Kinetic Contributions to Gating by Interactions Unique to N-methyl-D-aspartate (NMDA) Receptors*

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Background: NMDA receptor deactivation is characteristically slow and depends on unique intersubunit contacts in the ligand binding domain.

Results: These additional contacts slow current deactivation mainly by increasing gating.

Conclusion: Slow deactivation reflects higher open probability due to more stable heterodimers.

Significance: A firmer heterodimer interface supports basic functional differences between NMDA and non-NMDA glutamate-gated channels.

Among glutamate-gated channels, NMDA receptors produce currents that subside with unusually slow kinetics, and this feature is essential to the physiology of central excitatory synapses. Relative to the homologous AMPA and kainate receptors, NMDA receptors have additional intersubunit contacts in the ligand binding domain that occur at both conserved and non-conserved sites. We examined GluN1/GluN2A single-channel currents with kinetic analyses and modeling to probe these class-specific intersubunit interactions for their role in glutamate binding and receptor gating. We found that substitutions that eliminate such interactions at non-conserved sites reduced stationary gating, accelerated deactivation, and imparted sensitivity to aniracetam, an AMPA receptor-selective positive modulator. Abolishing unique contacts at conserved sites also reduced stationary gating and accelerated deactivation. These results show that contacts specific to NMDA receptors, which brace the heterodimer interface within the ligand binding domain, stabilize actively gating receptor conformations and result in longer bursts and slower deactivations. They support the view that the strength of the heterodimer interface modulates gating in both NMDA and non-NMDA receptors and that unique interactions at this interface are responsible in part for basic differences between the kinetics of NMDA and non-NMDA currents at glutamatergic synapses.

Within the ionotropic glutamate receptor (iGluR) family, tetrameric channels have similarly layered architectures but distinct kinetics and synaptic functions. All iGluRs have large extracellular domains composed of stacked N-terminal and ligand binding domains (LBD) that connect to a pore-forming transmembrane domain and extend C termini into the cytoplasm (1). After a brief, synaptic-like exposure to saturating glutamate (1 ms, 1 mM), AMPA receptor currents rapidly deactivate ($t_{\text{deact}}$, 1–2 ms) and thus can relay faithfully the presynaptic firing pattern. In contrast, NMDA receptor currents deactivate much slower ($t_{\text{deact}}$, 50–500 ms) and thus can integrate stimuli according to frequency (2). To investigate the structural basis for these notable and physiologically salient kinetic differences, we examined interactions unique to NMDA receptors for their roles in the receptor’s reaction mechanism.

The atomic structures of separated LBD monomers and dimers have been mapped for several iGluR homologues in the presence and absence of agonists, antagonists, and allosteric modulators (3), and more recently, the positions of LBD residues within functional tetrameric assemblies were reported (4–6). These studies confirmed previous x-ray crystallography results identifying that iGluRs are organized as dimers of dimers and that within the LBD layer, each dimer represents a genetically detachable functional unit (6–10). Furthermore, they revealed that the LBD of each subunit consists of two hinged lobes, D1 and D2, which form a narrow agonist binding cleft (3, 8). The dimensions and stability of the cleft depend on the nature of the bound ligand and the geometry and chemistry of the residues that face the cleft; in turn, they determine the affinity and efficacy of a receptor-ligand pair. Within LBD dimers, interactions between D1 and D2 lobes belonging to separate subunits position protomers into a back-to-back arrangement but may have distinct functions in AMPA and NMDA receptors.

In AMPA receptors the strength of the intersubunit D1-D1 interface controls desensitization and allosteric modulators that stabilize the hinge region slow deactivation (11–14). Similarly, the dimer interface of NMDA receptors is stabilized through D1-D1 interactions that occur at two symmetry-related sites, termed Site I and III (15). However, in contrast to AMPA receptors where strengthening these contacts prevents desensitization, in NMDA receptors flexibility at this interface, mediated by hydrophobic residues, appears to be required for receptor gating (16). In addition to these conserved interactions, in NMDA receptors the LBD dimer interface is braced by unique contacts. These occur between the D1 and D2 lobes.
within the conserved sites I (N1 Gln-696) and III (N2A Asn-693 and Asn-697) of the adjacent subunits and at a novel site II (N1 Tyr-535) between residues located at the D1-D2 hinge (15). It has been proposed that these latter interactions control NMDA receptor deactivation kinetics by controlling the stability or geometry of the closed-cleft conformation.

