C-terminal Domains of NMDA (N-Methyl-d-aspartic Acid) Receptor Modulate Unitary Channel Conductance and Gating

Bruce A. Maki¹, Teresa K. Aman², Stacy A. Amico-Ruvio², Cassandra L. Kussius² and Gabriela K. Popescu¹,²

From the Neuroscience Program¹ and Department of Biochemistry², University at Buffalo, State University of New York, Buffalo, New York 14214

Running title: NMDA receptor CTD truncations

Correspondence should be addressed to: Gabriela K. Popescu, Department of Biochemistry, University at Buffalo, State University of New York, 636 Biomedical Research Building, 3435 Main Street, Buffalo, NY 14214, Tel: (716) 829-3807, Fax: (716) 829-2661, E-mail: popescu@buffalo.edu

Keywords: Ionotropic glutamate receptors; NMDA receptors; gating mechanism; single-channel kinetics; allosteric modulation

Background: The C-terminal domains (CTDs) of NMDA receptors are essential for normal brain function.

Results: We developed kinetic mechanisms for receptors lacking CTDs using single-channel methods.

Conclusion: GluN1 CTDs control primarily unitary conductance and GluN2 CTDs control gating kinetics.

Significance: Results afford quantitative insight into how intracellular perturbations can change the time course of NMDA receptor currents.

SUMMARY

NMDA receptors (NRs) are glutamate-gated calcium-permeable channels that are essential for normal synaptic transmission and contribute to neurodegeneration. Tetrameric proteins consist of two obligatory GluN1 (N1) and two GluN2 (N2) subunits, of which GluN2A (2A) and GluN2B (2B) are prevalent in adult brain. The intracellularly located carboxy-terminal domains (CTDs) make a significant portion of the receptors’ mass and are essential for plasticity and excitotoxicity but their functions are incompletely defined. Recent evidence shows that truncation of the N2 CTD alters channel kinetics, however the mechanism by which this occurs is unclear. Here we recorded activity from individual NRs lacking the CTDs of N1, 2A or 2B and determined these receptors’ gating mechanisms. Receptors lacking the N1 CTDs had larger unitary conductance and faster deactivation kinetics; receptors lacking the 2A or 2B CTDs had longer openings and longer desensitized intervals; and the first 100 amino acids of the N2 CTD were essential for these changes. In addition, receptors lacking the CTDs of either 2A or 2B maintained isoform-specific kinetic differences and swapping CTDs between 2A and 2B had no effect on single-channel properties. Based on these results, we suggest that perturbations in the CTD can modify the NR-mediated signal in a subunit-dependent manner; in 2A these effects are most likely mediated by membrane-proximal residues; and the isoform-specific biophysical properties conferred by 2A and 2B are CTD-independent. The kinetic mechanisms we developed afford a quantitative approach to understanding how the intracellular domains of NR subunits can modulate the receptor’s responses.
NMDA receptors (NRs), AMPA receptors and kainate receptors represent the three glutamate-activated receptors of the ionotropic glutamate receptor (iGluR) family that mediate the vast majority of rapid excitatory synaptic transmission in the mammalian central nervous system (reviewed by (1)). They form tetrameric ion channels with similar overall architecture, consisting of extracellular N-terminal (NTD) and ligand-binding domains (LBD), a transmembrane domain (TMD), and an intracellular C-terminal domain (CTD) (2).

Among iGluRs, NRs have several unique features; they are obligate heteromers, have high channel conductance, high Ca\textsuperscript{2+} permeability, voltage-dependent Mg\textsuperscript{2+} block, and slow kinetics (3-7). NRs comprise of two obligatory GluN1 (N1) subunits, which are ubiquitously expressed from a single gene and two GluN2 (N2) subunits, whose expression from four separate genes (2A-2D) is temporally and specialty regulated (8-16). In adult, the 2A and 2B subunits are the most highly expressed N2 isoforms; however throughout development 2B dominates early while 2A does so in adulthood. A developmental switch in N2 subtype expression underlies a shift in synaptic plasticity and this is critically dependent on the CTD (17).

The CTDs act as conduits by which intracellular modulators can alter the NR response. N1 CTDs are comparable in size to those of AMPA and kainate receptors (~100 aa) (18), and are crucial for Ca\textsuperscript{2+}-dependent inactivation of NR currents (19,20). N2 CTDs are ~6 fold larger (~600 aa), and are the largest within the iGluR family (18). All iGluR CTDs are predicted to adopt an intrinsically disordered structure (21). Knock-in mice expressing 2A receptors with truncated CTDs survive into adulthood but have synaptic plasticity deficits, while those expressing truncated 2B CTDs die perinatally (22-24). These mutant receptors maintain ionotopic functionality and are trafficked to the plasma membrane, however their synaptic localization is impaired (22,24-28), suggesting that the deficits arise from insufficient synaptic signaling and/or altered channel gating/modulation. Changes in receptor gating could arise from loss of phosphorylation sites, interaction deficits with intracellular proteins, or changes in structural requisites. Consistent with these possibilities, specific residues within the CTD represent molecular targets for modification by kinase/phosphatase systems (reviewed by (29)) and for binding by cytoplasmic proteins such as calmodulin, α-actinin and PSD-95 (19,30,31).

