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Residues in the GluN1 C-terminal Domain Control Kinetics and Pharmacology of GluN1/GluN3A N-methyl-D-aspartate Receptors

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Abstract

N-methyl-D-aspartate (NMDA) receptors assembled from GluN1 and GluN3 subunits are unique in that they form glycine-gated excitatory channels that are insensitive to glutamate and NMDA. Alternative splicing of the GluN1 subunit mRNA results in eight variants with regulated expression patterns and post translational modifications. Here we investigate the role of residues in the GluN1 C-terminal splicing in receptor gating and modulation. We measured whole-cell currents from recombinant GluN1/GluN3A receptors expressed in HEK293 cells that differed in the sequence of their GluN1 C-terminal tail. We found that these residues controlled the level of steady-state activity and the degree to which activity was facilitated by zinc and protons. Further, we found that the phosphorylation status of sites specific to certain variants can also modulate channel activity. Based on these results we suggest that GluN1 C-terminal domain splicing may confer cell-specific and activity-dependent regulation onto the level and pharmacologic sensitivity of GluN1/GluN3A currents.

Keywords

N-methyl-D-aspartate receptors; splice variants; glycine; zinc; pH; whole-cell electrophysiology; kinetics

1. INTRODUCTION

GluN1 and GluN3 are homologous subunits in the N-methyl-D-aspartate (NMDA) receptor family of tetrameric ionotropic glutamate receptors (iGluRs). In contrast to all other iGluR subunits, they are insensitive to glutamate and instead bind glycine with high affinity. They can combine to form excitatory glycine-gated channels that are insensitive to the neurotransmitter glutamate, and are insensitive to the eponymous agonist NMDA [1]. The GluN3 subunit participates in fundamental physiological and pathological processes [2–15]. However, the literature on the biophysical and pharmacologic properties of GluN3-containing NMDA receptors remains sparse [1, 16–23].

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

Functional NMDA receptors are obligate heterotetramers composed of two GluN1 and two GluN2(A-D) or/and GluN3(A, B) subunits. The GluN1 subunit is expressed ubiquitously in the central nervous system (CNS) whereas the GluN2/3 subunits have cell-type and development- specific expression patterns [24]. Recombinant receptors expressed in heterologous cells showed that molecularly defined NMDA receptor isoforms have distinctive functional properties [5, 25]; therefore, regulated subunit expression represents a major mechanism for adjusting the amplitude and time course of NMDA receptor responses during development, following activity- dependent synaptic plasticity, and in certain acute and chronic disorders [7, 9, 10, 13, 26–28].

The molecular composition of native NMDA receptors is still unclear, and ascertaining the complement of NMDA receptor isoforms in a native preparation remains challenging for two main reasons. First, several subunits have substantial temporal and regional expression overlap [26, 27, 29, 30], such that although identifying the protein subunits expressed limits the possible receptor combinations substantially, this approach by itself does not determine definitively which combinations generate the observed electrical signal. Second, the activation mechanisms and thus functional signatures of NMDA receptors have been delineated in sufficient detail for only a handful of molecularly defined receptors, with the best studied being the diheteromeric glutamatergic GluN1-1a/GluN2A and GluN1-1a/GluN2B receptors [31–37]. Here we describe functional properties for four diheteromeric glycinergic GluN1/GluN3A receptors.

GluN1 is ubiquitous across brain regions, is essential for the assembly of functional NMDA receptor channels, and controls their correct cellular localization [29, 30, 38, 39]. It occurs naturally as eight variants produced by alternative splicing of the GluN1 RNA [40, 41]. Two types, denoted a and b, differ in their extracellular N-terminal sequences; and for each, four variants, denoted 1 – 4, differ in their intracellular C-terminal domain (CTD). GluN1 splicing changes developmentally and regionally; following neuronal activity; and in several neurological disorders [30, 42–47]. GluN3A expression surges visibly shortly after birth [27], when GluN1-1 is prominent in striatum, hippocampus, cortex, olfactory bulb, and dorsal septum; GluN1-2 is prominent throughout the forebrain; GluN1-3 is restricted to cortex, thalamus, and hippocampus; and GluN1-4 is observed in thalamus, colliculi, ventral septum, brainstem, and the CA3 region of the hippocampus [30]. Despite this clear differential expression, the functional relevance of GluN1 splicing remains unclear. Here we delineate functional properties for four naturally occurring GluN1-a/GluN3A receptors.