We examined these unique interactions for their role in the NMDA receptor glutamate binding and receptor gating. We found that changing the strength of interactions at site II mainly affected steady-state gating, and aniracetam, an AMPA receptor positive modulator, restored the kinetics of NMDA receptors that had side-chain truncations at this site. Our results argue for a similar role of the hinge region in AMPA and NMDA receptor function and provide evidence that these unique interactions support class-specific gating properties within the ionotropic glutamate receptor family.

**Experimental Procedures**

*Molecular Biology*—Plasmids encoding rat GluN1–1a (N1) (U08261) and rat GluN2A (N2A) (M91562) were gifts from R. Wenthold (National Institutes of Health, Bethesda, MD), and A. Auerbach (University at Buffalo, SUNY, Buffalo, NY), respectively. The coding cDNA within each plasmid was subcloned into pcDNA3.1(+) for expression in mammalian cells. Plasmids were linearized using the QuikChange method (Stratagene, Amsterdam, The Netherlands) and verified by sequencing. Human embryonic kidney 293 cells (ATCC CRL-1573), a gift from A. Auerbach (University at Buffalo, SUNY), were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Becco’s modified Eagle’s medium (Invitrogen) was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and a 5% CO2 atmosphere at 37 °C. Cells between passages 20–40 were grown in 35-mm dishes and used for transfections with 10% fetal bovine serum and 1% penicillin-streptomycin.

**Molecular Biology—**Plasmids encoding rat GluN1–1a (N1) (U08261) and rat GluN2A (N2A) (M91562) were gifts from R. Wenthold (National Institutes of Health, Bethesda, MD), and A. Auerbach (University at Buffalo, SUNY, Buffalo, NY), respectively. The coding cDNA within each plasmid was subcloned into pcDNA3.1(+) for expression in mammalian cells. Plasmids were linearized using the QuikChange method (Stratagene, Amsterdam, The Netherlands) and verified by sequencing. Human embryonic kidney 293 cells (ATCC CRL-1573), a gift from A. Auerbach (University at Buffalo, SUNY), were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and a 5% CO2 atmosphere at 37 °C. Cells between passages 20–40 were grown in 35-mm dishes and used for transfections with the calcium phosphate precipitation method after reaching an ∼30–50% density (17). 1 μg of each NMDA receptor subunit cDNA along with 1 μg of green fluorescent protein cDNA were used for transfection of four 35-mm dishes. After a 2-h incubation period the medium was discarded, and cells were gently washed and transferred into medium supplemented with 2 mM MgCl2. Transfected cells were used 24–48 h after transfection for electrophysiological experiments.

Excised patch recordings were obtained with electrodes pulled from borosilicate glass capillaries polished to a final resistance of 3–8 megaohms when filled with an intracellular solution containing 135 mM CsCl, 22 mM CsOH, 2 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, 11 mM EGTA, and adjusted to pH 7.4 (CsOH). Once the outside-out patch clamp configuration was established, membrane patches were clamped at −70 mV and perfused with extracellular solutions containing 150 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl2, 0.01 mM EDTA, 0.1 mM glycine, and 10 mM HEPBS adjusted to pH 8.0 (NaOH) without (wash) or with 1 mM glutamate (saturating solution). Whole-cell currents were recorded with intracellular solutions containing 150 mM CsCl, 22 mM CsOH, 2 mM MgCl2, 1 mM CaCl2, 11 mM EGTA, and 10 mM HEPES adjusted to pH 7.4 (CsOH) and clamped at −70 mV. Clamped cells were perfused with extracellular solutions containing 150 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl2, 0.01 mM EDTA, 0.1 mM glycine, and 10 mM HEPBS adjusted to pH 8.0 (NaOH) without (wash) or with 1 mM glutamate (saturating solution). Solution exchange was connected to extracellular solution reservoirs with pinch valves digitally controlled with a micro-manifold (VC 6, Warner Instruments, Hamden, CT). Excised patches were positioned in the wash solution near the flow interface created by the simultaneous stream of both the wash and saturating solutions through the theta tube. The theta tube was driven upward via a piezoelectric translator (Burleigh LSS-3100/3200, Thorlabs, Newton, NJ), allowing patches to be moved quickly into and out of the adjacent saturating solution. Solution exchange rates were evaluated at the end of each recording by measuring the open-tip potential and were considered reliable if 10–90% exchange between solutions occurred within 0.2–0.5 ms. Currents were low-pass-filtered at 5 kHz (Axopatch 200B; 4-pole Bessel), sampled at 50 kHz (Digidata, 1440A, Molecular Devices, Sunnyvale, CA), and digitized in Clampex 10.2 (pClamp 10.2 software, Molecular Devices). 10–30 traces were recorded from each patch in response to 1- or 10-ms pulses of saturating glutamate and were analyzed in Clampfit 10.2 (pClamp 10.2 software). The time course of deactivation (τdeact) was calculated by fitting the decay phase of the current from its peak (Ipeak) to baseline with a mono-exponential declining function (Table 1). Current responses were normalized to Ipeak and superimposed in Origin 10.0 (OriginLab Corp., Northampton, MA) for visualization.

