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Deletion of the N-terminal Domain (NTD) Alters the Ethanol Inhibition of NMDA Receptors in a Subunit-Dependent Manner

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Abstract

Background—Ethanol inhibition of NMDA receptors is poorly understood due in part to the organizational complexity of the receptor that provides ample locations for sites of action. Among these the N-terminal domain of NMDA receptor subunits contains binding sites for a variety of modulatory agents including zinc, protons and GluN2B selective antagonists such as ifenprodil or Ro-25–6981. Ethanol inhibition of neuronal NMDA receptors expressed in some brain areas has been reported to be occluded by the presence of ifenprodil or similar compounds suggesting that the N-terminal domain may be important in regulating the ethanol sensitivity of NMDA receptors.

Methods—Wild-type GluN1 and GluN2 subunits and those in which the coding sequence for the N-terminal domain was deleted were expressed in HEK293 cells. Whole-cell voltage-clamp recording was used to assess ethanol inhibition of wild-type and mutant receptors lacking the N-terminal domain.

Results—As compared to wild-type GluN1/GluN2A receptors, ethanol inhibition was slightly greater in cells expressing GluN2A subunits lacking the N-terminal domain. In contrast, GluN2B N-terminal deletion mutants showed normal ethanol inhibition while those lacking the N-terminal domain in both GluN1 and GluN2B subunits had decreased ethanol inhibition as compared to wild-type receptors. N-terminal domain lacking GluN2B receptors were insensitive to ifenprodil but retained normal sensitivity to ethanol.

Conclusions—These findings indicate that the N-terminal domain modestly influences the ethanol sensitivity of NMDA receptors in a subunit-dependent manner. They also show that ifenprodil's actions on GluN2B containing receptors can be dissociated from those of ethanol. These results suggest that while the N-terminal domain is not a primary site of action for ethanol on NMDA receptors, it likely affects sensitivity via actions on intrinsic channel properties.

Keywords

Ethanol; NMDA receptors; N-terminal domain; glutamate

INTRODUCTION

The voluntary consumption of ethanol by humans and animals results in a broad spectrum of neurophysiological and neuropathological changes, many of which are correlated with the degree of perturbation of N-Methyl-D-Aspartate (NMDA) receptor function (Alfonso-Loeches and Guerri, 2011; Krystal et al., 2003). Given this correlation, the mechanism(s) of

exactly how acute ethanol interrupts NMDA receptor function has attracted considerable attention from the alcohol research community. Currently, ethanol is thought to inhibit NMDA receptors in a non-competitive and voltage-independent manner (Peoples et al., 1997). Single channel studies show that ethanol decreases the frequency of channel openings and the channel mean open time with no change in single channel conductance (Wright et al., 1996). These actions are consistent with ethanol acting as an allosteric modulator of NMDA receptors although the site of this allosteric interaction remains unknown.

NMDA receptors are hetero-multimeric proteins that are composed of four subunits arranged in a dimer of dimer formation (for review see Traynelis et al., 2010). In this arrangement, pairs of GluN1/GluN2 dimers assemble to generate the functional receptor that contains binding sites for glutamate (GluN2) and the co-agonist glycine (GluN1). Receptor complexity is increased by the existence of eight splice variants for the GluN1 subunit, four different GluN2 subunits, and the recently discovered GluN3 subunit that can substitute for one of the GluN1 subunits in the mature receptor (Pachernegg et al., 2012). Structurally, the NMDA receptor is similar to other ionotropic glutamate receptors, and consists of an extracellular N-terminus [N-terminal domain (NTD); a ligand-binding domain (LBD)], 3 membrane-spanning domains (TM1, TM3, TM4), a reentrant loop (M2), and a cytoplasmic C-terminus (Sobolevsky et al., 2009).