2. MATERIALS AND METHODS

2.1 Molecular Biology

We used plasmids encoding rat GluN1-1a (GenBank: U08261) (R. Wenthold, National Institutes of Health, Bethesda, MD), GluN1-2a (Genbank: U08262) and GluN1-3a (GenBank: U08265) (J. Woodward, Medical University of South Carolina), GluN1-4a (GenBank: U08267) (R.S. Zukin, Albert Einstein College of Medicine, Yeshiva University) and GluN3A (GenBank: NM_138546) (S. Lipton, University of California, San Diego). We sub-cloned, amplified, and sequenced the cDNA inserts as detailed elsewhere [48]. We introduced residue-specific substitutions using the QuikChange site-directed mutagenesis kit

(Stratagene, Amsterdam, Netherlands) and verified these by full-insert sequencing. GluN1, GluN3A, and GFP plasmids, in a 1:2:1 ratio, were transiently transfected into human embryonic kidney (HEK) 293 cells (ATCC CRL-1573; passages 25–35) using the calcium phosphate precipitation method [49]. We incubated cells for 3 hours with the transfection mixture, washed, and incubated for 12–24 hours in DMEM supplemented with 2 mM MgCl₂ and 100 μM D-serine before using them for experiments.

2.2 Western Blotting

Cell lysates were prepared from HEK293 cells transfected with the indicated plasmids. We used a plasma membrane protein extraction kit (Abcam) to isolate proteins expressed at the cell surface; we quantified extracted proteins with the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology), and resolved mixtures on a 10% reducing sodium dodecylsulfate polyacrylamide gel, for ~3 hours at 100 V before transferring to a polyvinylidene difluoride (PVDF) membrane. We blocked membranes with 5% BSA (w/v) in TBS/Tween-20 buffer and probed them with mouse anti-calnexin (1:5000) at room temperature for 1.5 hours or rabbit anti-GFP (1:1000) and mouse anti-pan-GluN1 (1:1000) (Millipore) primary antibody in TBS/Tween-20 at 4°C overnight. We revealed specific bands by incubating with appropriate secondary antibody (goat anti-mouse or goat anti-rabbit) conjugated to horseradish peroxidase 1 hour at room temperature, followed by ECL reagent (Pierce Biotechnology) to activate horseradish peroxidase before being exposed for 5–30 seconds to X-ray film. Following X-ray exposure, we stained the PVDF membrane with amido black to verify equal protein loading across lanes [50], and performed densitometry analysis in ImageJ (National Institutes of Health).

2.3 Immunocytochemistry

Cells transfected with indicated subunits were plated on coverslips; grown for 1 day, and live- stained with wheat germ agglutinin conjugated to Alexafluor 647 (1:350) for 10 mins at 4°C. Stained cells were fixed with 4% paraformaldehyde at 25°C for 15 mins; permeabilized with 0.1% Tween in phosphate-buffered saline (PBS) for 10 mins at 25°C; and treated with blocking buffer (2% BSA, 20% FBS, 0.01% sodium azide in PBS) for 1 hr at 25°C. We probed blots with mouse anti-pan-GluN1 (1:1000) at 4°C overnight in a humid chamber, washed with PBS, and incubated with secondary antibody (donkey anti-mouse conjugated to Alexafluor 488; 1:500) at room temperature for 1 hour. Following incubation with secondary antibody, cells were washed with PBS, incubated with DAPI for 5 minutes at room temperature, washed, and mounted on slides with Vectashied (Vector Labs, Burlingame, CA). Controls in which the primary antibody was omitted were stained with secondary antibodies in parallel with experimental samples. All antibodies were from Millipore (Darmstadt, Germany). We used an inverted Zeiss Axio Observer fluorescence microscope for imaging of coverslips and ImageJ for analyses (National Institutes of Health).