Whole-cell currents were recorded with intracellular solutions containing 135 mM CsCl, 22 mM CsOH, 2 mM MgCl2, 1 mM CaCl2, 11 mM EGTA, and 10 mM HEPES adjusted to pH 7.4 (CsOH) and clamped at −70 mV. Clamped cells were perfused with extracellular solutions containing 150 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl2, 0.01 mM EDTA, 0.1 mM glycine, and 10 mM HEPBS adjusted to pH 8.0 (NaOH) without (wash) or with 1 mM glutamate (saturating solution). Solution exchange was controlled through a lightly pressurized pinch valve system (BPS-8, ALA Scientific Instruments Inc., Westbury, NY), and solution exchange protocols were generated in Clampex; these generally consisted of several rounds of a 5-s wash followed by 5-s pulse of glutamate. Currents were low-pass-filtered at 2 kHz, and analog signals were sampled at 5 kHz (Digidata 1440A, Molecular Devices) into digital files using Clampex. Between 3 and 15 current traces were obtained per condition and were analyzed in Clampfit. The macroscopic desensitization time constant (τdes) was calculated by fitting the declining phase of the whole-cell current from its peak (Ipeak) to its steady-state (I∞) value with a mono-exponential declining function; the extent of desensitization was expressed as the I∞/Ipeak ratio.

