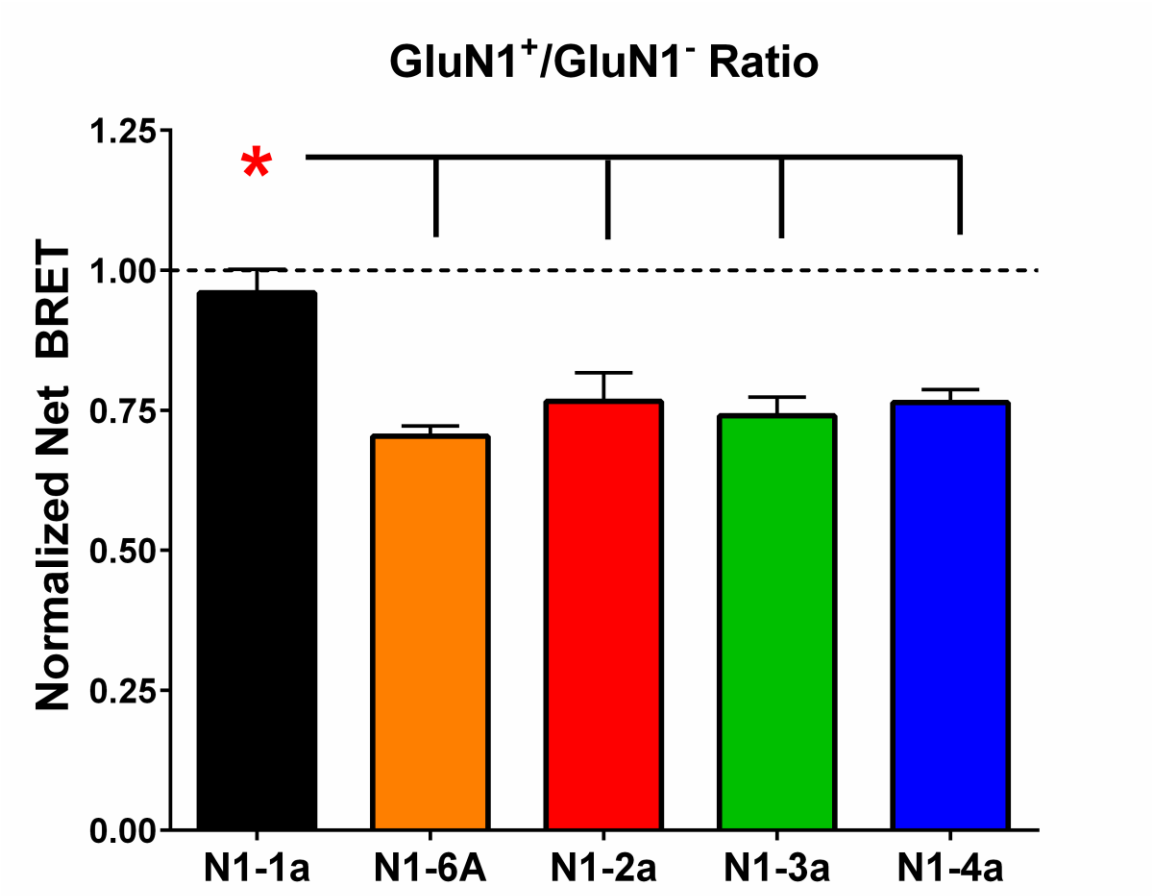


Figure IV.3: By taking a ratio of GluN1+ to GluN1- net BRET, we see that all splice variants except GluN1-1a exhibit a significant decrease in signal likely reflective of reduced association of GluN2A-YFP and GluN2B-Rluc as the fully complemented receptors are trafficked out of the ER and to the membrane. Indeed, it is inferred that since a reduction in signal is observed with the GluN1-6A mutant that significant retention of receptors in the ER is occurring with the GluN1-1a variant. (***** = $p < 0.01$; One-way ANOVA with Tukey's post hoc test; $F_{4, 50} = 7.858$)

When normalized to GluN1-lacking values, the data appear to show that triheteromeric GluN1/GluN2A/GluN2B receptors constitute nearly 75% of the receptor pool as shown in Figure IV.3. Importantly, however, triheteromericity seems to occur irrespective of GluN1 splice variant type. Furthermore as shown in Figure IV.4, expressing the "B" GluN1 variants that contain the N-terminal Exon 5, appears to have no effect either on triheteromeric assembly or trafficking. Thus, it appears that triheteromeric expression occurs regardless of the GluN1 splice variant, although retention motifs within GluN1-1a may help act as a preliminary sorting mechanism.