NRs lacking N2 CTDs produce whole-cell responses that are distinct from wild-type receptors (WT). Specifically, 2A receptors with a truncated CTD exhibit increased macroscopic desensitization (26). Additionally, 2A and 2B receptors lacking CTDs have whole-cell responses with lower peak open probability and faster deactivation (28). However, swapping the CTDs of 2A and 2B subunits did not change macroscopic responses, an indication that domains other than the CTDs are responsible for subtype-dependent differences in gating kinetics.

Currently, neither the mechanistic basis for these observed changes in macroscopic behaviors nor the portions of the CTD responsible for influencing the response kinetics are known. To investigate these aspects, we examined single-channel activity recorded from individual NRs that lacked the CTDs of the N1, 2A and 2B subunits. Based on these results we conclude that: 1) the CTDs of N1 and N2 subunits influence primarily unitary conductance and receptor gating, respectively; 2) the first 100 amino acids of the N2 CTD control the observed gating effects; and 3) isoform-dependent differences in kinetics and unitary conductance are maintained upon swapping CTDs between 2A and 2B subunits.

**EXPERIMENTAL PROCEDURES**

*Cells and plasmids* – Rat GluN1-1a (N1) (U08261), GluN2A (2A) (M91561) or GluN2B (2B) (M91562) were expressed in HEK293 cells along with GFP as previously described (32). Plasmids encoding CTD-truncated GluN1-1a (N1\textsuperscript{Δ}) or GluN2A (2A\textsuperscript{Δ}) were generously provided by G. Westbrook (19,26). We generated additional truncations by introducing stop codons at residue 845 in GluN2B (2B\textsuperscript{Δ}) and at residue 944 in GluN2A (2A\textsuperscript{Δ944}) with standard molecular biology.
procedures. Chimeric receptors with CTDs swapped between 2A and 2B subunits were generously provided by M. Constantine-Paton (17). N1/2A2B had 1-837 of 2A fused with 839-1483 of 2B; N1/2B2A had 1-838 of 2B fused to 838-1465 of 2A. As controls for wild-type N1/2A receptors, we used data files recorded previously in our lab (n = 14) (32), whereas for wild-type N1/2B, we recorded new traces (n = 8) to be consistent with the sampling rate used in this study (40 kHz). Both these data sets were re-analyzed to be consistent with the shorter dead times imposed in this study (0.075 ms).

**Electrophysiology** – Single-channel currents were recorded continuously from cell-attached patches containing only one active receptor with glass pipettes filled with extracellular solution (in mM): 150 NaCl, 2.5 KCl, 1 EDTA, 10 HEPES (pH 8.0 with NaOH) and supplemented with 1 glutamate and 0.1 glycine. Inward Na$^+$ currents were elicited by applying +100 mV through the recording pipette, were amplified and low-pass filtered (10 kHz, Axopatch 200B), digitally sampled (40 kHz, National Instruments PCI-6229 A/D board) and stored into digital files (QuB software, www.qub.buffalo.edu). For single-channel conductance and reversal potential measurements, we stepped in each patch the applied pipette potentials in 20 mV increments, between +100 mV and +20 mV, at two-minute intervals. Traces were subsequently separated by voltage and analyzed individually with QuB (33).

Whole-cell currents were recorded using glass pipettes filled with intracellular solution (in mM) 135 CsCl, 33 CsOH, 2 MgCl$_2$, 11 EGTA, 1CaCl$_2$ and 10 HEPES (pH 7.4 with CsOH). Cells were perfused with extracellular solution (in mM): 150 NaCl, 2.5 KCl, 0.5 CaCl$_2$, 0.1 glycine, 0.01 EDTA, and 10 HEPBS (pH 8.0 with NaOH), and were supplemented with glutamate (1 mM) when indicated. Cells were held at -70 mV and inward Na$^+$ currents were elicited by perfusing the cell with a glutamate-containing extracellular solution. Macroscopic currents were amplified and low-pass filtered (2 kHz, Axopatch 200B), sampled (5 kHz, Digidata 1440A), and stored as digital files (pClamp10 software, Molecular Devices). Excised-patch experiments were done with the same solutions used for whole-cell experiments. To mimic a synaptic stimulation, solutions with or without glutamate were switched rapidly by moving a double-barrel glass theta tube back-and-forth across the patch using a piezoelectric translation system (Burleigh LSS-3100/3200) (34). For each patch, open-tip potentials were measured at the end of the experiment and data were retained for analyses only when the 10 - 90% solution exchange occurred within 0.15 - 0.25 ms.

**Kinetic Modeling** – Selection, processing, idealization and modeling of single-channel data were done in QuB as described in detail previously (32). Briefly, records were minimally processed to correct for artifacts detected visually and baseline shifts, filtered digitally (12 kHz) and idealized with the SKM algorithm (35). Idealized data in each record contained between 2.5 x 10$^5$ and 9 x 10$^6$ events. After imposing 0.075 ms as the dead time, these data were fit directly with a MIL algorithm by state models that had increasing numbers of closed and open states (36). Best fitting models were selected with an arbitrarily set threshold for log-likelihood improvement of 10 units per added state. Time constants and areas of individual kinetic components, as well as rate constants for the transitions considered in each model, were calculated for each data file and are reported for each data set as mean ± SEM (Supplementary Tables 1, 2).