Single-channel currents were recorded with cell-attached patch clamp, and electrodes were pulled from borosilicate glass capillaries and polished to a final resistance of 12–25 megaohms. Electrodes were filled with extracellular solution containing 150 mM NaCl, 2.5 mM KCl, 10 mM HEPES (pKw 8.3), 1 mM EDTA, 1 mM Glu, 0.1 mM Gly, adjusted to pH 8.0 (NaOH). The extracellular solution contained saturating glutamate concentrations (1–5 μM) as noted. Aniracetam (1-(4-methoxybenzoyl)-2-pyrrrolidinone) (Sigma) was added as noted to extracellular solutions from DMSO stocks (100 mM, final DMSO 1–10%) stored at −80 °C. Recordings were done after applying +100 mV through the recording electrode; currents were low-pass-filtered at 10 kHz, digitally sampled at 40 kHz (PCI-6229, M Series card, National Instruments, Austin, TX) into
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digital files using QuB acquisition software (University at Buf-
falo, SUNY), and stored for off-line processing and analyses.
Processing and analyses were performed on recordings that
tained one active channel, and recordings from patches
with more than one channel, as ascertainment with the method
of Colquhoun and Hawks (18), were discarded. Idealization was
performed with the segmental k-means (SKM) algorithm in
QuB and digitally low-pass-filtered at 12 kHz before analysis
(19). State modeling and kinetic analyses were performed with
the maximum interval log likelihood algorithm in QuB with an
imposed dead time of 0.15 ms (20, 21). The number of distinct
kinetic states required to describe the single-channel behavior
of each individual recording was determined with a log-likeli-
hood threshold method. A minimal two-state model depicting
linked closed (C) and open (O) states was constructed first;
additional C and O states were sequentially added to the CO
model until the log-likelihood increased by <10 units/added
state. This value is derived from previous theoretical work using
Akaike’s formulation of asymptotic information criteria (AIC)
in which the model is rejected if the difference in dimension
values is less than the natural logarithm of the likelihood ratio
(LLR). Using this criterion, a value of 10.55 was experimentally
defined in comparing Markovian and non-Markovian models
(22). Recordings obtained with saturating agonist concentra-
tions were all typically described by models including 5C and
between 2 and 4 O states, as previously reported for wild-type
N1/N2A receptors (23, 24). Several different state arrange-
ments that included linear, cyclic, and branched models were
examined for each condition; the model that consistently pro-
duced the highest overall log-likelihood value across all record-
ings was selected to compare gating across conditions. Several
of the top ranking models were used to simulate macroscopic
responses (described below) and are compared with experi-
mental data for validation.
For each condition, equilibrium open probability (P_o), mean
open time, mean closed time, and the time constants and cor-
responding fractional areas of exponential components were
calculated from best-fitting models. To compare rate constants
across conditions, we used a simplified 5C1O state model
where all the open states were aggregated, so that it was appli-
cable to all files; values were determined for each individual
recording and are reported as the mean ± S.E. except for rate
constants, which are given as rounded means for each condi-
tion. Statistical significance was assessed with a paired two-tail
Student’s t test and deemed significant when p < 0.05.
Microscopic glutamate association (k_{on}) and dissociation
(k_{off}) rate constants were estimated from models fitted to sin-
gle-channel current traces recorded in the presence of sub-sat-
urating concentrations of glutamate (1, 3, and 5 μM). Three to
five recordings for each glutamate concentration (total, n = 12)
were fitted globally in QuB as previously described (25–27).
Global fits were performed by first constructing a basic 5C1O
state model derived from individual recordings using the max-
imum interval log likelihood function; two identical and inde-
pendent binding steps representing the unliganded (C_U) and
mono-liganded (C_A) receptor states were appended to the C_3
state in the model (28), where the k_{on} rates were concentration-
dependent and the k_{off} rates were concentration-independent;
this global modeling approach reports only mean-weighted val-
ues for the files used; K_d was calculated as k_{off}/k_{on} (Fig. 3).
Macroscopic simulations were performed in QuB as previ-
ously described with noted minor adjustments (24, 27). C_U and
C_M states were appended in turn to each C state with either
previously determined rates for N1/N2A (k_{on} = 1.7 × 10^7
m^{-1}s^{-1}, k_{off} = 60 s^{-1}) (25) or experimentally estimated gluta-
matate k_{on} and k_{off} rate constants. Simulation protocols were
created to generate a macroscopic response to either a prolonged
(5-s) or brief (1- or 10-ms) pulse of saturating glutamate. I_{ss}/I_{pk}
and τ_{sw} were calculated for 5-s macroscopic simulations in QuB
and compared with experimentally determined values to evaluate
the validity of the chosen model (16). The deactivation
time constant was calculated for the 1- or 10-ms simulated cur-
rent as for the experimental data using a mono-exponential
declination function. To predict relative I_{ss} values at various gluta-
tamate concentrations for each of the corresponding kinetic
models using either previously reported or experimentally
determined k_{on} and k_{off} rate constants, we used the “dose
response” function in QuB. The EC_{50} values calculated from
simulated dose-response curves were calculated in Origin
using: y = y_0 + A_1e^{-x/s_1} + A_2e^{-x/s_2}.
Molecular docking simulations were performed using Au-
oDock software (29). The N1/N2A LBD heterodimer structure
(PDB code 2A5T) (15) was used as a template for molecular
docking of aniracetam (PubChem Compound Identification
2196). The in silico substitution of N1 Y/S was created using the
“replace sequence” function. Both WT and N1 Y/S of the
N1/N2A dimer structure were first prepared for docking by 1)
removing all H_2O molecules, 2) adding hydrogen atoms to the
entire protein, and 3) adding charges throughout the entire
structure. Binding sites used for docking were determined by
manually selecting residues equivalent to those that define the
aniracetam binding site in GluA2 LBD homodimer: N1 Pro-
532, Tyr-535, Arg-755 and N2A Pro-527, Glu-530, Thr-758
(PDB code 2AL5) (11). Docking parameters were set to 10 poses
per molecule, and ligand poses were evaluated by their corre-
sponding estimated free energy of binding. Docked ligands with
the two highest binding energies were further examined for
residues engaged in binding. Images depicting crystal struc-
tures were generated with PyMOL software (PyMOL Mole-
cular Graphics System, Version 1.5.0.4, Schrödinger, LLC) using
the corresponding PDB file.
Results
Site II Contacts Control NMDA Receptor Current Deac-
tivation—The atomic structure of the N1/N2A receptor LBD
heterodimer revealed a group of intersubunit interactions that
is absent in AMPA receptors (15). These unique interactions
occur along the 2-fold symmetry axis of the dimer at the clam-
shell hinge (Fig. 1A). Such unique Site II interactions occur
between N1 Tyr-535, which is located directly at the D1-D2
hinge region, and two conserved proline residues located on the
D1 lobes of both subunits: N1 Pro-532 and N2 Pro-527 (Fig.
1B). Notably, N1 Tyr-535 plays a critical role in NMDA recep-
tor deactivation, with increased hydrophobic contacts in the
N1 Y535W (N1 Y/S) mutant, dramatically slowing macroscopic
current deactivation and reducing contacts as in the N1 Y535S
(N1<sub>Y/S</sub>) mutant, substantially accelerating deactivation (15). Based on these observations it was proposed that these mutations modulate current deactivation by changing the glutamate dissociation rate, although the exact mechanism is unknown. Consistent with this hypothesis, fast deactivating AMPA and kainate receptors lack this critical tyrosine residue but instead accommodate at this site allosteric modulators that also decrease deactivation (11, 30, 31). Therefore, it was proposed that the tyrosine residue uniquely present in NMDA receptors acts as a “natural” break in deactivation and renders NMDA receptors insensitive to AMPA receptor-specific positive modulators.