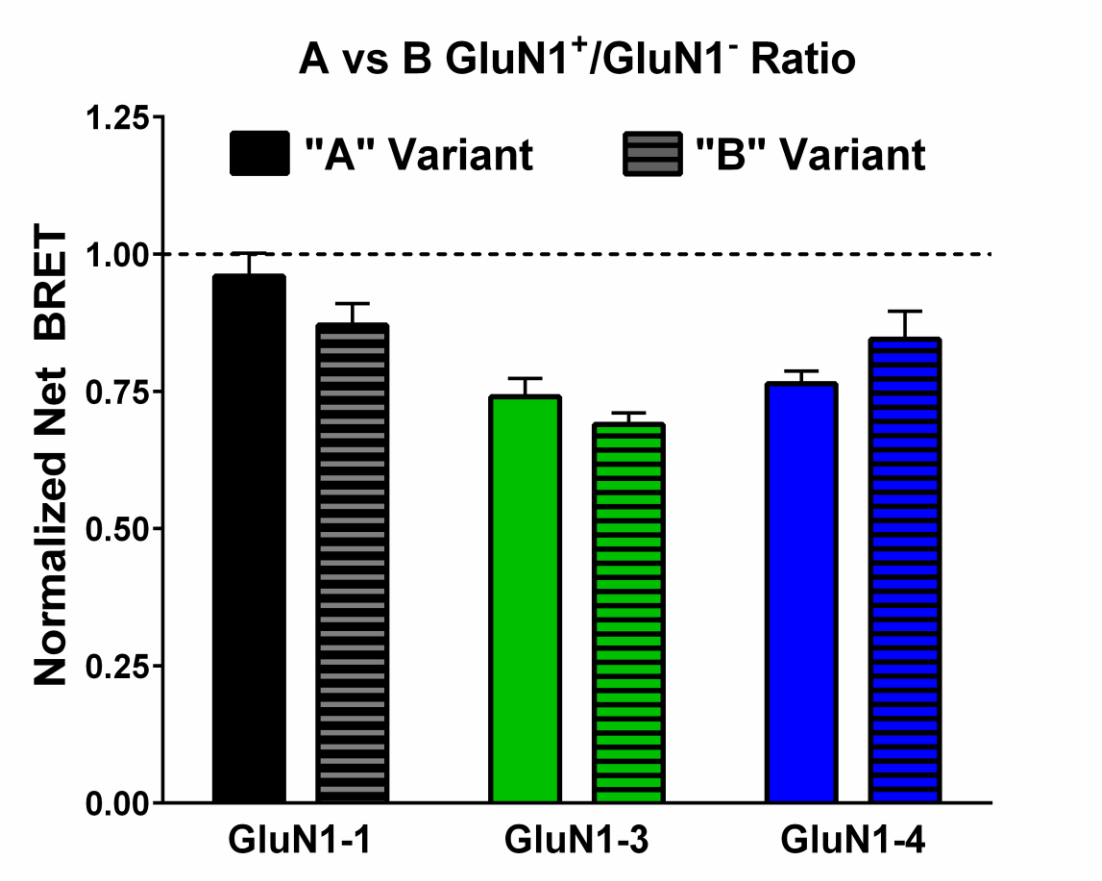


Figure IV.4: The "B" GluN1 variants containing Exon 5, an ~25 amino acid inclusion in the N-terminal domain, were also evaluated due to the known role of N-terminal domain alignment in receptor complex assembly. Statistical comparison of A and B variant GluN1+/GluN1- ratios yielded no significant results suggesting the presence of Exon 5 does not affect triheteromeric NMDA receptor assembly. (Two-way ANOVA; interaction $p > 0.05$, $F_{2,60} = 3.022$; A/B subtype $p > 0.05$, $F_{1,60} = 0.422$; splice variant $p < 0.0001$, $F_{2,60} = 15.18$)