To represent the receptors’ activation reaction we selected events that occurred within bursts by defining a $\tau_{\text{crit}}$ that misclassified an equal number of events belonging to the $E_3$ and $E_4$ components, as described previously (37). The selected 'active' portions were fit by a kinetic model containing 3 closed and 2 open states (3C2O). The two open states represented a fast open state (O$^+$), which is common to all gating modes, and a 'global' long open state (O$^-$), which represented the average of all long open components occurring in each file. Next, we focused on microscopic desensitization by modeling all events in each data file with a 5C2O model. Rate constants within activation were not different from those obtained by fitting 'active' periods only. In addition, this complete
model estimated microscopic desensitization rate constants.

Simulations – Macroscopic responses were simulated in QuB software from 100 channels using the experimentally determined single-channel amplitude of each construct. Traces were computed as the time-dependent accumulation of receptors in the open states using the models indicated to which we appended two identical glutamate-binding steps. For wild-type 2A receptors we used values reported previously for the association and dissociation rate constants: \(1.7 \times 10^7 \text{M}^{-1}\text{s}^{-1}\) and 60 s\(^{-1}\), respectively (38). For the simulated traces we measured the 10–90% rise time and the peak current amplitude \((I_{pk})\). Time constants for current decay \((\tau_d)\) and desensitization \((\tau_D)\) were estimated by fitting a single exponential function to the declining portion of the trace.

Statistics – Results are presented as means ± SEM for each data set; statistical differences were determined using the Student’s t-test and were considered significant for \(p < 0.05\).

RESULTS

CTD contributions to channel conductance. To investigate the role of the large intracellular domain of NRs, we examined the activities of recombinant NRs that lacked the entire CTD modules of N1 \((N1^\Delta)\), 2A \((2A^\Delta)\) or 2B \((2B^\Delta)\) subunits (Fig 1). WT or CTD-truncated subunits were co-expressed in HEK293 cells in the following combinations: N1\(^\Delta\)/N2, N1/N2\(^\Delta\) or N1\(^\Delta\)/N2\(^\Delta\) for either 2A or 2B isoforms. For each receptor type we obtained long-duration recordings (> 10\(^5\) events) of steady-state currents from cell-attached membrane patches containing only one active channel (Fig 2). Inward sodium currents were obtained in the presence of saturating levels of agonists (glutamate, 1 mM and glycine, 0.1 mM) in conditions that minimized inhibitory effects of contaminating divalent ions (EDTA, 1 mM) (3-6,39-42) and protons (pH 8.0, HEPBS, 10 mM) (43-45).

All NRs examined produced unitary currents of uniform amplitudes, with no obvious sub- or supra-level conductances; currents occurred as bursts of frequent openings separated by long silent periods. In addition to this prevalent bursting behavior, receptors lacking N2 CTDs produced frequently isolated openings (Fig 2). All events, including these isolated openings, were considered in subsequent analyses. Whether truncated or not, 2B receptors always produced currents with ~10% larger current amplitudes compared to 2A receptors. Relative to their wild-type (WT) counterpart, 2A and 2B receptors lacking the N1 CTD produced currents with ~30% and ~20% larger unitary amplitudes, respectively (Table 1).

To test whether these observed changes in unitary current amplitudes reflected greater unitary conductances, we examined the current-voltage relationships for these constructs and estimated their slope conductances (Fig 3). Both 2A and 2B receptors lacking the N1 CTD had significantly higher single-channel conductance compared to their WT equivalent. Unitary conductance increased from 74 ± 2 pS to 91 ± 3 pS for N1\(^\Delta\)/2A receptors \((p < 0.05)\); and from 87 ± 2 pS to 98 ± 3 pS for N1\(^\Delta\)/2B receptors \((p < 0.05)\). All receptors tested had similar reversal potential values \((V_r = 12 – 18 \text{ mV}, p > 0.05)\), an indication that differences in resting membrane potential between cells did not contribute to the observed changes in unitary current amplitudes.

CTD contributions to activation gating. Next, we used the accumulated single-channel data to estimate channel open probability \((P_o)\), mean open time \((\text{MOT})\) and mean closed time \((\text{MCT})\), as global measures of gating kinetics for each receptor. By these metrics, receptors lacking the N1 CTD had WT-like kinetics regardless of the N2 subunit with which they were paired (Table 1).

In contrast, receptors lacking the N2 CTD had ~2-fold lower \(P_o\) relative to the corresponding WT receptor. For either 2A or 2B, the lower activity was due solely to longer closures (MCT ~10 fold or ~3 fold, respectively) because the openings were also significantly longer in each case (MOT ~ 2 fold or ~1.5 fold, respectively) (Table 1).

To investigate in further detail how the absence of CTD modules influenced NR gating kinetics, we examined the relative distributions of event durations observed for each receptor type. Previous work established that NRs have complex
open and closed event duration distributions. For agonist bound receptors, closed-intervals recorded from WT 2A or 2B receptors have durations that distribute around five components: three shorter components, E₁ – E₃, occur within bursts and make the majority of closed events during brief (1 – 10 ms) receptor activations (38,46-48); the two longest components, E₄ and E₅, occur between bursts and become populated to an observable degree only when receptors are stimulated with longer (>100 ms) agonist pulses (32,49,50). Open events have duration distributions that include at least two components: one short and one long when the record examined is kinetically homogeneous; and can have up to four components when the record captures shifts between low-, medium- and high- kinetic modes, for which the long component adopts mode-specific values (O₁, O₃, O₅) (47,51). As observed for WT receptors, all constructs investigated in this study produced distributions with five closed and four open components, a clear indication that receptors with truncated CTDs retained the machinery necessary for all gating transitions, including modal shifts. Next, we compared time constants and relative areas of the individual kinetic components.