Crystallographic studies of glutamate receptors suggest that the NTD of NMDA receptors provides binding sites for a wide range of molecules that influence receptor function (Farina et al., 2011; Karakas et al., 2011). The NTD is known to contain binding sites for divalent cations such as zinc, protons, and subunit-selective antagonists such as ifenprodil and related analogs (Brimecombe et al., 1997; Fayyazuddin et al., 2000; Gallagher et al., 1997; Saglietti et al., 2007). The finding that ifenprodil inhibition of GluN2B receptors is controlled by the NTD is especially interesting in light of studies suggesting that ethanol may preferentially inhibit GluN2B receptors in neurons (Kash et al., 2008; Roberto et al., 2004; Wills et al., 2012). Functionally, the NTD plays a role in channel gating and agonist potency (review Hansen et al., 2010). For instance, Madry et al. (2007) demonstrated that the high glycine affinity of the GluN2B containing receptor could be transferred to the GluN2A subunit by exchanging their NTDs. In addition, the properties influenced by the NTD such as alteration of channel gating and a preference for subunit-specific inhibition are roughly similar to those produced by ethanol, (Lovinger, 1995; Smothers et al., 2001; Wright et al., 1996). This suggests that the ethanol inhibition of NMDA receptors may be modulated by the NTD. In this study, mutant GluN1 and GluN2 subunits lacking the NTD were expressed in HEK293 cells and ethanol sensitivity was analyzed by whole-cell voltage-clamp electrophysiology. The findings of these experiments suggest that NTD domains exert a subunit-selective effect on the ethanol sensitivity of recombinant NMDA receptors.

MATERIALS AND METHODS

Molecular

Drs. S. Nakanishi (NR1-1a and NR2A), and P. Seeburg (NR2B) kindly provided the wild-type NMDA receptor cDNA used in these experiments. Drs. C. Madry and P. Paoletti kindly provided the N-terminal deleted (amino acids 5 – 358) GluN1 (GluN1^{NTD}) and GluN2 NTD deletion (GluN2^{NTD}) constructs, respectively (Madry et al., 2007; Rachline et al., 2005).

HEK293 cell maintenance and receptor expression

HEK293 cells were obtained from American Type Culture Collection (Manassas, VA) and grown according to the provided protocol. In brief, cells were cultured in Dulbecco's

Minimum Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Hyclone, Logan, Utah) and grown at 37°C in a 5% CO₂ environment. Twenty-four hours following plating of low-density cultures (approximately 5×10^4 cells per dish) onto 35 mm dishes coated with poly-L-lysine, cells were transfected with equal amounts of subunit cDNA (1 µg) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). To identify transfected cells, 0.25 – 0.5 µg of pmaxGFP was added to transfection. Cells were used for electrophysiological recordings 24 to 48 hours following transfection.

Electrophysiological recording conditions

All recordings were performed as previously described (Smothers and Woodward, 2009). Briefly, cells were perfused with an external solution containing (in mM): NaCl 135, KCl 5.4, CaCl₂ 1.8, HEPES 5, and glucose 10 (pH adjusted to 7.2 with NaOH and osmolarity adjusted to 290–300 mOsm/kg with sucrose). The pipette filling solution was composed of (in mM): N-methyl-D-glucamine 100, CsCl 40, NaATP 2, MgCl₂ 2, HEPES 10, EGTA 10 (pH was adjusted to 7.2 with KOH and osmolarity was adjusted to 325 mOsm/kg with sucrose). In experiments examining current-voltage relationships, the internal solution contained (in mM): KCl 140, CaCl₂ 1, NaATP 4, MgCl₂ 6, HEPES 10, EGTA 5 (pH was adjusted to 7.2 with KOH and osmolarity was adjusted to 290–300 mOsm/kg with sucrose). All internal solutions used for each experiment were from frozen stocks. All drug solutions were prepared fresh for each experiment from frozen stocks in external solution. Stock solutions of glycine and glutamate were prepared in water and diluted into external solution. Ethanol was purchased from Pharmco-Aaper (Shelbyville, KY).

Whole-cell voltage-clamp recordings (Hamill et al., 1981) were performed at room temperature using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). Cells were voltage-clamped at –55 or –60 mV and current records were filtered at 1 kHz (eight-pole Bessel filter) and digitized at 2 kHz using an ITC-16 interface (Instrutech Corp., Port Washington, NY). Software control of data acquisition was provided by Pulse Control running within the Igor Pro program (version 4.03; WaveMetrics, Portland, OR) on an Apple Macintosh G3 computer (Apple Computer Inc. Cupertino, CA). Patch electrodes were fabricated from thick-walled borosilicate glass (B150; WPI, Sarasota, FL) and filled with internal solution (tip resistance 3–6 MΩ). A 3-barrel perfusion apparatus (barrel ID 0.6 mm; Warner Instruments, SF-77B) was used to switch between control and drug-containing solutions. Solution exchange times were determined to be between 6 – 8 ms as calculated by measuring changes in the liquid junction current across an open electrode when switching between solutions with different ionic strength.

