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Pharmacology of Triheteromeric N-Methyl-D-Aspartate Receptors

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Abstract

The N-Methyl-D-Aspartate Receptors (NMDARs) are heteromeric cation channels involved in learning, memory, and synaptic plasticity, and their dysregulation leads to various neurodegenerative disorders. Recent evidence has shown that apart from the GluN1/GluN2A and GluN1/GluN2B diheteromeric ion channels, the NMDAR also exists as a GluN1/GluN2A/GluN2B triheteromeric channel that occupies the majority of the synaptic space. These GluN1/GluN2A/GluN2B triheteromers exhibit pharmacological and electrophysiological properties that are distinct from the GluN1/GluN2A and GluN1/GluN2B diheteromeric subtypes. However, these receptors have not been characterized with regards to their inhibition by conantokins, as well as their allosteric modulation by polyamines and extracellular protons. Here, we show that the GluN1/GluN2A/GluN2B triheteromeric channels showed less sensitivity to GluN2B-specific conantokin (con)-G and con-R1B, and subunit non-specific con-T, compared to the GluN2A-specific inhibitor TCN-201. Also, spermine modulation of GluN1/GluN2A/GluN2B triheteromers switched its nature from potentiation to inhibition in a pH dependent manner, and was 2.5-fold slower compared to the GluN1/GluN2B diheteromeric channels. Unraveling the distinctive functional attributes of the GluN1/GluN2A/GluN2B triheteromers is physiologically relevant since they form an integral part of the synapse, which will aid in understanding spermine/pH-dependent potentiation of these receptors in pathological settings.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

JC performed the experiments and analyzed the data. JC, RDB and FJC conceived the experiments and wrote the manuscript. KBH supplied the plasmid constructs. All authors reviewed the manuscript.

Keywords

NMDAR; Contanokins; Triheteromers; Diheteromers; Pharmacology

1. Introduction

The N-Methyl-D-Aspartate Receptors (NMDAR) are ligand-gated ionotropic glutamate receptors present throughout the central nervous system, and are activated by the co-agonists glutamate (Glu) and glycine (Gly). The NMDARs are ligand- and voltage-gated ion channels, that contains a Mg^{2+} block which is released when neurons are depolarized [1]. Functionally, the NMDAR is involved in synaptic plasticity, learning and memory, and their expression is developmentally regulated [1]. However, these receptors also play a central role in several neurodegenerative disorders, *e.g.*, stroke, Alzheimer's Disease, Parkinson's Disease, and traumatic brain injury, as well as, depression that is a psychiatric disorder [2,3]. The majority of NMDARs in the central nervous system are heterotetramers consisting of two mandatory GluN1 subunits that bind to Gly and two GluN2 subunits that bind to Glu. GluN3 subunits can also assemble with GluN1 and GluN2 subunits, but the structure and function of these receptors are unresolved [4,5]. The mandatory GluN1 subunit can consist of any of the eight splice variants, denoted as GluN1(a–h), and the GluN2 subunits can be composed of four independent gene products, GluN2(A–D). Thus, assembly of different combinations of the GluN1 splice variants and GluN2 subunits lend considerable diversity to NMDARs, resulting in many distinct receptor subtypes. Specifically, the different GluN2 subunits impart distinct electrical, biochemical, and pharmacological properties to the ion channel [6], including channel deactivation kinetics, strength of Mg^{2+} block, and sensitivity to antagonists and modulators [7,8]. Furthermore, in addition to the heterogeneity of the GluN1/GluN2A or GluN1/GluN2B diheteromers, NMDARs can also exist as GluN1/GluN2A/GluN2B triheteromers in neurons [9, 10, 11, 12, 13]. These triheteromeric receptors are composed of two GluN1, one GluN2A, and one GluN2B subunit. Currently, there are no specific reagents available to pharmacologically distinguish the diheteromers from the triheteromers within neuronal cells. However, the GluN1/GluN2A/GluN2B triheteromers have been characterized as having distinct kinetic and pharmacological properties from either GluN1/GluN2A or GluN1/GluN2B diheteromers [12, 14]. Utilizing acute brain sections from wild-type (WT) mice, as well as GluN2A- and GluN2B-gene inactivated mice (GluN2A^{-/-} and GluN2B^{-/-}, respectively), it was demonstrated that the NMDA-evoked excitatory postsynaptic current (EPSC) decay time in WT neurons was intermediate to that of neurons derived from GluN2A^{-/-} and GluN2B^{-/-} cells [11,13]. Also, WT cells had significantly decreased sensitivity toward a GluN2B-specific pharmacological antagonist, CP-101606, in the presence of Mg^{2+} compared to GluN2A^{-/-}-derived cells [11]. Similarly, utilizing kinetic, genetic, and pharmacological approaches, it was observed that EPSC deactivation kinetics of WT mouse neurons was not the simple consequence of only GluN1/GluN2A and GluN1/GluN2B diheteromers present in the synapses. By calculating the probability of channel opening at peak EPSC (P_O^*) it was estimated that triheteromers were 5.8× and 3.2× more abundant at the synapses compared to the GluN1/GluN2A and GluN1/GluN2B receptors, respectively [12]. Additionally, although Zn^{2+} , GluN2B-specific ifenprodil, or the competitive GluN2A antagonist, NVP-AAM007, deactivated GluN1/