2A and 2B receptors containing N1Δ had WT-like open distributions consistent with the overall WT-like kinetic parameters reported in Table 1 (Supplementary Table 1). For receptors lacking the 2A or 2B CTD, the increases observed in MOTs were entirely explained by across-the-board increases (1.5 – 3-fold) in the component durations (Fig 4), while the relative component areas were unchanged (Supplementary Table 1). This suggests that the longer MOTs represent changes in the stabilities of open states rather than an alteration in mode distribution (34,52). In contrast to this generalized effect on open state duration, CTD truncations increased MCTs by increasing preferentially the longer closed intervals, E₄ and E₅ (Supplementary Table 2), an indication that truncated receptors spent more time in desensitized conformations.

To examine the channels’ activation kinetics, we selected from each record the events that occurred within bursts by using a τ_{cr} that excluded closures associated with the long E₄ and E₅ components (37) (Fig 5A, B). Based on the observation that receptors lacking CTD modules maintain WT-like modal behavior, we chose to represent their activations with a simplified kinetic scheme composed of three closed and two open states (47). This model describes the activation reaction as a series of kinetic transitions that start with the first fully-ligated closed state C₁ and culminates with two coupled open states, O₁-O₂. In this model, modal shifts are not incorporated explicitly; rather the O₂ state represents globally all long openings observed. Rate constants were estimated for all transitions explicit in the model by fitting the model to the entire sequence of openings and closures within bursts in each record, and values obtained for a particular transition were averaged within each data set. Results showed a complex pattern of changes suggesting that CTDs influenced the receptor activation pathway for all combinations tested (Fig 5C).

We noted that, although 2A and 2B receptors containing N1Δ showed overall kinetic parameters that were not significantly different from WT (P₀, MOT and MCT) (Table 1), the modeling results indicated that the activation reaction was significantly faster for both these receptors. In contrast, all constructs lacking N2 CTDs had slower activation and deactivation rates (Fig 5C). We used the kinetic models and the measured channel conductances (Table 1) to simulate population responses to brief synaptic-like agonist applications (10 ms, 1 mM). The resulting traces were compared with macroscopic responses recorded from receptors residing in outside-out patches (Fig 5D). Below, we present the results we obtained for 2A receptors.

Our simulations predicted an increase in peak current for N1Δ/2A and N1/2AΔ relative to WT (20% and 10%, respectively). This prediction is not testable because in outside-out patches the number of receptors generating the observed currents is variable and unknown. However, we were able to compare the kinetics of simulated and measured traces and observed that for receptors lacking CTDs displayed changes in activation and
deactivation time course. Specifically, receptors lacking only the N1 CTDs produced currents that decayed faster while receptors lacking 2A CTDs produced currents that were slower to reach peak and also slower to decay (Fig 5D, Table 2).

**CTD contributions to desensitization.** To examine the effects of CTD-truncations on desensitization kinetics, we compared the time constants and amplitudes of the two longest-duration closed components, E₁ and E₃ (Fig 6A). Except for N1/2AΔ, which had a smaller area for E₃, all truncated receptors had time constants and areas of equal or greater magnitude for these two components (Fig 6B, Supplementary Table 2).

Next, we fit the entire sequence of events observed in each file with a model that, in addition to the 3C2O states that describe the activation sequence, also included two desensitized states, D₁ and D₂ (Supplementary Fig 1). The estimated rate constants for the 3C2O sequence were not different from those estimated by fitting bursts; in addition, with this procedure we were able to determine microscopic desensitization and resensitization rates. We used the 5C2O models to simulate macroscopic responses to long glutamate pulses (5s, 1 mM) and compared these to responses we recorded experimentally from whole-cells exposed to similar stimuli (Fig 6C).

We observed a close match in the extent of macroscopic desensitization (I₅₋₇/Iₚ₃) between the simulated and experimentally measured responses, with receptors containing 2AΔ desensitizing more deeply (smaller I₅₋₇/Iₚ₃ ratio) (Fig 6C, Table 3). The macroscopic desensitization time constant of whole-cell currents decreased for receptors lacking the 2A CTD, and was in fact faster than what was predicted by our results obtained from cell-attached measurements. However, even for WT receptors macroscopic desensitization was faster in whole-cell recordings relative to values expected from cell-attached measurements (32).

**CTD residues proximal to the membrane control channel gating.** Given that CTD truncations in N2 subunits produced a much more robust change to gating than in N1, we wondered whether the magnitude of the effect correlates with the conspicuously larger size of N2 CTDs. To investigate this aspect, we introduced a stop codon at residue 944 of 2A (2AΔ944) (Fig 1B) to produce a CTD of comparable length to that of WT N1 subunits. We co-expressed 2AΔ944 with WT N1 and we recorded on-cell single-channel steady-state currents (data not shown). We were surprised to observe that N1/2AΔ944 currents had Pₚ, MOT or MCT similar to those of wild-type N1/2A (Table 1). This result strongly supports the conclusion that, as with N1 subunits, the gating effects of 2A CTDs are largely mediated by membrane proximal residues.