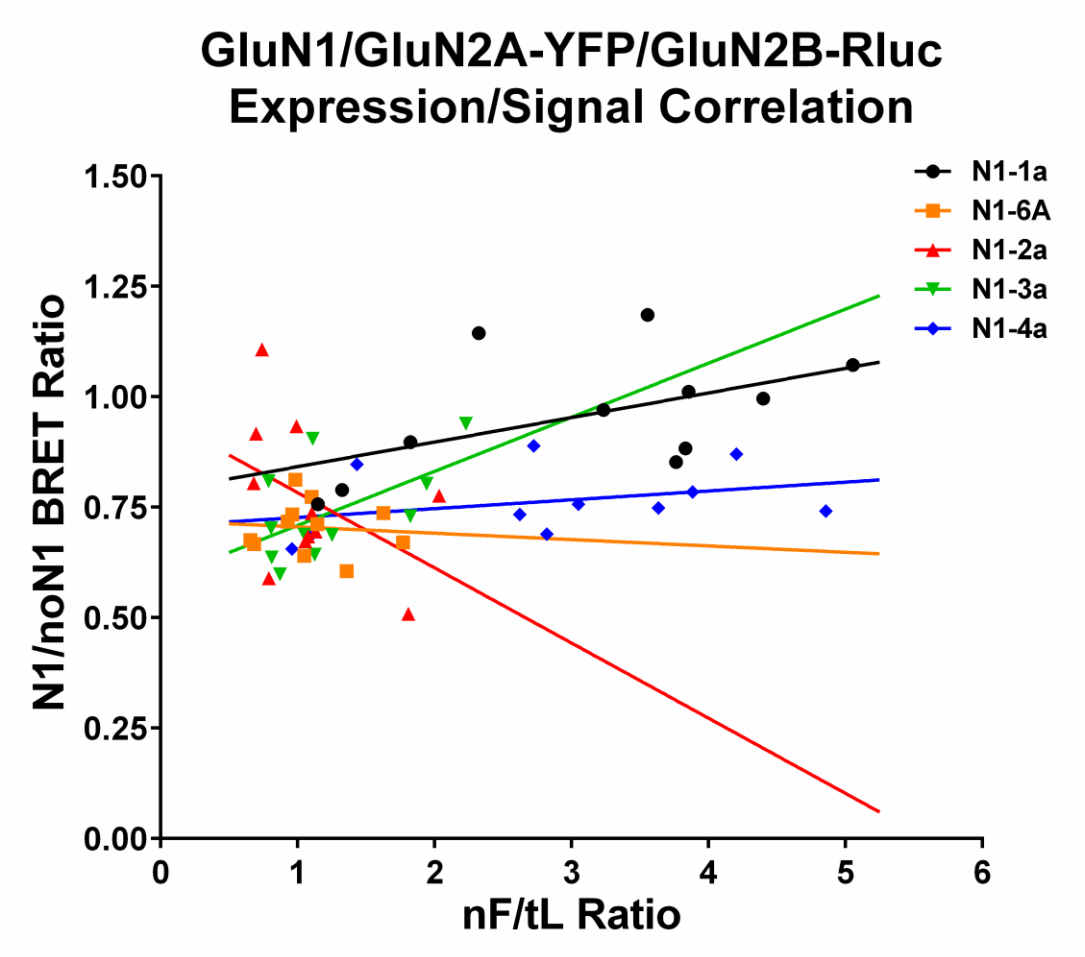


Figure IV.5: Linear regression analysis of Acceptor/Donor expression and Net BRET signal reveals no significant correlation, indicating that the signal observed is reflective of a specific interaction. Furthermore, expression of 2B-Rluc with soluble YFP elicits minimal BRET signal (data not shown), lending further support for the specificity of the BRET signal.