GluN2A/GluN2B triheteromer-directed EPSCs, these agents displayed a lower potency than the GluN1/GluN2A and GluN1/GluN2B containing diheteromers. It has been suggested that the distinct kinetics of the GluN2A/GluN2B-containing triheteromers was due to the molecular differences of fast Glu deactivation kinetics arising from GluN2A, with GluN2B being the rate limiting factor for channel opening [12].

Employing molecular and pharmacological approaches, the triheteromeric NMDARs were further characterized by isolated heterologous expression of the GluN1/GluN2A/GluN2B subunits to be able cleaner interpretation of the results. Similar to the observations in hippocampal neurons, it was observed that the triheteromers displayed tonic sensitivity to Zn^{2+} that was comparable to GluN1/GluN2A-containing channels, as well as, phasic sensitivity to Zn^{2+} similar to the GluN1/GluN2B diheteromers. Additionally, the GluN1/GluN2A/GluN2B ion channel exhibited reduced sensitivity to ifenprodil with slower ($\sim 2\times$) *on*-rates (τ_{on}) and twice as fast *off*-rate (τ_{off}) compared to GluN1/GluN2B diheteromers [15]. However, the challenge still remained to authentically selectively express the triheteromeric channels in a heterologous system with minimal expression of the GluN1/GluN2A or GluN1/GluN2B diheteromers. This issue was elegantly solved by molecularly engineering an expression system to allow robust triheteromeric surface expression of GluN1/GluN2A/GluN2B [14,16]. Both of these independent studies reported that triheteromeric receptors showed functional and pharmacological properties that were intermediate to diheteromeric channels with either GluN2A or GluN2B.

While these, and other, studies reported outcomes using GluN2A- and GluN2B-specific small molecule inhibitors on the triheteromers [12,14,15], we investigated the effects of peptide antagonists, namely the conantokins, and potentiation by spermine, on the GluN1/GluN2A/GluN2B channels. Conantokins are small peptides that contain the unusual amino acid residue, γ -carboxyglutamic acid (Gla), and are naturally found in the venom of marine snails of the genus *Conus* [17]. The conantokins are NMDAR antagonists, and the Gla moieties confer structural and functional integrity to these peptides. As part of the marine snail venom, conantokins are used as defense/predatory agents. However, since some of these toxins, *e.g.*, conantokin-G (con-G), con-RIB, and con-Br display inherent GluN2 subunit specificity, they have been exploited to study molecular mechanisms of NMDAR-directed function [18,19]. We utilized the triheteromeric NMDAR expression system as described to study the effect of conantokins, con-RIB, con-G and con-T, on these receptors. We report herein that the pharmacology of the GluN1/GluN2A/GluN2B channel is closer to that of GluN2A containing diheteromeric channels with respect to antagonism induced by conantokins, and that spermine potentiation time constants of NMDAR triheteromeric channels are slower compared to diheteromeric channels, and could potentially be modulated by pH changes in triheteromeric channels.