**Isoform-specific gating kinetics are independent of CTDs.** We noted that CTD truncations in 2A and 2B subunits affected channel kinetics in a similar manner and that N1/2AΔ and N1/2BΔ receptors retained isoform-specific reaction mechanisms, despite lacking the entire N2 CTDs. Given the well documented differences in kinetics between WT 2A and 2B receptors and the physiologic importance of this difference, we asked whether CTDs of N2 contribute at all to isoform-specific gating. To address this question, we examined the reaction mechanisms of receptors with chimeric N2 subunits: N1/2A₂B had 2A subunits that contained the CTD of 2B, and N1/2B₂A had 2B subunits that contained the CTD of 2A (17). N1/2A₂B receptors produced currents with similar amplitudes and kinetics as N1/2A, while N1/2B₂A receptors produced currents with similar amplitudes and channel kinetics as N1/2B (Table 4). Together, these results indicate that isoform-specific conductance and gating properties of NRs are independent of the N2 CTD modules.

**DISCUSSION**
The results reported here describe the mechanism by which the N1 and N2 CTDs impact the amplitude and time course of NR responses. We used stationary single-channel recordings and computational methods to delineate the single channel conductance and gating reaction of receptors lacking CTD modules and receptors with swapped C-termini and we validated these results by comparing the predicted macroscopic responses with those measured in whole-cell and excised-patch preparations. Our results demonstrate the
following: 1) N1 CTDs make significant contributions to channel conductance and to macroscopic deactivation kinetics; 2) N2 CTDs control the peak macroscopic current and the kinetics of macroscopic deactivation and desensitization; 3) the first 100 residues of N2 CTDs are mainly responsible for the observed gating effects; and 4) CTDs do not contribute to isoform-specific conductance or gating differences between 2A- and 2B-containing NRs.

We were surprised to note that N1 CTD truncations increased NR single-channel conductance. Among all iGluRs, NRs have the largest single-channel conductance and have remarkably slow kinetics (1). A main function of NRs in synaptic plasticity is to detect coincident pre- and post-synaptic activity and the frequency of pre-synaptic firing (38,53) and to respond with large excitatory fluxes, of which calcium represents a significant fraction (54). The molecular determinants and regulatory mechanisms of high NR conductance and calcium permeability are just beginning to be revealed (55,56). Our results suggest the possibility that perturbations in the N1 CTD modulate NR conductance. Importantly, however, note that in this study we detected channel activity as Na\(^+\) fluxes and thus we have no information regarding possible changes in channel Ca\(^{2+}\) conductance. Also, since in all our single-channel measurements Ca\(^{2+}\)-influx was absent and the membrane integrity was preserved, our results lack information regarding Ca\(^{2+}\)-dependent modulatory processes.

Previous reports demonstrated that N1 CTDs mediate Ca\(^{2+}\)-dependent modulation of NR kinetics. Alpha-actinin and/or calmodulin modulate single-channel gating of NRs via binding-sites located within the N1 CTD (30,57,58). These protein-protein interactions, as well as the phosphorylation status of N1 CTD residues, influence Ca\(^{2+}\)-dependent inactivation of NR macroscopic currents (19,20). More specifically, the N1 CTD contains two PKC phosphorylation sites (S890, S896) and one PKA phosphorylation site (S897) (59). In the cell-attached preparation used here, it is not clear whether these proteins are bound to NRs at basal levels, thus we cannot directly identify whether the loss of either of these interactions is responsible for the changes we observed in receptors lacking N1 CTDs. Our analyses showed no changes in the global single-channel kinetic parameters and only minor changes in the open and closed time distributions for N1\(^{Δ}\)/N2 constructs. However, these receptors had faster deactivation rates raising the possibility that protein phosphorylation and/or protein-protein interactions can modulate the NR signal through the N1 CTD. Alternatively, residues in the N1 CTD may be required for the recognition and Ca\(^{2+}\)-dependent binding of these proteins, whereas the gating effect may also demand additional interactions with N2 CTDs.

N2 CTDs had the greatest effect on NR gating, and this effect was largely mediated by the membrane proximal segment. Of all iGluR subunits, N2A and N2B have the longest C-termini and also have the largest number of post-translational modification sites (1). Consequently, we were not surprised to observe robust changes to NR gating following full truncation of the N2 CTDs. Consistent with previous reports (24,27,28), we observed an overall decrease in \(P_o\), which, based on our results, we attribute primarily to increased entry into desensitized states. This is an important observation because, in contrast to AMPA-type iGluRs (60), the molecular determinants of NR microscopic desensitization are not known (61). Interestingly, along with the increase in desensitization, there was a simultaneous increase in open durations. In some ion channel families (62-64), phosphorylation/dephosphorylation events are thought to modulate channel \(P_o\) by controlling transitions between gating modes. In our study, however, following truncation of the N2 CTD, modal transitions were largely intact. This suggests that protein phosphorylation is not responsible for modal shifts in NRs, but instead may regulate the lifetime of open conformations in all modes.