As a prelude to testing these hypotheses, we aimed to replicate the previously reported macroscopic behaviors of the N1<sup>Y/W</sup> and N1<sup>Y/S</sup> mutations (15) in the same conditions required by single-channel gating measurements. For this, we recorded macroscopic currents from excised patches exposed briefly (1 ms) to saturating glutamate concentrations (1 mM) in minimal concentrations of protons and divalent blocking cations (pH 8, EDTA). In these conditions as well, relative to wild-type N1/N2A receptors (WT), currents recorded from N1<sup>Y/W</sup>/N2A (N1<sup>Y/W</sup>) deactivated slower, and those from N1<sup>Y/S</sup>/N2A (N1<sup>Y/S</sup>) deactivated faster (Fig. 1 and Table 1), which was similar to responses elicited by 3-ms glutamate pulses (15). Given that the time course of current deactivation after rapid agonist withdrawal is the result of several kinetic pathways, including agonist dissociation and stationary gating (32, 33), we next sought to more precisely determine the mechanism(s) by which site II residues control the shape of the NMDA receptor macroscopic response.

**Site II Residues Set Stationary Gating Kinetics**—We recorded stationary single-channel currents from WT, N1<sup>Y/W</sup>, and N1<sup>Y/S</sup> receptors with saturating agonist concentrations (1 mM Glu, 0.1 mM Gly, pH 8, and 1 mM EDTA). Global single-channel properties of N1<sup>Y/W</sup>, including channel amplitude, open probability (P<sub>o</sub>), mean open time, and mean closed time were not significantly different from WT (Table 2). During steady-state gating, NMDA receptors visit multiple closed and open states (34–36). This behavior can be quantified by statistically separating closed and open dwells into discrete kinetic components. For WT receptors, closed dwells distribute into five distinct components (E<sub>i</sub>–E<sub>o</sub>), whereas the open dwells can distribute into two, three, or four open components depending on which modes of gating are probabilistically represented in a given recording; all records present fast openings (O<sub>f</sub>) and a combination of low (O<sub>l</sub>), medium (O<sub>m</sub>), and high (O<sub>H</sub>) openings indicative of eponymous modes (24, 25, 27, 37, 38). Recordings from N1<sup>Y/W</sup> receptors had similar activity patterns (Fig. 2A), and correspondingly, a detailed inspection of the closed and open dwell distributions revealed only minor differences relative to WT (Fig. 2, B and C).

We organized these kinetic data using an established scheme for NMDA receptor gating that consists of five closed and one aggregated open state (5C1O) (23, 25, 27, 39). This model describes gating as successive reversible transitions between three pre-open states and a collective open state (C<sub>1</sub>⇔C<sub>2</sub>⇔C<sub>3</sub>⇔O), and desensitization as two separate transitions branching off from C<sub>2</sub> and C<sub>3</sub>. With this scheme we found that relative to WT receptors, for N1<sup>Y/W</sup> receptors the C<sub>3</sub>⇔C<sub>1</sub> equilibrium was shifted toward the C<sub>1</sub> state, from 0.3 to 0.5, consistent with more active, slower deactivating kinetics for this mutant (Fig. 2D). These rates also matched well the desensitization time course (τ<sub>des</sub>) and extent (I<sub>inf</sub>/I<sub>inf</sub>) measured from whole-cell currents elicited with long pulse (3 s) of glutamate, thus indicating that the models capture the basic features of the gating mechanism.