IV.4 Discussion

As revealed by the BRET data, it appears that splice variants of GluN1 that possess alternative C-termini do not significantly contribute to triheteromeric GluN1/GluN2A/GluN2B assembly within the ER. While reports have shown that significant ER retention motifs within the C-termini of GluN1 splice variants can

greatly impact surface delivery of NMDA receptors, the presence of the C1 cassette appears to largely dictate retention of NMDARs as total C-terminal truncation does not alter receptor membrane expression (Ehlers et al., 1998; Scott et al., 2001; Meddows et al., 2001). Indeed, our data as well as studies showing mutation of select residues within the C1 cassette or masking of C1 retention motifs by additional cassettes effectively relieve ER sequestration (Ehlers et al., 1998; Riou et al., 2012). Taken together, this data argues that the C-terminal alternative splicing of GluN1 may largely be a mechanism to dictate post-assembly targeting of fully complemented receptors, and does not significantly participate in GluN1/GluN2 co-assembly.

Further work has shown that N-terminal residues may instead be essential regulators of GluN1/GluN2 subunit association. While experiments have shown that N-terminal regions of GluN1 and GluN2 contain additional ER retention motifs, observations that their progressive deletion correlates with both blunted surface delivery and heightened presence of GluN1 homodimers argues that their presence is essential in the recognition and resulting co-assembly of GluN1 and GluN2 (Meddows et al., 2001; Qiu et al., 2009). Strangely, however, we did not observe any differences in net BRET between the “A” and “B” variants suggesting that such N-terminal recognition between GluN1 and GluN2 occurs regardless of the GluN2 subtype or does not involve the residues coded by Exon 5.

Thus, while discrete regions within GluN1 appear to be essential in both the assembly of complete NMDA receptor complexes as well as the exit of these receptor complexes from the endoplasmic reticulum, they do not appear to participate in the directed formation of triheteromeric GluN1/GluN2A/GluN2B receptors. Given the role of GluN1 and GluN2 C-termini in facilitating interactions with synaptic scaffolding proteins such as neuronal filaments and α-actinin, it seems much more likely that triheteromeric assembly is a stochastic process, and that post-assembly interactions in the secretory pathway govern synaptic enrichment of triheteromeric NMDARs (Wyszynski et al., 1997; Ehlers et al., 1998; Scott et al., 2001; Scott, Blanpied, & Ehlers, 2003). It remains unknown, however, if the presence of subtype-specific C-termini of GluN2 subunits can either regulate triheteromeric assembly or influence post-assembly trafficking distinct from diheteromeric NMDARs, and represents an interesting future direction for understanding the process of triheteromeric NMDA receptor expression.

Chapter V: Discussion

V.1 Summary of Findings

In Section I, the essential role of the N-methyl-D-aspartate receptor in neurotransmission was detailed with special emphasis on outlining the dichotomous function of NMDAR-mediated activity in synaptic signaling. Specifically, the relationship between intrinsic magnesium sensitivity, high calcium permeability, and high propensity for calcium-dependent inter-molecular signaling of NMDARs were discussed to impress the importance of this receptor in a number of synaptic processes including long term potentiation, understood to be the molecular basis for learning and memory. Such pains were taken in order to demonstrate how the preferential activity of alcohol, its predilection for NMDAR antagonism in particular, at this receptor can elicit profound behavioral manifestations such as acute intoxication, and that with continued exposure compel the induction of pathological behaviors that drive further alcohol abuse.

While much is now known both of the role of NMDARs in synaptic plasticity as well as the actions of ethanol on NMDA receptor function, gaps in knowledge nevertheless remain. One such glaring example is a persistent disregard for NMDA receptor subtype in teasing the actions of alcohol both on intrinsic receptor function and on NMDAR-dependent inter-molecular interactions. Thematically, then, the research I have conducted and presented here has sought to further parse the mechanisms of ethanol action on NMDA

receptors by discriminating them according to subtype, GluN2A- and GluN2B-containing types in particular.