An important advance afforded by the work reported here is the delineation of gating mechanisms that account for the entire kinetic repertoire of receptors with CTD perturbations.
We used these models to predict in silico macroscopic responses to two physiologically-relevant stimulation patterns: brief pulses of glutamate, which reproduce stimuli experienced by synaptic receptors, and seconds-long pulses which reproduce stimuli experienced by extrasyaptic receptors during pathologic conditions (65,66). The results, which we also validated by measuring responses from excised patch and whole-cell preparations, showed that receptors lacking N2 CTDs produced currents with slower deactivation, and much deeper desensitization. Similar changes to macroscopic desensitization have been reported previously for receptors with 2A CTD-truncations. In these studies, the authors correlated the progression of calcineurin-dependent desensitization with the availability for phosphorylation at position of S900 of 2A (26). Preliminary data from our lab also support a modulatory role for S900, however this residue is not solely responsible for the observed phenotype of N1/2AΔ (RF Cole, personal communication), suggesting that additional sites along the first 100 residues of the N2 CTD contribute to NR gating.

The structural mechanism by which the N2 CTD exerts its influence on channel kinetics cannot be fully inferred from the data presented in this paper. However, two potential mechanisms are possible: perturbations in the CTD may be transmitted to the desensitization gate through the M4 helix, with which CTD is continuous in sequence; alternatively, such perturbations may be transmitted through putative interactions with the M1-M2 and/or M2-M3 intracellular linkers between helices in the TMD. Our observation that the membrane proximal segment is critical for the modulatory effects of the N2 CTD is consistent with both these two scenarios.

Our finding that that currents produced by N1/2AΔ receptors were slower to reach peak and also slower to deactivate is in contrast with a recent study which shows that the current rise time was unchanged and deactivation time was faster following truncation of the N2 CTD (28). This apparent discrepancy is likely due to differences in experimental conditions as well as differences in Ca2+-dependent inactivation. Specifically, we used the outside-out configuration and a minimal extracellular calcium concentration (0.5 mM) whereas Kohr et al used the lifted whole-cell protocol and physiologic calcium concentrations (1.8 mM). Thus, the slower current rise and decay kinetics we observed for N1/2AΔ most likely reflects a change in channel gating rather than a difference in sensitivity to intracellular signaling. Closer examination of Ca2+-dependent changes in channel kinetics will likely provide valuable information regarding intracellular mechanisms of NR modulation.

The effects of N1 CTD truncations on conductance and gating were distinct from those observed for N2 CTD truncations. Thus, regulatory mechanisms mediated through the obligatory N1 subunit are likely applicable to all NRs, whereas those through the differentially expressed N2 subunits may vary across cells, subcellular locations and developmental stages. This inference is consistent with the idea that N2 subunits, in addition to controlling kinetic differences between NR isoforms, also control sensitivity to intracellular signaling. Specific biochemical pathways which can execute these modifications, as well as individual residues along the CTDs that can serve as molecular substrates for such proteins, have yet to be determined. Our demonstration that truncation of the CTDs of either 2A or 2B had similar effects on gating and that swapping CTDs of 2A and 2B have no effect on single-channel channel properties suggest that although intracellular mechanisms acting through N2 C-termini may be different, the mechanisms through which they affect channel deactivation kinetics and the extent of desensitization are similar. Both these aspects of channel response are of physiologic significance and pharmacologic interest.
References

NMDA receptor CTD truncations

Acknowledgements - We thank Dr. Gary Westbrook for providing CTD-truncation constructs for N1 and 2A subunits, Dr. Martha Constantine-Paton for providing chimeras with swapped CTDs, constructs, Tom Ruffino for contributing single-channel recordings, and Eileen Kasperek for molecular biology and tissue culture procedures.

Author contributions – BAM recorded and analyzed all data for CTD truncated receptors; TKA contributed single-channel results for wild-type N1/2B controls; CLK and SAA contributed single-channel results for receptors with swapped CTDs; BAM and GKP designed the experiments, interpreted the results and wrote the paper.
FOOTNOTES
Abbreviations used: NR, N-methyl-D-aspartate receptor; iGluR, ionotropic glutamate receptor; NTD, N-terminal domain; LBD, ligand binding domain; TMD, transmembrane domain; CTD, C-terminal domain; \( V_r \), reversal potential; \( P_o \), open probability; MOT, mean open time; MCT, mean closed time; RT, rise time; \( I_{ss} \), steady state current; \( I_{pk} \), peak current; \( \tau_{Dec} \), decay time constant; \( \tau_{Des} \), desensitization time constant.
FIGURE LEGENDS

FIGURE 1: Schematic representation of NR subunits A) Scaled illustration of major functional domains and their respective relative sizes in N1 and N2 subunits. B) Cartoon illustrates membrane topology and overall shape for each subunit; indicated are truncations used in this study (red arrows) and putative phosphorylation sites (lines).

FIGURE 2: NR single-channel activity Each panel illustrates a continuous 50-s portion of current recorded from a one-channel HEK293 attached patch expressing wild-type receptors (A) and CTD-truncated receptors (B, C, D). P_o values indicated in each panel were calculated for the entire file from which the segment was selected. Arrows point to isolated openings that occur only in receptors lacking N2 CTDs.

FIGURE 3: Current-voltage relationships of single NRs Amplitudes of single-channel currents recorded from on-cell patches were measured at several applied voltages for receptors lacking the N1 CTD and for wild type N1/2A and N1/2B. *, indicates significant difference relative to the corresponding wild-type receptor; n = 5 for each condition (p < 0.05).