Data and Statistical Analysis

Axograph 4.9 (Axograph.com) was used to measure current waveform parameters. Currents were evoked by a 5 second agonist application and were measured at two time points, peak and steady-state. Peak current amplitude was determined as the difference between the current immediately prior to agonist application and at the point where inward current was maximal. Steady-state current (I_{ss}) amplitude was determined as the difference between current immediately prior to agonist application and that 4.5 seconds into the agonist application where currents were stable. Percent desensitization was calculated relative to agonist-induced peak current using the formula $[1 - (I_{\text{steady-state}}/I_{\text{peak}})] \times 100$. Drug induced inhibition of receptor currents (I_{Control}) was calculated using the formula $[1 - (I_{\text{Agonist+drug}}/I_{\text{Control}})] \times 100$, where $I_{\text{Agonist+drug}}$ represents the response to co-application of agonist + drug, and I_{Control} represents the mean of two responses to agonist, one before and one after the co-application of the drug. Leak currents were continually monitored as an indicator of seal and cell integrity. Cells that showed unstable leak currents were not included in the data analysis.

Statistical analyses were performed using the software program Prism 4.0 (Graphpad Software Inc., San Diego, CA). All inhibition data values are expressed as percent inhibition and represent the mean $\pm$ S.E.M and *N* represents the number of cells. The statistical significance was determined using ANOVA or Student's *t*-test, two-tailed, unpaired.

RESULTS

Previous studies have shown that cells transfected with subunits lacking the NTD (GluN1^{NTD}, GluN2A^{NTD} and GluN2B^{NTD}) produce functional NMDA receptors (Gielen et al., 2009; Madry et al., 2007; Paoletti et al., 2000). To determine whether the NTD of either the GluN1, GluN2A, or GluN2B subunit influences ethanol inhibition, cells expressing recombinant receptors with or without NTD domains were exposed to the agonists glutamate (10 μ M) and glycine (100 μ M) in the presence or absence of 100 mM ethanol. All subunit combinations tested resulted in functional receptors when expressed in HEK293 cells except for GluN1^{NTD}/GluN2A that generated no measurable currents during application of agonists. Current amplitude differed between wild-type and mutant receptors (Table 1). Mean steady-state current from GluN1/GluN2A^{NTD} receptors was significantly smaller than that of wild-type GluN1/GluN2A. In GluN2B containing receptors, mean current amplitude was significantly greater for GluN1^{NTD}/GluN2B^{NTD} receptors compared to wild-type. Channel function was similar between wild-type and mutant receptors lacking an NTD as measured by the extent of macroscopic receptor desensitization (ratio of steady-state to peak current; Table 1). GluN1/GluN2A^{NTD} and GluN1^{NTD}/GluN2A^{NTD} receptors displayed a non-significant ($p > 0.1$; one-way ANOVA) 7% increase in desensitization compared to wild-type receptors.

Effect of GluN2 NTD on ethanol inhibition

Since the GluN2 NTD has been shown to be required for modulation of NMDA receptor function by various allosteric modulators, we first examined ethanol inhibition of receptors lacking the GluN2 NTD (Herin and Aizenman, 2004). Application of agonists resulted in robust currents in HEK293 cells expressing GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2A^{NTD} and GluN1/GluN2B^{NTD} receptors (Figure 1A, 1B). Ethanol (100 mM), co-applied with agonists significantly inhibited currents from all receptor combinations. The magnitude of ethanol inhibition was significantly increased in mutant GluN1/GluN2A^{NTD} receptors as compared to that of wild-type receptors (Figure 1C; $p = 0.0007$; unpaired *t*-test). However, ethanol (100 mM) inhibited currents from GluN1/GluN2B^{NTD} receptors to the same extent as those from wild-type GluN1/GluN2B receptors (Figure 1D).

Effect of GluN1 NTD on ethanol inhibition

Given the increase in inhibition observed in GluN2A^{NTD} containing receptors, we investigated whether receptors lacking the GluN1 NTD would also display altered ethanol inhibition. No currents were observed in cells transfected with GluN1^{NTD} and the wild-type GluN2A subunit (data not shown) while those transfected with both subunits lacking the NTD showed robust agonist-evoked responses (Figure 2A). However, ethanol inhibition was unchanged in GluN1^{NTD}/GluN2A^{NTD} receptors as compared to wild-type GluN2A receptors (Figure 2B). In contrast to non-functional GluN1^{NTD}/GluN2A receptors, GluN1^{NTD}/GluN2B receptors were fully functional as were those formed from GluN1^{NTD}/GluN2B^{NTD} subunits (Figure 2C). As compared to wild-type GluN1/GluN2B receptors, responses from receptors lacking the NTD in both GluN1 and GluN2B subunits were less inhibited by ethanol (Figure 2D; Dunnett post-hoc, $p < 0.01$).