2. Methods

2.1. Cell line and plasmid constructs

The genetically engineered constructs encoding GluN1, GluN2A, and GluN2B, fused with C1 or C2 tags to the intracellular C-termini of GluN2, were transfected in HEK Tet-On Advanced cells as described [14]. EGFP was expressed from the same construct as GluN1 as

previously described [14]. At a time 48 hr prior to transfections, the cells were plated on poly-D-lysine-coated 35 mm tissue coated dishes and grown in DMEM/GlutaMax-I (Invitrogen, Carlsbad, CA), containing 10% fetal bovine serum, 10 U/ml penicillin, and 10 µg/ml streptomycin. The calcium phosphate method was employed to transfect cells with plasmid cDNAs encoding GluN1 and the GluN2 subunit at a ratio of 1:2. At 4 hr post-transfection, NMDAR antagonists, 200 µM D,L-2-amino-5-phosphonovalerate and 200 µM 7-chlorokynurenic acid were added to the medium to prevent NMDAR expression cytotoxicity. Simultaneously, 5 ng/ml doxycycline was also added to the medium to induce low levels of GluN1 expression.

2.2. Whole-cell patch-clamp recordings

Whole-cell voltage clamp recordings were performed 24 hr post-transfection using an Axopatch 200B amplifier at a holding potential of -70 mV. The intracellular solution was composed of: 140 mM CsF, 1 mM CaCl_2 , 2 mM MgCl_2 , 10 mM EGTA, 10 mM Cs-Hepes, 2 mM tetraethylammonium chloride, and 4 mM Na_2ATP (pH 7.35). The extracellular solution contained: 140 mM NaCl, 10 mM HEPES, 3 mM KCl, 2 mM CaCl_2 and 20 mM dextrose (pH 7.35). Data were analyzed using ClampFit (Molecular Devices, Union City, CA). NMDA (100 µM) and glycine (10 µM) were perfused over the cells after patching to induce NMDAR currents. Solutions were applied using a rapid changer equipped with a 9-barrel straight-head (RSC-200, BioLogic Science Instruments, Knoxville, TN) positioned ~ 200 µm from the cell that is clamped. The concentrations of various pharmacological agents were as follows: conantokins 10 µM, NVP 60 nM, ifenprodil 10 µM, TCN-201 10 µM, spermine 100 µM. Recordings were made before and after 1 min perfusion of conantokins and the remaining compounds were co-applied along with Glu/Gly. 100 µM Glu/10 µM Gly was used to activate NMDAR currents for experiments using spermine. For spermine potentiation, one set of experiments were performed continuously at pH 7.3. Another set of experiments were performed, where the first part of the traces shows current at pH 7.3 and then spermine potentiation was performed at pH 6.4 (middle part of the trace), and the latter part of the trace was current recorded after spermine was removed at pH 7.3.

2.3. Conantokin synthesis

The conantokins were chemically synthesized and purified on a solid phase peptide synthesizer (Applied Biosystems, Model 433A, Foster City, CA) [20]. The amino acid sequences of the conantokins utilized in this study were ($\gamma = \gamma$ -carboxyglutamate):

con-G: $\text{GE}\gamma\gamma\text{L}^5\text{Q}\gamma\text{NQ}\gamma^{10}\text{LIR}\gamma\text{K}^{15}\text{SN}(\text{NH}_2)$

con-T: $\text{GE}\gamma\gamma\text{Y}^5\text{QKML}\gamma^{10}\text{NLR}\gamma\text{A}^{15}\text{EVKK}^{20}\text{A}(\text{NH}_2)$

con-RIB: $\text{GE}\gamma\gamma\text{L}^5\text{A}\gamma\text{KAO}^{10}\gamma\text{FAR}\gamma^{15}\text{LAN}(\text{NH}_2)$

2.4. Ligands and reagents

Glutamate, glycine, spermine, and ifenprodil were purchased from Sigma (St. Louis, MO) and TCN-201 was purchased from Tocris/R & D Systems (Minneapolis, MN). NVP-AAM007 (NVP) was a gift from Y.P. Auberson (Novartis; Basel, Switzerland).