Though the overarching hypothesis of Section II sought to determine what, if any, role extracellular linker domains had in dictating intrinsic sensitivity of NMDARs to ethanol, a corollary posited that manipulation of these domains could reveal essential differences in ethanol sensitivity between GluN2A- and GluN2B-subtypes. Indeed, while the data presented show that linker domains are not major determinants of receptor sensitivity to ethanol, the experimental methods employed did reveal essential differences in gating between the GluN2 subtypes that do affect the innate sensitivity of the receptor to alcohol. As these results are among the first to describe this phenomenon, their significance remains to be determined.

Section III similarly sought to parse GluN2A- and GluN2B-intrinsic ethanol actions by seeking to reveal whether the subunit-specific post-translational modifications of GluN2B described in Sections I.9-10 and III.1 could account for the differential potencies of ethanol between these subtypes. As previously discussed, the dephosphorylation of select tyrosine residues on the distal C-terminus of GluN2B is correlated with inhibition of NMDAR-mediated current by alcohol in neurons, and these sites are not found in homologous positions in GluN2A. Mutation of these residues to a non-phosphorylatable phenylalanine showed that these sites to do not significantly affect either basic receptor function or alcohol sensitivity. Indeed, this data corroborates results observed in Section

II; namely, that essential differences in channel gating between GluN2A- and GluN2B-containing NMDARs dictate their differential sensitivities to ethanol, not extraneous, subunit-specific sites of action.

Finally, Section IV.1 detailed the accumulating evidence suggesting that not only do NMDA receptors exhibit myriad different GluN2 subunit types, but as well that different GluN2 subunits heterogeneously assemble within individual receptors, adding further complexity to an already diverse receptor pool. Many significant questions remain regarding these novel triheteromeric receptors, however, we wished to determine whether the obligate GluN1 subunit, and its eight potential splice variants, could bias the formation of diheteromeric or triheteromeric GluN1/GluN2A/GluN2B receptor types. From the data presented, we conclude that such a case is unlikely, and that diheteromer/triheteromer receptor sorting occurs at a level beyond initial ER assembly.

V.2 Future Directions

While linker domains do not appear to participate significantly in defining the intrinsic sensitivity of NMDA receptors to ethanol, results observed in Section II nevertheless reveal new insights into the inherent function of and alcohol action on different NMDAR subtypes. That intrinsic differences in channel gating may partially define the idiosyncratic sensitivities to ethanol observed between GluN2 subtypes is especially interesting when considering the fundamentally different roles of NMDA receptor types in eliciting plastic phenomena such as long term potentiation and depression. It would be especially interesting to probe whether

such innate differences in alcohol sensitivity between the subtypes not only is a key determinant in driving the GluN2B-centric metaplastic mechanisms detailed in Sections I.9-10 and III that occur with chronic alcohol exposure, but as well these innate differences participate, if at all, in propagating the hedonic valence of alcohol intoxication. With the development of CRISPR-Cas9 technology, engineering knock-in mice that can display altered sensitivities of specific GluN2 subunits could be an invaluable resource to probe these very questions.

In a similar vein, it is unclear as to whether the metaplastic events detailed in Section III.1 are in any way related to the acute inhibition of NMDAR-mediated current by ethanol. Specifically, whether or not the effect of alcohol on NMDAR current, modest as it is, alters receptor signaling in such a way as to recruit GluN2B/STEP interactions, or if instead induction of STEP is an unrelated, upstream process. Again, creating a viable knock-in mouse expressing an alcohol insensitive mutant GluN2B subunit would allow for this phenomenon to be tested. Recent efforts by our laboratory to do this have so far been unsuccessful.

In Section IV the intriguing question was posed as to the functional significance of novel triheteromeric GluN1/GluN2A/GluN2B NMDA receptors in the pathogenesis of alcohol use disorders. Since relatively little is known about the function and expression of these receptor populations, reflected by a dearth of studies specifically addressing them in the literature, they represent fertile ground for research. While the expression of these receptors appears dependent

on a process distinct from GluN1-directed assembly, more elementary questions abound. Indeed, it remains unknown as to whether this receptor population is predominantly synaptic or extrasynaptic, much less whether chronic alcohol exposure alters their presence on the membrane.