2.4 Whole-cell currents

Fire-polished electrodes (3–10 MΩ) were filled with intracellular solution containing (in mM): 135 CsCl, 22 CsOH, 2 MgCl₂, 1 CaCl₂, 11 EGTA, and 10 HEPES adjusted to pH 7.4. Cells were clamped at –70 mV and perfused with extracellular solutions containing (in mM): 150 NaCl, 2.5 KCl, 0.5 CaCl₂, and 10 PIPES adjusted to pH 6.8 without (wash) or

with 0.5 mM glycine. For calcium block experiments, 1.8 mM free Ca^{2+} was achieved by buffering with 1.5 mM EGTA (MaxChelator, Stanford University). Solution applications were controlled through a lightly pressurized pinch valve system (BPS-8, ALA Scientific Instruments Inc., Westbury, NY). For each cell, 3–10 traces were averaged and analyzed in Clampfit. Currents were low-pass filtered at 2 kHz and digitally sampled at 5 kHz (Digidata 1440A, Molecular Devices, Sunnyvale, CA) using Clampex. The extent of desensitization was evaluated by calculating the I_{ss}/I_{pk} ratio. The time constant of current desensitization (τ_D) was calculated by fitting the relaxation phase of the current, between peak (I_{pk}) and steady-state (I_{ss}) levels with a bi-exponential decay function.

2.5 Statistical analyses

We report results as rounded means $\pm$ standard error. We compared these values with one-way ANOVA and Bonferroni post-hoc test in Graphpad Prism software and considered statistical differences significant for $P < 0.05$. Notations in figures indicate: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

3. RESULTS

3.1 Expression of GluN1/GluN3A receptors in HEK293 cells

We co-expressed GluN3A with each of the four naturally occurring GluN1-a variants (1 through 4) in HEK293 cells (Figure 1A). We recorded maximal whole-cell excitatory glycinergic currents using 0.5 mM glycine, in external solutions containing 150 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl_2 , and 10 mM PIPES, adjusted at pH 6.8 [16]. To compare functional expression across cells, we normalized the observed peak current amplitudes (I_{pk}) to the measured whole-cell capacitance, and calculated the current density in each condition. We found that relative to cells transfected with GluN1-1 or GluN1-2 variants, those transfected with GluN1-3 and GluN1-4 had current densities that were up to ~4-fold larger; densities were lowest with GluN1-1/GluN3A (14 ± 4 pA/pF, $n = 5$) and highest for GluN1-4/GluN3A (57 ± 12 pA/pF, $n = 8$) ($F_{3,19} = 3.32$, $P = 0.04$) (Figure 1 B). These results are in agreement with the range of values reported previously [23].

Next, we examined whether this pattern of observed current correlated with the levels of GluN1 protein present in the whole-cell lysate and at the plasma membrane. We measured GluN1 protein levels in total cell lysates and in isolated plasma membrane proteins by performing densitometry on Western blots probed with a pan anti-GluN1 antibody. We found that, in whole-cell lysates, GluN1-1, -2, and -3 occurred at similar levels, whereas GluN1-4 was substantially more prominent ($F_{3,8} = 10.62$, $P = 0.004$) (Figure 1C), consistent with the higher level of current measured in cells co-transfected with GluN1-4 and GluN3A. Similarly, when probing plasma membrane proteins, we found lowest levels of GluN1-1 (2.1 ± 0.6 , $n = 3$), followed by GluN1-2 and GluN1-3 (4.0 ± 0.9 , $n = 3$; and 4.6 ± 0.9 , $n = 3$); and highest levels for GluN1-4 (13.2 ± 1.6 , $n = 3$) ($F_{3,8} = 21.44$, $P = 0.0004$). Fluorescence imaging of immunostained cells (Figure 1D) were consistent with these measured differences in the abundance of GluN1 variants at the plasma membrane, with GluN1-1 appearing to be least abundant, GluN1-2 and GluN1-3 having intermediate levels, and GluN1-4 most abundant.

Together, these results indicate that in HEK293 cells transfected with GluN1 and GluN3A, the magnitude of the recorded whole-cell glycinergic current varies with the C-terminal tail of GluN1. These differences in current amplitude can be attributed at least in part to differences in protein expression, and/or in trafficking to the plasma membrane, with GluN1-4/GluN3A channels likely being ~5-fold more abundant than GluN1-1/GluN3A channels in the membrane ($P < 0.001$). We next examined whether these receptors also exhibit differences in biophysical properties such as kinetics and permeability.