2.5. Data analyses

Changes in the steady state current amplitudes, before and after treatment with the peptides and drugs, were used to calculate the percentage of NMDAR inhibition as described previously [21]. Mean $\pm$ SEM of the calculated percentages are presented as bar diagrams. The statistical significance between groups was calculated by unpaired t test. N values of each of the groups are indicated along with the data. Analyses and data plotting were performed using GraphPad Prism software. Time constants for spermine-mediated potentiation were determined by fitting the region on the trace with a standard single exponential equation using Clampfit software.

3. Results

Effect of conantokins on triheteromeric NMDARs

Whole-cell patch clamp recordings of NMDAR-evoked currents in HEK cells expressing GluN1/GluN2A/GluN2B receptors were compared to cells expressing either GluN1/GluN2A or GluN1/GluN2B diheteromers. It was observed that both peak and steady state NMDAR currents were inhibited by GluN2B-selective con-G (5.5%) or con-RIB (14.3%) in the GluN1/GluN2A/GluN2B channels to a lesser extent compared to the GluN1/GluN2B diheteromers [22,23,24] (Fig. 1 A,B). Similar data were obtained with the small molecule GluN2B-specific inhibitor, ifenprodil, which is in agreement with previously published data [14] (Fig. 1 A,B). The GluN2A-specific agents, TCN-201 and NVP, which at low concentrations (<100 nM) retain GluN2A-selective inhibition robustly inhibited current (~80%) of the triheteromeric channels that were similar to GluN1/GluN2A-containing channels (Fig. 1 C,D). Con-T, which is a subunit non-selective conantokin did not inhibit the GluN1/GluN2A/GluN2B channels as robustly as TCN-201 or NVP. This indicated that the inhibitory activity of con-T is more effective when either GluN2A or GluN2B subunits are present as diheteromeric channels compared to when both, the GluN2A and GluN2B subunits coexist in the same channels (Table 1). Thus, as far as inhibition by GluN2B-specific small peptides is concerned, the triheteromeric channels exhibited characteristics similar to GluN2A-containing diheteromers, with GluN2A component being the dominant subunit.

Effect of spermine on triheteromeric NMDARs

To test the effect of pH on spermine potentiation, we devised an assay, whereby NMDAR expressing cells were first continuously exposed to Glu/Gly or Glu/Gly/spermine at pH 7.3. In another set of experiments the cells were exposed to pH 7.3 followed by application of Glu/Gly/spermine at pH 6.4, before switching back again to Glu/Gly at pH 7.3. In HEK cells expressing GluN1/GluN2A diheteromers, presence of spermine inhibited NMDA/Gly-evoked current at pH 7.3 (Fig. 2A) and pH 6.4 (Fig. 2B). However, in the presence of Glu/Gly, spermine potentiation (100 μ M) was observed in GluN2B-containing diheteromers at pH 7.3 (Fig. 2C) and when the pH was changed from 7.3 to 6.4 (Fig. 2D). Our data is in consonance with previously reported studies demonstrating that maximum spermine potentiation was observed for GluN1/GluN2B ion channels [25]. The GluN1/GluN2A/GluN2B triheteromeric channels were potentiated by spermine in the continuous presence of Glu/Gly at pH 7.3 (Fig. 2E). On the other hand, under slightly acidic conditions spermine

inhibited Glu/Gly-mediated current at pH 6.4 (Fig. 2F) in cells expressing the GluN1/GluN2A/GluN2B channels. Thus, in case of spermine potentiation, the GluN2B subunit functionally dominates over the GluN2A subunit in a triheteromer at pH 7.3, but the GluN2A subunit characteristic predominates at pH 6.4. This distinctive property of triheteromers flipping their response towards spermine potentiation under different pH conditions could be exploited to identify this triheteromeric subunit combination. We also analyzed the potentiation trace for the channels, and found that the potentiation time constants for triheteromeric channels were found to be slower compared to GluN1/GluN2B channels at pH 7.3 (Fig. 3A–C). Quantification of potentiation or inhibition by spermine is shown in figure 2G.

