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Disruption of S2-M4 Linker Coupling Reveals Novel Subunit-Specific Contributions to N-methyl-D-aspartate Receptor Function and Ethanol Sensitivity

Benjamin A. Hughes and John J. Woodward

Department of Neuroscience and Addiction Sciences Division, Department of Psychiatry & Behavioral Sciences, Medical University of South Carolina, Charleston, SC 29425

Abstract

The N-methyl-D-aspartate (NMDA) receptor is a glutamatergic ion channel and is a known site of ethanol action. Evidence suggests that ethanol inhibits NMDA receptor activity by reducing channel open probability and mean open time potentially via interaction with specific residues within the transmembrane (M) domains 3 and 4 of GluN subunits. Recent models of NMDAR function demonstrate that extracellular residues near the M domains are key regulators of gating, suggesting that they may contribute to ethanol sensitivity. To test this, we substituted cysteines at key positions in GluN1 and GluN2 M3-S2 and S2-M4 regions previously shown to affect channel open probability and mean open time similar to ethanol treatment. Although crosslinking of these domains was predicted to restrict linker domain movement and occlude ethanol inhibition, only intra-GluN1 M1:M4 linker-crosslinked receptors showed a decrease in ethanol sensitivity. For the converse experiment, we expressed NMDARs with glycine substitutions in the S2-M4 domain of GluN subunits to enhance M4 flexibility and recorded currents in response to ethanol. Glycine substitution in the GluN1 S2-M4 region significantly decreased glutamate potency of GluN1(A804G)/GluN2A receptors, while GluN1(A804G)/GluN2B receptors exhibited no change in glutamate sensitivity. In contrast, GluN1/GluN2B(S811G) receptors showed a 10-fold increase in glutamate potency while GluN1/GluN2A(S810G) receptors showed no change. Surprisingly, while S2-M4 glycine substitutions modulated ethanol sensitivity, this was observed only in receptors that did not display a change in agonist potency. Overall, these results suggest that S2-M4 linkers strongly influence receptor function and modestly impact ethanol efficacy in a subunit- and receptor subtype-dependent manner.

Authorship Contribution

Participated in research design: Hughes and Woodward

Conducted experiments: Hughes

Contributed new reagents or analytic tools: None

Performed data analysis: Hughes

Wrote or contributed to the writing of the manuscript: Hughes and Woodward

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Introduction

The N-methyl-D-aspartate receptor (NMDAR) is a ligand-gated ion channel activated by the excitatory neurotransmitter glutamate and is highly enriched in the post-synaptic density where it is essential to synaptic signaling and plasticity. NMDARs are tetramers composed of two obligate GluN1 subunits and two GluN2 subunits, and exhibit vastly different channel kinetics, pharmacological profiles, and protein-protein interactions depending on the GluN2 subunit expressed (Traynelis et al., 2010). NMDA receptor dysfunction is implicated in the etiology of a number of disease states including schizophrenia, excitotoxic cell death, and addiction to drugs of abuse including ethanol (Cull-Candy, Brickley, & Farrant, 2001). As a primary site of action for ethanol, all NMDA receptor subtypes are inhibited by ethanol at doses associated with behavioral intoxication, and prolonged or repeated exposures result in compensatory adaptations that may contribute to further ethanol-seeking (Lovinger, White, & Weight, 1989; Masood, Wu, Brauneis, & Weight, 1994; Ron, 2004). Given the importance of NMDARs in mediating various forms of neuronal plasticity and associative learning, it is important to understand how ethanol alters NMDA receptor function.

Though its precise mechanism(s) of action remains unclear, results from initial reports showed that ethanol does not compete with agonist binding nor does it act as an open channel blocker (Peoples & Weight, 1992; Mirshahi & Woodward, 1995). Instead, ethanol inhibits channel gating by reducing open probability and mean channel open time (Wright, Peoples, & Weight, 1996), consistent with its role as an allosteric modulator of channel function. Work by our laboratory and others has implicated residues within transmembrane (TM) domains 3 and 4, key elements for channel gating, in mediating the acute effects of ethanol. Specifically, mutation of phenylalanine (F) 639 in the TM3 of GluN1 to a smaller alanine (A) residue rendered receptors significantly less sensitive to ethanol (Ronald, Mirshahi, & Woodward, 2001; Smothers & Woodward, 2006; den Hartog et al., 2013), and mutation of alanine 825 in TM4 of GluN2A to the much larger tryptophan (W) produced similar reductions in ethanol sensitivity (Ren, Honse, & Peoples, 2003). On a structural basis, it is tempting to conclude that these residues coordinate to define an ethanol interaction site within the primary gating elements. However, receptors with both mutations still exhibit ethanol inhibition, albeit significantly reduced compared to wild type receptors (Smothers & Woodward, 2006). Furthermore, when the F to A mutation was made at the analogous TM3 site in GluN2A (F637), no change in ethanol sensitivity was noted (Ren et al., 2008), suggesting differential roles for the GluN1 and GluN2 subunits in mediating ethanol sensitivity. Recent studies by our laboratory and others further support this observation and show that constitutively active NMDA receptors have reduced sensitivity to ethanol that varies in a GluN2 subunit-dependent fashion (Xu, Smothers, Trudell, & Woodward, 2012).