3.2 Kinetic properties of GluN1/GluN3 currents

To delineate kinetic properties of the macroscopic currents, we elicited whole-cell responses from cells expressing individual GluN1-a variants and GluN3A using prolonged (5 s) applications of glycine (0.5 mM) at pH 6.8. We found previously that this acidic external medium strongly facilitates GluN1/GluN3A activity, regardless of the differential splicing in the extracellular N-terminal domain (a vs. b variants) [16]. Therefore, here we evaluated and compared receptors containing GluN1-a subunits that differed in their intracellular C-terminal sequence (1–4). All four channels examined produced deeply desensitizing currents, such that the steady-state current levels (I_{ss}) represented less than 10% of the initial peak levels (I_{pk}). For all receptors, the decay phase of the current was best described with biphasic exponential functions, whose weighted time constants were similar (τ_D , ~0.2 – 0.4 s) ($F_{(3,25)} = 1.58$, $P = 0.22$). However, receptors containing the GluN1-2 or GluN1-3 subunit desensitized to a lesser extent (I_{ss}/I_{pk} , 0.08 ± 0.01 , $n = 5$; and 0.07 ± 0.01 , $n = 11$) than those containing GluN1-1a (0.03 ± 0.01 , $n = 8$) or GluN1-4 (0.02 ± 0.01 , $n = 7$) ($F_{(3,27)} = 7.26$, $P = 0.001$) (Figure 2A and Table 1).

3.3 Permeation properties of GluN1/GluN3A channels

GluN1 CTD contains PKA phosphorylation sites that, in glutamatergic GluN1/GluN2 receptors, control the receptor's calcium permeability [51]. To examine whether the C-terminal sequence of GluN1 subunits influences conductance and permeation properties for glycinergic GluN1/GluN3A receptors, we recorded whole-cell currents in response to glycine (0.5 mM) in the absence or presence of 1.8 mM free external Ca^{2+} and varied the applied pipette potential between –80 mV and +60 mV in 20 mV increments. From these data, we calculated the voltage dependency of macroscopic currents and the extent of voltage-dependent Ca^{2+} block.

The current reversal potential was ~–6 mV for each of the four isoforms tested, and the I/V slope, measured at voltages more positive than –20 mV, showed similar chord conductance in the 25–30 pS range, similar to calculations of unitary conductance (~38 pS) and reversal potential (~5–10 mV) in previous studies [1, 18]. Further, we found that, as expected for channels sensitive to voltage-dependent calcium-block, when Ca^{2+} (1.8 mM free Ca^{2+}) was present extracellularly, the I/V relationship deviated from linearity in the negative range of voltages (Figure 2B). We expressed the degree of Ca^{2+} block as a rectification index (I_{+40}/I_{-60}) and found this to be ~4 for all variants. These results indicate similar permeation properties for the four GluN1-a/GluN3A variants tested.

Together, these results indicate that the C-terminus of the GluN1 subunits affects the kinetics of the whole-cell GluN1/GluN3A receptor responses by modulating the extent of desensitization, but has no discernible effects on permeation properties such as conductance, reversal potential, and voltage-dependent Ca^{2+} block.

3.4 Pharmacological modulation of GluN1/GluN3A currents

Protons and zinc are endogenous ions that robustly potentiate GluN1/GluN3A currents [16, 17]. Given the likely physiological importance of this pharmacologic regulation, we asked the GluN1 CTD sequence affects receptor responses to these ions.

Protons and zinc strongly reduce the activation of glutamatergic GluN1/GluN2 receptors and this inhibition is mediated by residues in the N-terminal domain of GluN1 and GluN2 subunits respectively [52, 53]. In contrast, the strong facilitation of glycinergic GluN1/GluN3 current by protons and zinc depends on non-overlapping residues in the agonist-binding domain [16, 17]. Specifically, we reported that proton-dependent facilitation is independent of N-terminal domains [16]. Here, we recorded glycinergic currents from receptors containing GluN1-1a or GluN1-1b and found that they were similarly potentiated by free zinc (50 μM): $I_{\text{Zn}}/I_{\text{ctr}} = 12 \pm 5$ -fold, and 9.2 ± 1.5 -fold, respectively ($P = 0.6$, unpaired Student's t-test). Therefore, zinc-dependent potentiation is insensitive to splicing in the N-terminal domain of GluN1 subunits. Next, we describe experiments that ask whether C-terminal sequences alter the proton- and zinc-dependent effects.