Effect of NTD deletion on ifenprodil/ethanol sensitivity

Results from several studies have suggested that inhibition of GluN2B containing receptors with subunit-selective antagonists such as ifenprodil or R0-25-6981 may occlude further inhibition by ethanol (Criswell et al., 2003; Kash et al., 2008; Roberto et al., 2004; Wills et al., 2012). These compounds bind within the NTD domain and as predicted, deletion of the NTD in either GluN1 or GluN2B subunits eliminated ifenprodil inhibition of agonist-evoked currents (Figure 3A).

To investigate the putative interaction between ifenprodil and ethanol on GluN2B receptors, we examined the effect of applying ifenprodil in the presence of ethanol in wild-type and mutant GluN1/GluN2B receptors lacking the NTD. In wild-type GluN1/GluN2B receptors, ethanol (100 mM) inhibited agonist-evoked currents and responses were further inhibited during co-application of 10 μ M ifenprodil (Figure 3B). Although ifenprodil inhibition did not fully recover during the recording epoch, holding currents slowly returned to baseline values upon washout (data not shown). In GluN1/GluN2B^{NTD}, GluN1^{NTD}/GluN2B, and GluN1^{NTD}/GluN2B^{NTD} receptors, co-application of ifenprodil during ethanol exposure produced no further inhibition of the current (Figure 3B).

The magnitude of ifenprodil inhibition of wild-type GluN1/GluN2B receptors was not altered by the presence of ethanol. Thus, currents were inhibited by $59.1 \pm 4.4\%$ during application of ifenprodil alone while co-application of ifenprodil and ethanol inhibited wild-type GluN1/GluN2B receptor currents by $60.9 \pm 4.7\%$ (Figure 4A). In mutant GluN1/GluN2B^{NTD} receptors, ifenprodil alone had little effect on agonist-evoked currents ($6.6 \pm 2.6\%$) while inhibition produced during co-application of ifenprodil and ethanol was not significantly different from that of ethanol alone (Figure 4B).

DISCUSSION

The results of the present study show that deletion of the NTD from GluN1 or GluN2 subunits modestly alters the magnitude of ethanol inhibition of NMDA receptors. This was observed in two instances with GluN1/GluN2A^{NTD} receptors showing enhanced inhibition and GluN1^{NTD}/GluN2B^{NTD} receptor showing reduced inhibition.

With respect to GluN2A receptors, this effect was selective for the GluN2A NTD deletion mutant as no change was observed when the NTD of both GluN1 and GluN2A were removed. Nanomolar concentrations of zinc ions have been shown to modulate NMDA channel gating by interacting with residues in the GluN2A NTD (Choi and Lipton, 1999; Fayyazuddin et al., 2000; Paoletti et al., 2000). Thus, it is possible that the change in ethanol inhibition in GluN2A NTD deleted receptors found in this study could be secondary to the elimination of high-affinity zinc binding. However, findings from previous studies in this lab show that ethanol inhibition is not altered by a point mutation (GluN2A H128S) that renders these receptors zinc-insensitive (Woodward and Smothers, 2003). It is also possible that the increase in ethanol inhibition of GluN2A NTD deletion mutants could be a result of changes in channel kinetics that accompany removal of this domain. For example, Madry et al. (2007) reported differences in desensitization between wild-type receptors and those lacking the NTD in both GluN1 and GluN2A. In this study, we found a small, but statistically insignificant change in receptor desensitization in GluN2A-containing receptors lacking the NTD. Madry et al. (2007) also noted a slight increase in the EC₅₀ values for glutamate and glycine of GluN1/GluN2A^{NTD} and GluN1^{NTD}/GluN2A^{NTD} receptors that could potentially affect ethanol sensitivity. However, this effect is unlikely to explain our findings since the actions of ethanol are largely agonist and co-agonist independent (Mirshahi and Woodward, 1995; Peoples and Weight, 1992; Peoples et al., 1997) and we found no change in ethanol inhibition for GluN1^{NTD}/GluN2A^{NTD} receptors.