Kinetic modeling of NMDAR function has since shown that, while M3 and M4 are core elements in the gating cascade, other extracellular regions also participate significantly in regulating channel activity. For example, when amino acids within elements that link agonist binding sites to the transmembrane domains (e.g. S1-M1 and S2-M4 linker domains) were conformationally locked via cysteine substitution, significant effects on gating were observed. Restricting the movement of these linkers significantly blunted channel activity in

a subunit-dependent manner, revealing unequal contributions of the GluN1 and GluN2 subunits to different steps within the gating cascade (Kazi et al., 2013). Importantly, locking these linker domains resulted in changes in open probability and mean open time of the channel that resemble those observed with ethanol-treated wild type receptors (Kazi et al., 2013; Wright, Peoples, & Weight, 1996). Thus, the residual ethanol sensitivity observed for F639A/A825W mutant NMDARs (Smothers & Woodward, 2006) may reflect ethanol's interaction with other extracellular determinants of gating, consistent with reports showing that inhibition requires extracellular application of ethanol (Peoples & Stewart, 2000).

Recently, results from our preliminary studies show that glycine substitution of discrete residues at the end of the S2-M4 linker elicits profound changes in agonist sensitivity (Hughes BA & Woodward JJ. Functional modification of NMDA receptors by cysteine crosslinking S2-M4 linker domains: Implications for ethanol action. *Alcoholism Clin. Exp. Res.* 38(Supplement S1):8A, Abs. 0032, 2014.). In the present study, we use cysteine crosslinking and glycine substitutions to differentially impede and enhance extracellular linker flexibility to determine the role of the S2-M4 linker in dictating the function and ethanol sensitivity of GluN2A- and GluN2B-containing NMDA receptors.

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% CO₂. 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:GluN2A 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 MgCl₂ 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), CaCl₂ (1.8), HEPES (5), glucose (10), (pH adjusted to 7.4 with 1 M NaOH, and osmolality adjusted to 315–325 mOsm with sucrose). Patch pipettes (2–5 MOhms) were pulled from standard wall borosilicate glass (1.5 × 0.85 mm) and filled with internal solution containing the following (in mM); CsCl (140), MgCl₂ (2), EGTA (5), HEPES (10), NaATP (2), NaGTP (0.3), (pH adjusted to 7.2 with 2 M CsOH, and osmolality 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-potential 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, Prism 6.0 software (GraphPad Software, San Diego, CA) was used to calculate EC₅₀ 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 EC₅₀ 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 intergroup 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 ($1/\tau_{\text{MK-801}}$).

Results

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

Figure 1A shows the amino acid sequence of S1-M1 and S2-M4 linkers of GluN1, GluN2A and GluN2B subunits and schematic (Fig. 1B) and molecular (Fig. 1C/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 2A, 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 2C), 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).

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 AI, Rosconi MP, and Gouaux E, 2009; Karakas and Furukawa, 2014; Kazi et al., 2013). As shown in Figure 3A, 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 3B; 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 4A). DTT treatment enhanced these currents with the largest DTT-induced current potentiation, over 900%, observed for intra-subunit GluN2B receptors (Figure 4B; one-sample t-test; $p < 0.05$).

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

The results shown in Figures 3 and 4 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 5, 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 5B–C). This effect persisted when GluN1 (A804G) was co-expressed with GluN2A (S810G) (EC_{50} values: GluN1/GluN2A wild type = 2.18 μ M, GluN1 A804G/GluN2A = 3.69 μ M, GluN1/GluN2A S810G = 2.72 μ M, GluN1 A804G/GluN2A S810G = 3.54 μ M; ANOVA and Dunnett's 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 5E–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 μ M, GluN1 A804G/GluN2B = 1.09 μ M, GluN1/GluN2B S811G = 0.12 μ M, GluN1 A804G/GluN2B S811G = 0.78 μ M; ANOVA and Dunnett's 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 6A). 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 6B). 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 2, there were no significant differences in the rate of MK801 block for any of the glycine mutants tested. Table 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).

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 KM, Mirshahi T, and Woodward JJ, 2001; Ren H, Honse Y, and Peoples

RW, 2003; Xu, Smothers and 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 7A–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 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 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).

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 GluNB-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 support 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.

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Highlights

- LBD-TM linker domains contribute significantly to NMDA receptor function.
- Functional effects of S2-M4 linkers are GluN2 subtype-dependent.
- Non-homology of linker roles partially explains subtype-specific gating profiles.
- LBD-TM linkers contribute to non-homologous ethanol actions between GluN2 subtypes.

Figure 1.

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).

Figure 2. 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 $\pm$ 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 $\pm$ 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.

Figure 3.

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 $\pm$ 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) $\pm$ 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 4.

Crosslinking M1 and M4 linker domains of GluN1/2B receptors alters receptor function. *A*, Bar graph shows mean amplitude $\pm$ 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 $\pm$ 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.

Figure 5.

Pre-TM4 glycine mutations in GluN1/GluN2A and GluN1/GluN2B receptors alter glutamate potency. *A*, Representative traces from a GluN1/GluN2A-expressing cell showing response to 0.1 μ M, 3 μ M, and 30 μ M concentrations of glutamate. *B/C*, Concentration-response curves (*B*) and summary graph (*C*) of glutamate EC₅₀ 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 $\pm$ S.E.M. EC₅₀ 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 μ M, 1 μ M, and 10 μ M concentrations of glutamate. *E/F*, Concentration-response curves (*E*) and summary graph (*F*) of glutamate EC₅₀ 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 $\pm$ S.E.M. EC₅₀ 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 6.

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 EC₅₀ values $\pm$ 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 EC₅₀ of GluN2B (S810G) mutant was not affected by also substituting a glycine at position 811. Data shown are mean $\pm$ S.E.M. EC₅₀ 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).

Figure 7. 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 $\pm$ 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 $\pm$ 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).

Table 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 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 <i>Potentiation</i>	178% ↑	318.6% ↑	653% ↑ ****	4.5% ↑	151% ↑	902% ↑ ****
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 ****
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)