We elicited whole-cell currents with glycine in physiologic proton concentrations (10 μM , pH 7.4); after the currents equilibrated to a steady-state level ($I_{7.4}$), we exposed cells to slightly acidic solution (pH 6.8) until the surge produced by acidification subsided and the current equilibrated to a new steady state-level ($I_{6.8}$) (Figure 3A and Table 1). With this protocol, we found that protons strongly potentiated steady-state currents from receptors containing GluN1-1 (12 ± 5 -fold, $n = 4$) or GluN1-4 (20 ± 9 -fold, $n = 4$); and to a lesser extent those from receptors containing GluN1-2 (2 ± 0.2 -fold, $n = 5$) or GluN1-3 (4 ± 0.9 -fold, $n = 5$) ($F_{3,14} = 3.51$, $P = 0.04$).

Next, we evaluated the extent of current potentiation by zinc. We recorded glycinergic whole-cell currents (0.5 mM, pH 7.4) in the absence or presence of 50 μM free zinc (Figure 3B and Table 1). We found greatest potentiation for GluN1-2 (~16-fold) and smallest, although still substantial for GluN1-4 (~7-fold) ($F_{3,15} = 3.74$, $P = 0.04$).

These results demonstrate that the degree of potentiation by allosteric ligands (protons and zinc) depends on the identity of the GluN1 CTD, whose sequence is controlled by differential splicing. Ionotropic glutamate receptors are targets of *in situ* phosphorylation by specific signaling cascades and this is an important mechanism of regulating their activity and lifetime in the membrane. Next, we investigated phosphorylation as a possible mechanism by which differences in the GluN1 CTD sequence may affect the kinetics and allosteric properties of GluN1/GluN3A receptors.

3.5 Effects of phosphorylation on GluN1/GluN3A currents

PKA phosphorylates GluN1-1a at S897 and PKC phosphorylates S890 and S896, in neurons *in vivo* [54, 55]. Given that these residues are located on the C1 cassette, which may or may not be spliced out, we investigated whether dynamic phosphorylation at these sites can confer distinct functional properties onto GluN1/GluN3A receptors, as a possible means of splice variant-specific regulation.

To examine potential effects of phosphorylation at these sites, we introduced triple substitutions in GluN1a-3 at S890, S896, and S897, to mimic receptor states that are constitutively dephosphorylated (3S/3A) or phosphorylated (3S/3D). We co-expressed each mutant with GluN3A and recorded glycinergic whole-cell currents (0.5 mM, 5 s, pH 6.8). We found that relative to WT receptors (0.07 ± 0.01 , $n = 11$) ($F_{2,19} = 5.92$, $P = 0.01$), the 3S/3A phosphodeficient mutant desensitized much less ($I_{ss}/I_{pk} = 0.16 \pm 0.04$, $n = 4$, $P < 0.01$) while the 3S/3D phosphomimic mutant was not different (0.06 ± 0.02 , $n = 7$, $P > 0.05$). We observed no measurable differences in the desensitization time constant (3S/3D, $\tau_D = 0.20 \pm 0.06$ s, $n = 7$, $P > 0.05$; 3S/3A, $\tau_D = 0.24 \pm 0.04$ s, $n = 4$, $P > 0.05$) compared to WT (0.34 ± 0.03 s, $n = 11$) ($F_{2,19} = 3.38$, $P = 0.06$) (Figure 4B and Table 2). These results indicate that the phosphorylation state of these sites can control the amplitude of the steady-state GluN1-a/GluN3A current. Therefore, these findings reveal yet another distinctive property of glycinergic NMDA receptors. For all glutamatergic NMDA receptors investigated so far phosphorylation *elevates* current levels; in contrast, our result indicate that phosphorylation *attenuates* glycinergic currents. This observation is notable because it may indicate a general pattern where modulatory actions of signaling pathways, by diffusible allosteric modulators or metabolic cues, exert opposing actions on glutamatergic and glycinergic excitatory currents [16, 17, 53, 56, 57].

As expected from the observed lack of effect of GluN1-a splicing on permeation properties of GluN1/GluN3A receptors, we found that all four mutants had similar current - voltage relationships in the presence of 1.8 mM external calcium (I_{+40}/I_{-60} , ~4–5) (Figure 4C).