In addition to changes in receptor kinetics and agonist sensitivity of NTD deletion mutants, removing this domain in GluN2A subunits reduces the open probability of the receptor (Gielen et al., 2009). With respect to ethanol, most studies report that wild-type GluN2A receptors are less sensitive to inhibition by ethanol than those containing Glu2B (Lovingier, 1995; Smothers et al., 2001) and GluN2A receptors have a substantially higher open probability than GluN2B receptors (Chen et al., 1999). By removing the GluN2A NTD and lowering its open probability to a level similar to that of GluN2B-containing receptors (Gielen et al., 2009), ethanol's efficacy as an allosteric inhibitor of GluN2A receptors may be increased. An inverse relationship between ethanol inhibition and open probability is supported by findings from a recent report from this lab that investigated the ethanol sensitivity of constitutively active NMDA receptors (Xu et al., 2012). In that study, a cysteine was substituted at the alanine 7 position (A7C) in the highly conserved TM3 domain (SYTANLAAF) of GluN1 and GluN2 subunits that is involved in receptor gating. While ethanol IC₅₀ values were not altered in agonist-gated A7C receptors tested under control conditions, IC₅₀ values for ethanol were dramatically increased (7–14 fold rightward shift in the dose-response curve) when these receptors were treated with a thiol-modifying agent that induces agonist-independent gating. Since thiol-modification of A7C receptors produces receptors that are at or near maximal open probability, these results suggest that domain-induced changes in gating properties such as those produced by NTD deletion can influence the ethanol sensitivity of NMDA receptors.

In the current study, receptors composed of wild-type GluN2A and NTD-deleted GluN1 subunits were non-functional. In addition to its effects on receptor function, the NTD is also known to be involved in receptor assembly (Farina et al., 2011), subunit trafficking and surface expression (Meddows et al., 2001). The lack of functional responses in GluN1^{NTD}/GluN2A receptors suggests that these receptors were identified as mis-formed and were not trafficked to the surface (Qiu et al., 2009). This is consistent with findings from a recent study showing that the GluN2A NTD contains an endoplasmic reticulum retention signal that prevents surface expression unless masked by the GluN1 NTD (Qiu et al., 2009). However, this control mechanism may be cell-type specific as, unlike with HEK cells used in this study, Madry et al., (2007) reported that, GluN1^{NTD}/GluN2A receptors are functional when expressed in oocytes.

In contrast to that observed for GluN1/GluN2A^{NTD} receptors, ethanol inhibition of GluN2B containing receptors was reduced only when the NTD was deleted from both GluN1 and GluN2B subunits. Similar to that discussed for NTD-deleted GluN2A receptors, this change may also reflect alterations in receptor kinetics and/or gating. However, in the present study, no changes were seen in the extent of macroscopic desensitization of NTD-deleted GluN2B-containing receptors. In addition, the open probability of GluN1/GluN2B^{NTD} receptors is reported to not be different from wild-type receptors (Gielen et al., 2009) although we note that the gating characteristics of the double NTD deletion receptor have not been reported. Although the mechanism underlying the reduction in ethanol inhibition of NTD deleted GluN1/GluN2B receptors is currently unknown, the opposing actions of NTD deletion on ethanol modulation of GluN2A and GluN2B receptors suggests that this domain is not the major site mediating the antagonistic actions of ethanol on NMDA receptors.

Finally, the NTD provides binding sites for modulators such as the polyamines, zinc (Rachline et al., 2005), and on GluN2B receptors, the phenylethanolamine antagonist ifenprodil and related compounds such as Ro-25–6981 (see review by Traynelis et al., 2010). As mentioned previously, several studies report that ethanol has no effect on residual neuronal NMDA currents measured in the presence of GluN2B selective antagonists. This has been taken as evidence that, in these preparations, non-GluN2B containing NMDA

receptors are not sensitive to ethanol inhibition. In the present study, both ethanol and ifenprodil inhibited wild-type GluN2B currents but this inhibition was not additive when these compounds were co-applied. Since no other GluN2 subunits are present in the transfected HEK cells, these data suggest that ethanol and ifenprodil either share a site or mechanism of action for inhibiting GluN2B receptors. It is unlikely that they share the same site since NTD deletion mutants that are ifenprodil insensitive still show normal ethanol inhibition. However, recent single channel studies show that ifenprodil decreases mean open time and open probability of GluN1/GluN2B receptors (Amico-Ruvio et al., 2012; Bhatt et al., 2013). These changes in single channel properties are similar to those reported for ethanol (Wright et al., 1996) suggesting that NTD-selective antagonists and ethanol share convergent mechanisms of NMDAR inhibition that are mediated through distinct and non-overlapping sites.