One-channel current recordings obtained from N1<sup>Y/S</sup> receptors revealed a substantially altered current pattern (Fig. 2A). This behavior translated into significantly lower P<sub>o</sub> (Table 2), which arose primarily from changes in the closed dwell duration distribution. Similar to WT receptors, N1<sup>Y/S</sup> dwell distributions had five closed and three or four open components. Open distributions were unchanged, indicating wild-type-like open state stabilities and modal-gating kinetics (Fig. 2C). However, the duration and distribution of several closed components were altered dramatically (Fig. 2, B and C). The most notable changes occurred in the E<sub>3</sub> and E<sub>4</sub> time constants (τ<sub>E3</sub> and τ<sub>E4</sub>), which were ~14- and ~11-fold longer compared with the same components in recordings from WT receptors. Using the same 5C1O model to organize these data, we observed sta-


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TABLE 2

Microscopic kinetic properties of D1-D2 mutants

| Receptor          | n  | \(K_p\) | MOT | MCT | \(K_{d1}\) | \(K_{d2}\) | \(K_{d12}\) |
|-------------------|----|---------|-----|-----|----------|----------|----------|
| N1/N2A            | 18 | 0.50 ± 0.03 | 7.1 ± 0.6 | 6.9 ± 0.7 | 3.5 | 3.3 | 0.31 | 13 |
| N1\(^{YW}\)/N2A   | 10 | 0.56 ± 0.04 | 7.9 ± 0.3 | 6.3 ± 1.8 | 4.5 | 2.8 | 1.2 | 14 |
| N1\(^{YW}\)/N2A   | 10 | 0.06 ± 0.01 | 7.6 ± 1.0 | 198 ± 38 | 0.14 | 0.07 | 14 |
| N1\(^{YW}\)/N2A + DMSO | 3 | 0.015 ± 0.004 | 5.2 ± 0.3 | 575 ± 196 | 0.49 | 0.03 | 0.04 | 0.68 | 0.04 | 0.05 |
| N1\(^{YW}\)/N2A + ANI | 4 | 0.18 ± 0.04 | 5.7 ± 1.1 | 46 ± 22 | 3.7 | 0.36 | 10 |
| N1\(^{YW}\)/N2A   | 6  | 0.46 ± 0.05 | 7.4 ± 0.6 | 6.4 ± 0.8 | 3.7 | 0.36 | 10 |
| N1/N2A\(^{NN/AA}\) | 6  | 0.13 ± 0.03* | 4.5 ± 0.6 | 34 ± 6 | 1.6 | 0.09 | 4.2 |
| N1/N2A\(^{NN/AA}\) | 6  | 0.24 ± 0.05* | 8.2 ± 1.2 | 33 ± 9 | 0.7 | 0.12 | 11 |

* \(p < 0.05\) relative to WT (Student’s t test).

\(p < 0.05\) relative to no drug (Student’s t test).

FIGURE 2. N1 Tyr-535 side chain controls receptor gating kinetics. A, representative cell-attached one-channel currents recorded from WT (n = 18), N1\(^{YW}\) (n = 10), or N1\(^{WS}\) (n = 8) receptors (150 mM Na\(^+\), 1 mM EDTA, pH 8). Openings are downward, and expanded traces of the indicated regions (gray lines) illustrate openings to a single conductance level. D, gating mechanisms for fully liganded receptors with rate constants (s\(^{-1}\)) estimated from fits to single channel data; values (in s\(^{-1}\)) are the rounded means for the data set; \(p < 0.05\) relative to WT indicated in bold (Student’s t test); pie charts represent calculated relative occupancies for the states considered in the model. E, representative whole-cell traces from WT and N1\(^{WS}\) in response to a 5-s glutamate application.

There are statistically significant differences in several rate constants (Fig. 2D). Notably, along the activation pathway both the C\(_3\)→C\(_2\) and the C\(_2\)→C\(_1\) equilibria were substantially shifted away from active states, consistent with the faster macroscopic deactivation observed for this mutant (Fig. 1C); this change, which correlates with increased receptor occupancy in states C\(_3\) and C\(_2\), together with changes in desensitization equilibria also predicted faster and deeper macroscopic desensitization for N1\(^{WS}\).