Each GluN1 phosphorylation site makes unique contributions to glutamatergic GluN1/GluN2 receptor clustering and expression and are targets to independent signaling pathways [54]. Therefore, we sought to determine which residue(s) mediate the increased activity we observed for the triple mutants. We recorded glycine-elicited whole-cell responses and measured I_{ss}/I_{pk} ratios for receptors containing GluN1-3a subunits with the following single residue substitutions: S890A, S896A, and S897A (Figure 4D). Compared to WT receptors (0.07 ± 0.01 , $n = 11$) ($F_{(3,31)} = 3.02$, $P = 0.04$), we observed a 2-fold increase in I_{ss}/I_{pk} for S896A-containing receptors (0.13 ± 0.03 , $n = 6$, $P < 0.05$), and no statistically significant differences for receptors carrying either the S890A (0.05 ± 0.01 , $n = 6$, $P > 0.05$) or the S897A (0.10 ± 0.02 , $n = 5$, $P > 0.05$) substitution. These results show that specifically GluN1 S896, which is a PKC phosphorylation site, can control the level of GluN1/GluN3A steady-state current, and therefore may dictate their tonic levels *in vivo*.

4. DISCUSSION

NMDA receptors play myriad roles in the development and function of the mammalian CNS and have been implicated in neurologic and psychiatric dysfunctions. In part, this diversity reflects differential expression of receptor subtypes with distinct molecular composition and functional properties. Here we describe biophysical and pharmacologic properties of four recombinant GluN1-a/GluN3A receptor subtypes, which differ in the sequence of the intracellular tail of the GluN1-a subunit.

All four GluN1-a/GluN3A subtypes tested produced deeply desensitizing macroscopic currents in response to prolonged glycine applications, with decay time courses in the 0.2 – 0.4 s range. These kinetics are substantially faster than those reported for glutamatergic GluN1/GluN2 receptors, which desensitize with a time course of ~1-2 s but still two orders of magnitude slower than the desensitization kinetics of non-NMDA receptors [24]. Given that GluN1/GluN3A receptors are unlikely to experience brief pulses of glycine, but rather sustained ambient glycine levels, the level of residual or steady state current is a more physiologically relevant parameter. The steady state current will vary with the level of protein expression, and with each receptor's steady state open probability.

Here, we took advantage of a heterologous expression system to investigate molecularly defined subtypes of GluN1/GluN3A receptors; therefore, our measurements do not inform whether in neurons or glial cells, receptors with distinct GluN1 intracellular tails may have different levels of expression or surface trafficking. In HEK293 cells, we observed higher expression of receptors containing GluN1-3a and GluN1-4a, but whether these results apply to native preparations is unclear. Further, the absolute open probability of GluN1/GluN3A receptors remains unknown. However, we observed distinct differences in the extent of macroscopic desensitization for the four isoforms investigated, which is a strong indication that the C-terminal tail of the GluN1 subunit controls the level of steady state current, a biologically salient parameter of the GluN1/GluN3A response.

Relative to their respective peak current levels (I_{pk}), receptors containing GluN1-2 or GluN1-3 subunits produced 4-fold more steady state current (I_{ss}) than receptors containing GluN1-1 or GluN1-4 subunits (~8% vs ~2% residual current, respectively). Such changes will likely decrease the resting membrane potential, and possibly increase excitability in cells that express receptors with larger steady state currents. On the other hand, given that persistent depolarization can lead to decreased cellular excitability due to inactivation of voltage-gated sodium channels [58], it is possible that persistent GluN1/GluN3A activity may serve to decrease excitability. Regardless of directionality, our new results suggest that regulated splicing of the GluN1 subunit may control cellular excitability in cells expressing GluN1/GluN3A channels. The mechanism by which the intracellular domains of GluN1 subunits affect channel activity is unknown; however, our mutagenesis results are consistent with the view that perturbations in the C-terminal domain can alter receptor gating.