Overall, the results from this study show that the NTD of NMDA receptors has some ability to influence the extent of ethanol action on GluN2A and GluN2B receptors; albeit in opposite directions. Although NTD domains are not alternatively spliced and are thus always present in native receptors, modulating their activity via subunit selective compounds could represent a novel means of affecting the acute actions on ethanol on NMDA receptors.

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Figure 1.

Effect of GluN2 NTD deletion on ethanol sensitivity in GluN2A and GluN2B containing receptors. Trace currents from wild-type and mutant GluN1/GluN2A (A) and -GluN2B (B) NTD-deleted subunits expressed in HEK293 cells. Schematic beside traces shows domain organization of NMDA receptor GluN1 and GluN2 subunits and indicate which NTD is deleted from which subunit. NTD, N-terminal domain; LBD, ligand-binding domain; TMD, transmembrane domain; C-term, C-terminal domain. Currents were activated by co-application of agonists glutamate (10 μ M) and glycine (100 μ M) for 5 seconds. Ethanol inhibition was examined by co-application of 100 mM ethanol and agonists. The scale bars are 5 sec (horizontal) and 100 pA (vertical). (C) Comparison of ethanol inhibition in wild-type and GluN1/GluN2A^{NTD} receptors. Data represent the mean $\pm$ S.E.M. (N = 33 – 30 cells) of percent inhibition of steady-state current (I_{ss}). (D) Summary graph of ethanol inhibition in wild-type and GluN1/GluN2B^{NTD} receptors. Data represents the mean $\pm$ S.E.M. of the steady-state current from wild-type (N = 37 cells) and GluN1/GluN2B^{NTD} (N = 32 cells) containing receptors.

Figure 2.

Effect of GluN1 NTD deletion on ethanol sensitivity. (A) Representative currents from GluN1/GluN2A and mutant GluN1^{NTD} and GluN2A^{NTD} deleted receptors. Scale bars 2.5 s horizontal, vertical 200 pA (GluN1/GluN2A), 100 pA (GluN1^{NTD}/GluN2A^{NTD}). (B) Inhibition of GluN1/GluN2A and mutant GluN1^{NTD}/GluN2A^{NTD} receptors by 100 mM ethanol. Data represent the percent inhibition of steady-state current by ethanol and is expressed as the mean $\pm$ S.E.M. (N = 9 cells). (C) Examples of current traces from wild-type GluN1/GluN2B, and mutant GluN1^{NTD}/GluN2B, and GluN1^{NTD}/GluN2B^{NTD} receptors. Scale bars are 2.5 s horizontal, 50 pA (GluN1/GluN2B, GluN1^{NTD}/GluN2B) and 100 pA (GluN1^{NTD}/GluN2B^{NTD}). (D) Comparison of ethanol inhibition in GluN1/GluN2B, GluN1^{NTD}/GluN2B, and GluN1^{NTD}/GluN2B^{NTD} receptors. Data shows the percent inhibition expressed as mean $\pm$ S.E.M. of the steady-state current from the indicated subunit

combinations (GluN1/GluN2B N = 37, GluN1^{NTD}/GluN2B N = 6, and GluN1^{NTD}/GluN2B^{NTD} N = 8). Asterisks indicate significant difference between GluN1^{NTD}/GluN2B^{NTD} and GluN1/GluN2B (ANOVA, Dunnett's post hoc; $p < 0.01$).

Figure 3.