4. Discussion

The existence of GluN1/GluN2A/GluN2B triheteromeric channels in the adult mice hippocampus and cortex is now well established. However, their biochemical and pharmacological characterization is not well defined, as the presence of two GluN2 subunits confers to the triheteromeric channels pharmacological properties attributed to both, GluN2A and GluN2B. The triheteromeric NMDARs have been studied in isolation by heterologous expression in oocytes or HEK cells [14,15], and additionally they have been functionally isolated in hippocampal slices and cultured neurons [11,12]. Our data in this study also indicate that the triheteromers exhibited pharmacological properties that are reminiscent of both the GluN2 subunits. The GluN1/GluN2A/GluN2B ion channels were more pronouncedly inhibited by GluN2A-specific inhibitors (~80% by TCN-210 and ~60% by NVP) compared to the GluN2B-specific con-G or con-R1B (5.5% and 14.3% respectively). Similar to previous reports, we also observed that the GluN2B-specific pharmacological agent, ifenprodil, only weakly inhibits triheteromeric receptors, while GluN2A specific TCN-201-mediated inhibition is significantly higher [14,15]. Furthermore, we also show that the presence of GluN2A/GluN2B in the triheteromer decreased the sensitivity towards subunit non-selective con-T (23% inhibition), which otherwise exerts strong inhibition in both GluN1/GluN2A and GluN1/GluN2B ion channels in hippocampal and neuronal cells in culture [17,28] and in transfected HEK cells [29,30]. This reduced sensitivity to GluN2B-directed antagonists cannot be currently explained, but it is speculated that the binding affinity of these drugs is decreased in the presence of GluN2A. The fact that all GluN2B-selective inhibitors (i.e. ifenprodil and con-G/con-R1B) displayed a similar marked reduction in inhibition of triheteromers compared to GluN1/GluN2B diheteromers indicated that the sensitivity of these antagonists is independent of the mechanism of binding to GluN2B. Crystallographic data demonstrate that ifenprodil inhibition is mediated by binding deep within a hydrophobic region of the amino terminal domain, which increases the dwell time of the receptor in the closed conformation and restricting its transition to the open state [31,32]. Co-crystallization of NMDAR with conantokins has been unsuccessful, but based on mutational studies, it can be hypothesized that the conantokins bind to several residues of the ligand binding domain at the interface of the quaternary GluN1/GluN2B channel. Specifically, the S2 region of GluN2B spanning residues from E658 to I815 is sensitive to con-G inhibition [33]. Furthermore, preparing con-G variants it was molecularly

determined that GluN2B subunit specificity dwelled in the N-terminal region of the peptide. Glutamine at position 6 was found to be a key residue governing GluN2B specificity [34].

The ineffectiveness of GluN2B antagonists in the GluN1/GluN2A/GluN2B triheteromeric NMDARs appears to be a characteristic property of triheteromeric receptors, since one of the subunits, GluN2A in this case, plays a dominant role in controlling antagonist sensitivity. The orientation of subunits in a triheteromeric receptor may be different from that in a diheteromeric receptor, resulting in one type of subunit becoming more or less accessible to modulators. This view is supported by previous work showing a markedly slower rate of inhibition by ifenprodil as well as reduced binding affinity and efficacy at GluN1/GluN2A/GluN2B, suggesting structural variation between the ifenprodil binding site in triheteromers compared to GluN1/GluN2B diheteromers [14]. Here, we show markedly reduced inhibition by the non-selective Con-T at GluN1/GluN2A/GluN2B compared to both GluN1/GluN2A and GluN1/GluN2B diheteromers (Table 1). We speculate that Con-T binds a modulatory site, which is largely conserved in GluN1/GluN2A and GluN1/GluN2B diheteromers, but altered in GluN1/GluN2A/GluN2B triheteromers. Similar to the ifenprodil site, the binding site for Con-T can be located in an intra- or inter-subunit interface, which is modified in GluN1/GluN2A/GluN2B triheteromers compared to diheteromers.