FIGURE 4: Open duration distributions of single NR channels A) and B) Histograms of open durations detected in the entire records represented in Fig 2. Overlaid are probability density functions (thick lines) and individual kinetic components (thin lines) calculated by fitting 5C4O models to each data file; Inserts give the calculated time constants (τ) and areas (a) for each open component: E_f, E_L, E_M, E_h. C) and D) Summary bar graphs illustrate the relative changes in the duration of open components. * indicates statistically significant differences relative to wild-type (p < 0.05).

FIGURE 5: Kinetic analyses of activation events A) and B) Histograms of closed durations detected within bursts defined with τ_crit (see Methods and text). Overlaid are probability density functions (thick lines) and individual kinetic components (thin lines) calculated by fitting 3C4O models to each ‘active’ file. Inserts give the calculated time constants (τ) and areas (a) for each closed component: E_1, E_2 and E_3. C) State models fitted to the events occurring within bursts in each record. Values on each arrow are the calculated average, rounded to the first significant figure, for the respective rate constant (s⁻¹). * indicates rates that were significantly faster (red) or slower (blue) relative to the corresponding wild-type receptor (p < 0.05). D) Macroscopic responses to 10-ms applications of 1-mM glutamate were simulated with the models in panel C (left, absolute scale), or recorded experimentally from excised outside-out patches (right, normalized to peak current).

FIGURE 6: Kinetic analyses of desensitization events A) Histograms of closed durations for events that occurred outside of τ_crit defined bursts (see Methods and text) for WT receptors overlaid with the E_4 and E_5 components for WT (black lines) or CTD truncated (colored lines) receptors calculated by fitting 5C4O models to entire data files. Inserts give the calculated time constants (τ) and areas (a) for the E_4, E_5 closed components. B) Summary of measured time constants and areas for E_4 and E_5. * indicates significant differences relative to wild-type (p < 0.05). C) Macroscopic responses to 5-s applications of 1-mM glutamate were simulated with the full kinetic models in Supplementary Fig 1 (top, absolute scale), or recorded experimentally from whole-cells (bottom, normalized to peak).
### TABLES

<table>
<thead>
<tr>
<th>Condition</th>
<th>Amplitude (pA)</th>
<th>( P_0 )</th>
<th>MCT (ms)</th>
<th>MOT (ms)</th>
<th>Events</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1/2A</td>
<td>8.7 ± 0.3</td>
<td>0.54 ± 0.04</td>
<td>4.7 ± 0.5</td>
<td>5.4 ± 0.5</td>
<td>4.9 x 10^6</td>
<td>14</td>
</tr>
<tr>
<td>N1^Δ/2A</td>
<td>11.3 ± 0.4*</td>
<td>0.54 ± 0.05</td>
<td>4.2 ± 0.7</td>
<td>4.8 ± 0.5</td>
<td>3.3 x 10^6</td>
<td>6</td>
</tr>
<tr>
<td>N1/2A</td>
<td>9.7 ± 0.3</td>
<td>0.22 ± 0.04*</td>
<td>50 ± 10*</td>
<td>13 ± 2*</td>
<td>5.0 x 10^5</td>
<td>7</td>
</tr>
<tr>
<td>N1^Δ/2A^Δ</td>
<td>9.4 ± 0.5</td>
<td>0.08 ± 0.02*</td>
<td>120 ± 20*</td>
<td>9 ± 1*</td>
<td>2.0 x 10^5</td>
<td>8</td>
</tr>
<tr>
<td>N1/2A^Δ</td>
<td>8.6 ± 0.9</td>
<td>0.49 ± 0.04</td>
<td>5.4 ± 0.3</td>
<td>6.0 ± 0.7</td>
<td>6.0 x 10^5</td>
<td>6</td>
</tr>
<tr>
<td>N1/2B</td>
<td>10.0 ± 0.4</td>
<td>0.33 ± 0.07</td>
<td>14 ± 4</td>
<td>5.2 ± 0.7</td>
<td>2.0 x 10^6</td>
<td>8</td>
</tr>
<tr>
<td>N1^Δ/2B</td>
<td>12.3 ± 0.4*</td>
<td>0.29 ± 0.02</td>
<td>13.4 ± 0.9</td>
<td>5.6 ± 0.6</td>
<td>2.9 x 10^6</td>
<td>6</td>
</tr>
<tr>
<td>N1/2B^Δ</td>
<td>10.7 ± 0.3</td>
<td>0.12 ± 0.03*</td>
<td>80 ± 10*</td>
<td>8.7 ± 0.9*</td>
<td>5.7 x 10^5</td>
<td>8</td>
</tr>
<tr>
<td>N1^Δ/2B^Δ</td>
<td>10.5 ± 0.4</td>
<td>0.12 ± 0.03*</td>
<td>100 ± 20*</td>
<td>11 ± 1*</td>
<td>5.7 x 10^5</td>
<td>6</td>
</tr>
</tbody>
</table>