We compared glycinergic responses from receptors with targeted phosphomimic and phosphodeficient mutations in the C-terminal tail of the GluN1-3 subunit, and found that changes in the phosphorylation status of PKA/PKC targeted residues, but not of the putative

tyrosine kinase residues, can modify the fraction of residual current. This result is important on several levels. First, it demonstrates that receptor desensitization is sensitive to the phosphorylation status of residues known to be under kinase regulation *in vivo* [24, 40, 54, 57] and that this modulation is site specific. Second, it suggests that phosphorylation may be a physiologic mechanism of controlling residual current from GluN1/GluN3A receptors containing GluN1 variants 1 or 3, but not 2 or 4. Alternatively, differential splicing may serve to add or remove kinase-dependent sensitivity for glycinergic excitation.

Notably, relative to phosphorylation deficient mutations, phosphomimic mutations decreased the relative residual current. This is in contrast to glutamatergic NMDA receptors, for which as a general rule phosphorylation increases receptor activity [51, 57]. Our novel observation is consistent with an overall trend where modulators effective at both GluN1/GluN2 and GluN1/GluN3 receptors, such as protons, zinc and phosphorylation, have opposing effects on the currents generated by these receptors [16, 17, 53, 56].

All receptors we examined were sensitive to potentiation by zinc and protons, but showed different levels of modulation. Receptors with highest levels of residual current (less desensitization) also showed least potentiation by protons, consistent with the hypothesis that protons potentiate GluN1/GluN3A currents by reducing desensitization [16]. In contrast, the levels of potentiation by zinc showed no correlation with desensitization levels, or with the pattern of proton potentiation, consistent with the hypothesis that zinc and protons affect GluN1/GluN3A activity through separate mechanisms [16, 17].

Last, all receptors we tested had currents with similar reversal potentials, relative calcium to sodium permeability, and voltage-dependent Ca^{2+} block. These results indicate that the permeation pathway is insensitive to perturbations in the intracellular residues of the GluN1 subunit. These results further differentiate glycinergic NMDA receptors from their more intensely investigated glutamatergic relatives, for which perturbations in the GluN1 C-terminal domain, whether by splicing or phosphorylation, modify channel permeation properties [51, 59].

Throughout this study, we recorded GluN1/GluN3A activity receptors in slightly acidic conditions (pH 6.8) where they exhibit maximal activity [16]. Although this was for practical reasons, results have bearing on pathologic situations where the pH of the interstitial fluid decreases transiently, such as following acute ischemic or traumatic insults, or chronically, as in amyotrophic lateral sclerosis and schizophrenia [60–63].

In summary, our results show that perturbations in the C-terminal tail of the GluN1 subunit control the level of steady state GluN1/GluN3A current by changing receptor gating. This information is valuable in two ways. First, because at this time, the exact molecular composition of NMDA receptors expressed at specific locations in the mammalian CNS is unknown, information about subtype-specific kinetic and pharmacological properties can help evaluate which receptor subtypes contribute to the observed electrical response in native preparations during physiologic or pathologic conditions. Second, because the expression of receptors that differ in their GluN1 intracellular sequence is naturally controlled, our results indicate which properties of the native response are sensitive to this regulation. Last, the

ability of GluN1 C-terminal splicing to control the glycinergic current, may have important functional consequences under pathological conditions that influence the relative abundance of GluN1 splice variants, as for example in schizophrenia [44]. Together with emerging knowledge about the complement of subunits expressed *in vivo*, the new functional data we report here will help to delineate the roles played by glycinergic excitatory NMDA receptors in the health and disease of the CNS.

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HIGHLIGHTS

- GluN1/GluN3A receptors with alternatively spliced GluN1 C-termini have distinct electrophysiological and pharmacologic properties.
- GluN1 C-terminus modulates the level of GluN1/GluN3A steady-state current.
- GluN1 C-terminus modulates facilitation by H^+ and Zn^{2+}
- Residues in GluN1 C-terminus that are PKC phosphorylation targets in vivo can modulate steady-state current levels.

Figure 1.

Expression of GluN1/GluN3A receptors in HEK293 cells. (A) C-terminal sequence of four naturally occurring GluN1a subunits; known (blue arrowheads) phosphorylation sites are indicated. (B) Trace illustrates glycine-elicited whole-cell current (0.5 mM Gly, pH 6.8); bar graph summarizes calculated current densities. (C) Western blot of proteins in total cell lysates or in plasma membranes probed with pan-GluN1 and anti-calnexin (Cnx) antibodies or amido black (AB)-stained PVDF membrane. Bars, means $\pm$ SEM; n, within bars; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. (D) Confocal images of cells immunostained with nuclear stain (DAPI, blue), wheat germ agglutinin (WGA, red), and anti-GluN1 (green).