Effect of ifenprodil on NMDA receptors lacking the NTD. (A) Representative current trace from wild-type GluN1/GluN2B receptors in the presence and absence of 10 μ M ifenprodil, with the inhibitory trace overlaid onto the control current. Additional traces show ifenprodil inhibition of currents from GluN1/GluN2B Δ NTD, GluN1 Δ NTD/GluN2B, and GluN1 Δ NTD/GluN2B Δ NTD receptors. Scale bar 2.5 sec horizontal, 100 pA vertical, except for GluN1 Δ NTD/GluN2B (200 pA). (B) Ifenprodil inhibition of wild-type GluN1/GluN2B and mutant GluN1/GluN2B Δ NTD, GluN1 Δ NTD/GluN2B, and GluN1 Δ NTD/GluN2B Δ NTD receptors in the presence of ethanol. Current traces were activated by agonist in the absence (control trace) and presence of 100 mM ethanol (bar above trace) with 10 μ M ifenprodil co-applied as indicated by bar above traces. In wild-type GluN1/GluN2B receptors co-application of ifenprodil results in an inhibition of the current that remained when the solution was stepped back to agonist alone. Co-application of ifenprodil does not inhibit currents from GluN1/GluN2B Δ NTD, GluN1 Δ NTD/GluN2B, or GluN1 Δ NTD/GluN2B Δ NTD receptors. Scale bar 2.5 s horizontal and 200 pA vertical.

Figure 4.

Effect of co-application of ethanol and ifenprodil on NMDA receptors lacking the NTD. **(A)** Representative current traces and comparison of ethanol/ifenprodil inhibition in wild-type GluN1/GluN2B receptors. Current traces show control glutamate-activated current overlaid with that of 10 μ M glutamate + 100 mM ethanol (as indicated by arrow), glutamate + 10 μ M ifenprodil (Glu+IFN), and glutamate + ethanol + ifenprodil (Glu+Et+IFN). Scale bar 5 s horizontal and 100 pA vertical. Summary graph of ethanol/ifenprodil inhibition in wild-type GluN1/GluN2B receptors. Data represent the percent inhibition of steady-state current by ethanol, ifenprodil and co-application of ethanol and ifenprodil expressed as the mean $\pm$ S.E.M. (N = 7 cells). Asterisk indicates significant difference between EtOH and ifenprodil and EtOH+ifenprodil ($p < 0.001$; one-way ANOVA, Tukey's post-test). **(B)** Representative current traces and comparison of ethanol/ifenprodil inhibition in mutant GluN1/GluN2B^{NTD} receptors. Current traces show control glutamate-activated current overlaid with that of glutamate + ethanol (as indicated by arrow), glutamate + ifenprodil (Glu+IFN), and glutamate + ethanol + ifenprodil (Glu+Et+IFN). Scale bar 5 s horizontal and 100 pA vertical. Summary graph of ethanol/ifenprodil inhibition in mutant GluN1/GluN2B^{NTD} receptors. Data represent the percent inhibition of steady-state current by ifenprodil and co-application of ethanol and ifenprodil expressed as the mean $\pm$ S.E.M. (N = 3 cells). Asterisk

indicates significant difference between ifenprodil and EtOH and EtOH+ifenprodil ($p < 0.01$; one-way ANOVA; Tukey's post-test).

TABLE 1

Characteristics of NMDA receptors composed of wild-type and NTD-deleted subunits.

Subunit Combination	Mean SS Current (pA)	% Desensitization	N
GluN1/GluN2A	380.2 ± 47.2	33.0 ± 3.2	32
GluN1 ^{NTD} /GluN2A	N/A	N/A	N/A
GluN1/GluN2A ^{NTD}	241.0 ± 35.1*	40.4 ± 3.5	30
GluN1 ^{NTD} /GluN2A ^{NTD}	379.6 ± 85.6	40.6 ± 3.2	9
GluN1/GluN2B	230.4 ± 39.6	19.3 ± 3.0	37
GluN1 ^{NTD} /GluN2B	256.6 ± 174.9	25.4 ± 6.3	7
GluN1/GluN2B ^{NTD}	249.8 ± 31.0	19.7 ± 3.0	32
GluN1 ^{NTD} /GluN2B ^{NTD}	519.0 ± 97.3*	19.0 ± 3.2	6

N/A – nonfunctional receptors

Receptors were activated with 10 μ M glutamate + 100 μ M glycine. Mean steady-state (SS) current values were measured 4.5 seconds into agonist-induced current. Receptor desensitization was measured as the ratio of SS to peak agonist-induced responses. Data is the percentage of steady-state desensitization and values represent the mean $\pm$ S.E.M. from 6 to 37 cells per receptor subunit combination. No current was observed with the GluN1^{NTD}/GluN2A subunit combination. Asterisk indicate significance at $p < 0.05$ relative to wild-type control (ANOVA, Dunnett's post-hoc).