**Table 1:** Effects of CTD-truncation on global kinetic properties of individual NRs. Values represent means ± SEM for each data set; *, indicates significant difference (higher, in red and lower, in blue) relative to the corresponding wild-type value (p < 0.05).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rise Time (ms)</th>
<th>( \tau_\text{d} ) (fast) (ms)</th>
<th>( \tau_\text{d} ) (slow) (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1/2A</td>
<td>5.2 ± 0.4</td>
<td>45 ± 4</td>
<td>280 ± 40</td>
<td>11</td>
</tr>
<tr>
<td>N1^Δ/2A</td>
<td>4.9 ± 0.5</td>
<td>38 ± 3</td>
<td>130 ± 10*</td>
<td>9</td>
</tr>
<tr>
<td>N1/2A^Δ</td>
<td>9 ± 1*</td>
<td>60 ± 20</td>
<td>1500 ± 500*</td>
<td>6</td>
</tr>
<tr>
<td>N1^Δ/2A^Δ</td>
<td>13 ± 5*</td>
<td>70 ± 10*</td>
<td>500 ± 100*</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 2:** Effects of CTD-truncation on macroscopic activation and deactivation kinetics. Values represent means ± SEM for each data set; *, indicates significant difference (higher, in red and lower, in blue) relative to the full length receptor (p < 0.05).
<table>
<thead>
<tr>
<th>Condition</th>
<th>$\tau_D$ (ms)</th>
<th>$I_{ss}$ (pA)</th>
<th>$I_s/I_{pk}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1/2A</td>
<td>1000 ± 100</td>
<td>440 ± 60</td>
<td>0.67 ± 0.03</td>
<td>8</td>
</tr>
<tr>
<td>N1β/2A</td>
<td>700 ± 100</td>
<td>600 ± 200</td>
<td>0.49 ± 0.05*</td>
<td>10</td>
</tr>
<tr>
<td>N1/2A$^\Delta$</td>
<td>630 ± 50*</td>
<td>210 ± 50*</td>
<td>0.29 ± 0.02*</td>
<td>9</td>
</tr>
<tr>
<td>N1β/2A$^\Delta$</td>
<td>370 ± 20*</td>
<td>320 ± 40*</td>
<td>0.23 ± 0.01*</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 3: Effects of CTD-truncation on macroscopic desensitization kinetics. Values represent means ± SEM for each data set; *, indicates significant increase (red) relative to the full length receptor ($p < 0.05$).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Amplitude (pA)</th>
<th>$P_o$</th>
<th>MCT (ms)</th>
<th>MOT (ms)</th>
<th>events</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1/2A</td>
<td>9.0 ± 0.6</td>
<td>0.64 ± 0.06</td>
<td>6 ± 2</td>
<td>11 ± 1</td>
<td>$1.3 \times 10^6$</td>
<td>5</td>
</tr>
<tr>
<td>N1/2A$\beta$2B</td>
<td>9.7 ± 0.6</td>
<td>0.63 ± 0.03</td>
<td>5.9 ± 0.6</td>
<td>9.5 ± 0.9</td>
<td>$1.8 \times 10^6$</td>
<td>9</td>
</tr>
<tr>
<td>N1/2B</td>
<td>10.2 ± 0.4</td>
<td>0.21 ± 0.04</td>
<td>34 ± 9</td>
<td>5.4 ± 0.5</td>
<td>$2.5 \times 10^6$</td>
<td>11</td>
</tr>
<tr>
<td>N1/2B$\beta$2A</td>
<td>11.3 ± 0.5</td>
<td>0.21 ± 0.05</td>
<td>20 ± 3</td>
<td>4.2 ± 0.4</td>
<td>$1.6 \times 10^6$</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 4: Effects of switching CTDs between 2A and 2B subunits.
Figure 2

A

N1/2A

\( P_\text{g} : 0.44 \)

N1/2A

N1/2A

N1/2A

N1/2A

B

N1/2B

\( P_\text{g} : 0.35 \)

N1/2B

N1/2B

N1/2B

N1/2B

C

N1/2A

\( P_\text{g} : 0.54 \)

N1/2A

N1/2A

N1/2A

N1/2A

D

N1/2B

\( P_\text{g} : 0.30 \)

N1/2B

N1/2B

N1/2B

N1/2B

N1/2B

N1/2B

N1/2B

N1/2B

N1/2B
Figure 3

![Graph showing the relationship between applied voltage (mV) and current (pA). The graph includes data points for different conditions labeled N1/2A (n=5), N1/2A (n=5), N1/2B (n=5), and N1/2B (n=5). The axes are labeled as follows: X-axis: Applied voltage (mV), Y-axis: Current (pA).]
Figure 4

A

N1/2A

E_low, E_med, E_high

τ (ms) a(%)

N1^A/2A

τ (ms) a(%)

B

N1/2B

τ (ms) a(%)

N1^A/2B

τ (ms) a(%)

C

N1/2

N1^A/2

N1/2^A

N1^A/2^A

D

normalized time constants

WT (ms)

τ_f, τ_low, τ_med, τ_high

0.14, 2.6, 5.9, 11.1

0.19, 2.6, 6.4, 11.4
Figure 5

A

B

C

D

simulation

patch response

N1/2A

N1^A/2A

N1/2B

N1^A/2B

N1/2A^A

N1^A/2A^A

N1/2B^A

N1^A/2B^A

N1/2A

N1^A/2A

N1/2A^A

N1^A/2A^A

100 ms
C-terminal Domains of NMDA (N-Methyl-D-aspartic Acid) Receptor Modulate Unitary Channel Conductance and Gating
Bruce A. Maki, Teresa K. Aman, Stacy A. Amico-Ruvio, Cassandra L. Kussius and Gabriela K. Popescu

J. Biol. Chem. published online September 4, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.390013

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2012/09/04/M112.390013.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2012/09/04/jbc.M112.390013.full.html#ref-list-1