To test this prediction, we recorded whole-cell currents elicited with long (5 s) glutamate applications and found that indeed, N1\(^{WS}\) currents desensitized faster (\(\tau_{\text{ac}}\), 0.38 ± 0.04 s, \(n = 4\); \(\text{versus} \ 1.0 ± 0.1\) s, \(n = 6\) for WT, \(p < 0.05\)) and deeper (\(I_{\text{ac}}/I_{\text{pk}}\), 0.49 ± 0.03 \(\text{versus} \ 0.68 ± 0.04\) for WT, \(p < 0.05\) (Fig. 2E)). Therefore, based on this tested model, we conclude that the faster decay observed for N1\(^{WS}\) receptors originates from higher barriers to opening as compared with WT receptors; these energetic barriers result in substantially larger occupancy of state C\(_3\), from which the agonist can readily dissociate, thus producing currents that desensitize more (Fig. 2D).

Glutamate Dissociates Slower from N1\(^{WS}\)–In contrast to N1\(^{WS}\), for which faster deactivations were fully explained by gating changes, the relatively subtle gating effects we observed for N1\(^{WS}\) receptors did not exclude possible changes in glutamate dissociation as a factor in this mutation’s slower deactivation. Therefore, we set up to measure microscopic glutamate dissociation rate constants for N1\(^{WS}\) receptors. We recorded on-cell one-channel currents in the presence of several sub-saturating concentrations of glutamate (1, 3, and 5 \(\mu M\)) and in the presence of suprasaturating glycine concentrations (0.1 mM) (Fig. 3A). In lower glutamate concentrations, closed intervals in the record were visibly longer, consistent with the interpretation that they reflect dwells in mono-ligated or unliganded conformations. In contrast, open durations remain unchanged, consistent with a mechanism where glutamate dissociates from a closed state (25, 27, 38, 40–42). In previous reports glutamate dissociation rate constants were measured for WT and mutant receptors by fitting globally data obtained at several glutamate concentrations with the expanded version of the 5C10 model used here, which included glutamate binding reactions (Fig. 3B).

We used the same approach and fitted the N1\(^{WS}\) data set obtained in low glutamate concentrations with the extended model. Results show that the rate constants for the core 5C10 reaction were similar with those obtained in high glutamate concentrations (Fig. 2D), whereas the glutamate association and dissociation rate constants, \(1.0 \times 10^7\) \(M^{-1}\)s\(^{-1}\) and 45 s\(^{-1}\) (Fig. 3B), differed ~2-fold from those reported previously for WT receptors: \(1.7 \times 10^7\) \(M^{-1}\)s\(^{-1}\) and 60 s\(^{-1}\) (25, 26). With these
values, the calculated glutamate affinity of N1Y/W was only slightly lower ($K_D = 4.5 \mu M$) relative to WT (3.5 $\mu M$), and the $EC_{50}$ calculated from simulated macroscopic responses was only slightly different, although this latter approach also incorporates gating changes (data not shown). With this global fitting approach we cannot evaluate the statistical significance of these differences; however, these results suggest that slower glutamate dissociation may be an additional contributor to the slower current deactivation measured for this mutant.

To more precisely assign contributions from binding and/or gating changes to the measured slower deactivation of N1Y/W currents, we leveraged the mechanisms in Figs. 2D and 3B and simulated macroscopic responses with (1 ms) glutamate applications (1 mM) using four separate models that combined WT and N1Y/W binding/gating rates as follows: model I, WT binding with WT gating (WT-WT); model II, N1Y/W binding with WT gating (N1Y/W-WT); model III, WT binding with N1Y/W gating (WT-N1Y/W); and finally, model IV, which had N1Y/W binding and gating (Fig. 3C). Results show that whether we only considered gating changes (model III) or if we considered both binding and gating changes (model IV), $\tau_{deact}$ increased relative to WT receptors (model I) to a similar extent. In addition, simulations produced with either model III or model IV overlapped well with the experimentally recorded response (Fig. 3D). Based on these results, we suggest that the slower $\tau_{deact}$ of N1Y/W currents reflects in large part a change in gating, with negligible contributions from slower glutamate dissociation.