In the brain, polyamines, such as spermine, are released in high concentrations from presynaptic neurons in the synaptic cleft where the synaptic NMDARs are localized. Spermine is known to potentiate the excitability of the NMDAR *via* GluN2B subunits in an extracellular pH-dependent manner. It was previously reported that at physiological pH, spermine preferentially potentiates NMDARs that contain the GluN2B subunit [35,36]. We performed experiments to investigate the effect of spermine on triheteromeric channels. Our data showed that spermine potentiates triheteromeric NMDARs. In this respect, the GluN1/GluN2A/GluN2B channels function like GluN2B-containing receptors, but with slower potentiation time constants. The sensitivity of NMDAR to protons is important, as they are known to be inhibited at lower pH [37]. A recent study utilizing a similar system to study triheteromeric receptors have shown that spermine potentiates to a lesser extent on the triheteromeric channels [16]. We also obtained a similar result with regard to the potentiation induced by spermine on triheteromeric channels, with significantly slower time constants. Spermine potentiation is ~2.5 fold slower in triheteromeric channels compared to that in GluN2B diheteromeric channels. In addition, we also studied how a pH change can exert an effect on the potentiating ability of spermine on triheteromeric channels. In an experiment where the pH of the spermine solution was switched between two pH states, we observed a robust change in conductance in the triheteromeric channel, as opposed to no significant change of conductance in cells containing either GluN1/GluN2B or GluN1/GluN2A channels. Although this change of state induced by a change in pH is an inherent property of the channel, and not of spermine *per se*, the extent of change is more pronounced in triheteromeric channels as indicated in our data. The difference in spermine potentiation at two different pH values suggests that the pH sensitivity of triheteromeric NMDARs is significantly different from diheteromeric receptors. Hence, if there is a physiological drop in pH, the GluN2A/GluN2B-containing triheteromeric channels, that occupy >50% of the synapses [38], will not be potentiated by polyamines, and in such a scenario of increase in extracellular protonation, the triheteromers responds similar to GluN1/GluN2A

diheteromers. This feature can have physiological implications in the synaptic events that are likely to be accompanied by pH changes, and adds a new level of regulatory capability to these receptors.

Mixed electrophysiological traits for triheteromers were also observed wherein voltage dependency of EPSC decay is reflective of the GluN2A subunit, while, the slow deactivation kinetics of EPSC is comparable to a GluN2B-containing diheteromer [11]. Studying the functional peculiarities of the triheteromers is important, not only for their role in synaptic plasticity, but also to understand the predominant involvement of GluN2A and GluN2B subunits in neuronal hyperexcitability and signaling during various neuropathologies to aid in rational drug design.

Conclusion

Employing GluN2 subunit-specific conantokins and reagents we conclusively demonstrated that the GluN1/GluN2A/GluN2B triheteromeric channels are less sensitive to pharmacological inhibition by GluN2B-specific reagents, behaving more like GluN1/GluN2A diheteromers in that regard. However, the triheteromeric channel could be potentiated by spermine, which is otherwise not observed in the GluN1/GluN2A diheteromeric ion channels. Rapid acidification from pH 7.3 to pH 6.4 inhibited spermine-induced potentiation of the triheteromeric channels. Exploiting the distinctive properties of GluN1/GluN2A/GluN2B triheteromeric channels will aid in understanding physiological synaptic events, as well as, changes in synaptic activity during neuropathologies.

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Abbreviations

NMDAR	N-methyl-D-aspartate receptor
Glu	Glutamate
Gly	glycine
Con	Conantokin
EPSC	excitatory postsynaptic current
Gla	gamma (γ)-carboxyglutamic acid
NVP-AAM007	NVP

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Highlights

- Conantokins were employed to assess pharmacological properties of triheteromeric NMDAR ion channels containing GluN1/GluN2A/GluN2B.
- GluN2A pharmacology was dominant over GluN2B in triheteromeric NMDARs with respect to subunit specific antagonists.
- Spermine potentiation is slower in triheteromers compared to diheteromers and is dependent on pH.