V.3 Final Thoughts

The paramount reason for studying addiction is presumably in the noble pursuit of a cure. However, the ever-present lack of a “magic bullet” for alcohol use disorders even in the face of the decades of research outlined in Section I highlights the monumental difficulty in achieving such a goal. At its root, the problem is likely the sum of two factors, one being a research paradigm that has placed significant emphasis on small molecule treatments, thereby reducing complex cognitive disorders down to single molecules that target single receptors. The experimenters are not entirely to blame, as much of addiction research funding stems from publically supported institutions that expect extrinsic dividends for their support. The other, related factor stymying addiction treatment has been the rather slow development of effective non-invasive tools for treating cognitive disorders with minimal off-target effects. Indeed, such tools have only very recently come to the fore, in the form of transcranial magnetic stimulation (TMS) in its many iterations.

As to the first point, study of organic disorders often begins at the genetic or molecular level since many diseases are characterized by high-penetrance genetic mutations including Fragile X Syndrome, or diseases of acquired

receptor/protein dysfunction as observed with neurofibrillary tangles and aggregated amyloid-β protein that are the hallmarks of Alzheimer's Disease. While most clinicians agree that addiction is an organic disorder, and the NMDA-mediated metaplastic adaptations discussed in Section III.1 reflect this, the pathogenesis of the disease appears to begin at a much higher functional level. Indeed, divining the root cause of why people abuse ethanol in the first place highlights the intrinsically higher-order cognitive nature of addiction. To be sure, profound molecular adaptations are known to occur that proffer the addicted state, however targeting the symptoms rarely, if ever, cures the underlying disease. Thus, a tenable and lasting treatment for alcohol addiction likely lies in the implementation of novel therapies such as TMS whose therapeutic mechanisms seek primarily to re-engage and potentiate the activity of atrophied regions of executive function such as the dorso-lateral prefrontal cortex and as a result re-instill higher-order cognitive control on the patient's over-represented limbic drives. Collaborative pilot studies between the NIAAA and the University of Palermo have in fact demonstrated the power of this technique in treating cocaine addiction and likely portend a substantial increase in its clinical use (Terraneo et al., 2016).

This is not to say that molecular pharmacological research has nothing of value to offer; quite the contrary. As outlined in Section I.1, the value of addiction research is intrinsically two-fold. Explicitly, it incrementally leads experimenters towards a cure or solution for a costly, both emotionally and financially, problem.

As well, though, to echo the point made in Section I.1, it implicitly informs ourselves about ourselves. As the architects of computers, human beings have the luxury of stacks upon stacks of circuit diagrams, manuals, and procedures that display in exquisite detail the mechanics and coding that make up electronic computing, from the higher-order graphical user interface down to the level of elementary voltage-dependent floating gates that comprise flash memory. By comparison, our collective understanding of the human brain is woefully incomplete, and is perhaps best exemplified by the persistent, and false, belief on the part of the general public that at any given time a person uses merely 10% of their brain – something one would do well not to attempt! While our understanding of the machinations of the mind pale in comparison, our approach to problem solving when an error is encountered follows a similar procedure, whether it be an organic or electronic “brain.”

One first identifies at what level the error appears to originate. Alcohol intoxication is characterized by cognitive depression, leading to hypotheses that it inhibited excitatory neurotransmission that were ultimately confirmed. Researchers then focused their attention to the next level down, interrogating what molecular substrates elicited such depression of excitation. With the concerted efforts of an entire field, such a line of questioning not only parsed glutamatergic neurotransmission into three distinct receptor classes, identifying the NMDA receptor as a critical site of alcohol action, but also highlighted the

central importance of this receptor in the manifestation of learning and memory – a biological equivalent to the floating gates of flash memory.

By this analogy, we begin appreciate the latent value of molecular biological research, and by understanding the low-level intra- and inter-molecular interactions that percolate up into higher order cognitive processes, a view to treatment becomes clearer. These islands of insight discovered *a fortiori* in the pursuit of elevating the health of humanity legitimize and reinforce the argument that research is a universally sound investment – *quaerendo invenietis*.

Chapter VI: References

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