Figure 2.

Kinetic properties of GluN1/GluN3A whole-cell currents. GluN1 variants were expressed with GluN3A in HEK293 cells, as indicated. (A) Representative glycinergic whole-cell currents (0.5 mM, pH 6.8); insets summarize the time course (τ_D) and extent (I_{ss}/I_{pk}) of current desensitization. (B) Summary of current-voltage relationships recorded at pH 6.8 with 1.8 mM Ca^{2+} ; inset, rectification index (I_{+40}/I_{-60}); bars, means $\pm$ SEM; n, in bars; **, $P < 0.01$.

Figure 3.

GluN1 C-terminus modulates pharmacologic potentiation of GluN1/GluN3A currents.

GluN1 variants were expressed with GluN3A in HEK293 cells, as indicated. (A)

Representative glycinergic whole-cell currents were recorded at pH 7.4 and pH 6.8 as indicated; inset, summary of proton-dependent increase in steady-state current ($I_{6.8}/I_{7.4}$) (means ± SEM). (B) Representative glycinergic whole-cell currents recorded from the same

cell in the absence (black, I_{ctr}) or continuous presence of 50 μ M zinc (blue, I_{Zn}), are shown superimposed and normalized to the zero-zinc GluN1-1/GluN3A current; inset, summary of

zinc-dependent increase in peak current (I_{Zn}/I_{ctr}) (means ± SEM); *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Figure 4.

Specific perturbations at GluN1 intracellular residues affects GluN1/GluN3A steady- state current levels. (A) GluN1-3a sequence with substrates of PKC and PKA (blue); wild-type (WT) or mutated subunits were expressed with GluN3A in HEK293 cells. (B) Whole-cell currents were recorded at pH 6.8; bar graphs summarize the extent (I_{ss}/I_{pk}) of desensitization (means $\pm$ SEM). (C) Summary of current-voltage relationships recorded at pH 6.8 and 1.8 mM Ca^{2+} ; inset, rectification index (I_{+40}/I_{-60})(means $\pm$ SEM). (D) Summary of the extent (I_{ss}/I_{pk}) of desensitization for single mutants. Traces are WT (black) and S896A (blue); n, within bars; *, $P < 0.05$.

Table 1

Kinetic properties of GluN1/GluN3A receptors

	GluN1-1	GluN1-2	GluN1-3	GluN1-4
I_{pk} (pA)	214 ± 50	202 ± 70	595 ± 131	593 ± 200
I_{ss} (pA)	8.0 ± 4	14 ± 4	59 ± 39	8.0 ± 3
I_{ss}/I_{pk}	0.03 ± 0.01	0.08 ± 0.01 [*]	0.07 ± 0.01 [*]	0.02 ± 0.01
TauD (s)	0.24 ± 0.05	0.19 ± 0.06	0.34 ± 0.03	0.26 ± 0.07
I_{6,8}/I_{7,4} (fold)	12 ± 5.0	2.0 ± 0.2 [#]	4.0 ± 0.9 [#]	20 ± 9
I_{Zn}/I (fold)	13 ± 0.3	16 ± 2.5	12 ± 2.2	7.0 ± 2.3 [@]

^{*} P < 0.05 relative to GluN1-1a I_{ss}/I_{pk}

[#] P < 0.05 relative to GluN1-1a I_{6,8}/I_{7,4}

[@] P < 0.05 relative to GluN1-2a I_{Zn}/I

Table 2

Kinetics of mutant GluN1/GluN3A receptors

GluN1-3	3S/D	3S/A	S890A	S896A	S897A
Ipk (pA)	246 ± 87	211 ± 56	312 ± 45	267 ± 63	345 ± 97
Iss (pA)	16 ± 9	36 ± 11	15 ± 2	39 ± 14	32 ± 8
Iss/Ipk	0.06 ± 0.02	0.16 ± 0.04*	0.05 ± 0.01	0.13 ± 0.03*	0.10 ± 0.02
TauD (s)	0.20 ± 0.06	0.24 ± 0.04	0.31 ± 0.05	0.24 ± 0.06	0.32 ± 0.09

* P < 0.05 relative to WT GluN1-3a I_{ss}/I_{pk}