Fig. 1.

Pharmacological inhibition of GluN1/GluN2A/GluN2B triheteromers is similar to GluN1/GluN2A ion channels. (A) NMDA/Gly induced current traces from HEK Tet-On cells expressing triheteromeric NMDARs before and after 1 min conantokin incubation, and traces for TCN-201 co-application (10 μ M) with 100 μ M NMDA/10 μ M Gly. Conantokin were present at 10 μ M. (B) Percentage inhibition of NMDA induced currents from GluN1/GluN2A/GluN2B triheteromers in the presence of various inhibitors presented as bar graphs representing mean $\pm$ SEM. **p < 0.005, conantokin and ifenprodil inhibition values obtained are significantly lower than TCN-201 (10 μ M) or 60 nM NVP (con-RIB, N=4; con-G, N=5; con-T, N=1; ifenprodil, N=5; NVP, N=3; TCN, N=3). (C & D) Comparison

of ifenprodil and TCN inhibition in GluN1/GluN2A/GluN2B triheteromers (C) and GluN1/GluN2A diheteromers (D), *** $p < 0.0005$, $N = 3$.

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Fig. 2.

Altered sensitivity to pH in triheteromeric receptors leads to differential modulation by spermine. Potentiation by spermine was recorded in the continuous presence of either, pH 7.3 or 6.4. (A) Lack of spermine potentiation in GluN1/GluN2A diheteromeric channels at (A) pH 7.3 or (B) pH 6.4 after the pH was switched to pH 6.4 (N = 2). (C) Spermine potentiation in GluN1/GluN2B diheteromeric channels at (C) pH 7.3 or (D) where spermine potentiation was observed even after the pH was changed from 7.3 to 6.4 (N = 3). Spermine potentiation observed in triheteromeric channels at (E) pH 7.3 is abolished after (F) the pH

was changed to 6.4 ($N = 3$). Scale bar in each representative trace denotes current unit of 200 pA on y-axes and a time unit of 1 s on x-axes. (G) Bar graph shows negative potentiation of Glu/Gly-evoked current by spermine observed at pH 7.35 and at pH 6.4 in cells expressing GluN1/GluN2A diheteromers (dark grey bars). Positive spermine potentiation observed at the two different pH conditions in cells expressing GluN1/GluN2B channels (black bars). The triheteromeric channel expressing cells showed positive spermine potentiation at pH 7.35, but negative spermine potentiation at pH 6.4 (light grey bars). Unpaired t test shows $p < 0.0049$.

Fig. 3. Spermine potentiation is slower in triheteromeric NMDAR channels. (A) Representative trace showing a single exponential demonstrating spermine potentiation in GluN1/GluN2B diheteromeric channels. (B) Representative trace showing a similar single exponential curve fitting for spermine potentiation in a triheteromeric current trace. (C) Bar graph showing mean $\pm$ SEM values of the spermine potentiation time constants (τ_{on}) of triheteromeric

versus diheteromeric GluN1/GluN2B receptors. Triheteromeric mean = 139.3 ± 16.4 msec, N = 9; diheteromeric mean = 55 ± 6 msec, N = 9. Unpaired t test shows $p < 0.0001$.

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Table 1

Percentage inhibition values of NMDAR antagonists studied

	Con G	Con RIB	Con T	Ifen	TCN	NVP
2A/2A	None ²²	>40 ²⁴	92.9 ± 4.5 ²²	7.5 ± 3.8	79.6 ± 9.7	nd
2B/2B	91.3 ± 5.5 ²²	>95 ²⁴	94.9 ± 4.5 ²²	88 ± 2 ¹⁴	nd	nd
2A/2B	5.5 ± 3	14.3 ± 2	23 ± 5.7	19.14 ± 5.7	77.85 ± 2	59 ± 9.6

Data are calculated as mean ± SEM. None: no inhibition observed; nd: not determined