Site II Side-chain Truncations Impart Sensitivity to AMPA Receptor Positive Modulators—In AMPA receptors, the equivalent location of site II residues defines the binding pocket for small allosteric modulators, which potentiate currents by slowing macroscopic deactivation (11, 30, 31, 43–46). The “floor” of this binding pocket is lined by serine residues absent in NMDA receptors (Fig. 4A). Superimposing the N1/N2A LBD with that of aniracetam-bound GluA2 LBD dimer (cyan), as in panel A, superimposed with N1/N2A LBD dimer (green/blue) with conserved prolines and N1 Tyr-535 side chains depicted (PDB code 2AL5) (11). C, atomic model of N1Y/S/N2A LBD dimer with docked aniracetam in two high energy poses (orange and yellow). D, whole-cell currents elicited with Glu (1 mM) from WT, N1Y/S, or N1Y/W with three doses of aniracetam (Ani, 1, 5, and 10 mM) dose dependence of potentiation relative to DMSO calculated EC$_{50}$ values are 0.92 ± 0.05 mM for N1Y/S and 0.65 ± 0.07 mM for N1Y/W. D, N1Y/W currents recorded from excised-patches (1 ms Glu, 10 ms) in the absence (black) and presence of aniracetam (red, 5 mM); inset, traces normalized to peak amplitudes, and magnified open tip potential. F, single-channel N1Y/S currents from cell-attached patches in the presence of DMSO (top, 5%) and aniracetam (5 mM, bottom) and the associated open and closed interval distributions for DMSO (black) and aniracetam (red).
by defining search parameters between the conserved proline residues and the equivalent floor residues of the aniracetam-bound GluA2 structure (N1-Pro-532, Y535S, Arg-755, and N2A-Pro-527, Glu-530, Thr-758) (Fig. 1B). In these simulations the Y535S substitution produced a cavity large enough to accommodate aniracetam even though the docked positions revealed different orientations than observed in the bound GluA2 structure (11). Two poses of the highest binding energies predicted an upward orientation for the aromatic ring of the 1–4-methoxybenzoyl group toward the hydrophobic proline residues (Fig. 4C).

To determine whether site II truncated receptors are sensitive to aniracetam modulation, we recorded macroscopic current responses from WT, N1 Y/S, and N1 Y/A receptors first in the absence and then in the presence of aniracetam. Aniracetam has poor water solubility and is added from a DMSO stock. In our experiments final concentrations of 1, 5, and 10 mM aniracetam also contained 1, 5, and 10% DMSO, respectively. Given that NMDA receptor currents are inhibited by DMSO (48) we also tested the mutants for sensitivity to DMSO. As with WT, DMSO by itself inhibited N1 Y/S and N1 Y/A currents. However, when aniracetam was present, currents recorded from either N1 Y/S or N1 Y/A, but not WT, were potentiated by 1 and 5 mM aniracetam (Fig. 4D). At the highest concentrations of aniracetam (10 mM) tested, which also contained 10% DMSO, we were unable to detect aniracetam-mediated potentiation likely because DMSO-dependent inhibition overwhelmed the potentiating effects of aniracetam. To estimate the aniracetam-mediated effect, we normalized traces recorded with aniracetam to those obtained with DMSO alone. This analysis illustrates a dose-dependent potentiation of N1 Y/S and N1 Y/A currents; the aniracetam EC50 was similar for the two mutants, but the drug was more effective on N1 Y/S currents (Fig. 4D).

If this effect is mediated specifically by aniracetam binding to the pocket created by the Y535S mutation, then aniracetam should prolong the macroscopic current deactivation and should shorten closed duration(s) in microscopic responses. We tested these two corollaries by recording macroscopic N1 Y/S currents in excised patches and microscopic currents in cell-attached patches. We found that indeed relative to 5% DMSO, 5 mM aniracetam slowed τdeact ~1.6-fold (Fig. 4E), and this change was mediated exclusively by a decrease in closed durations (Fig. 4F, Table 2). Overall these results indicate that aniracetam potentiated N1 Y/S currents by reversing deficits produced by the Y535S substitution. This result argues for the functional conservation of site II residues between AMPA and NMDA receptors and an overall conserved gating mechanism for these two iGluRs.

Site III D1-D2 Contacts Control Steady-state Gating and Deactivation—Next, we took a similar approach to probe the roles of NMDA receptor-specific contacts within the conserved sites I and III. At these symmetry-related locations, a D1 backbone carbonyl forms polar contacts with D2 residues in the adjacent subunit (Fig. 5A). To abolish these interactions we produced N1 Q696A (N1 Q/A) and N2A N693A N697A (N2A NNN/AA) subunits.

Currents from one-channel cell-attached patches showed relatively normal activity for N1 Q/A/N2A receptors and lower steady-state gating levels for N1/N2A NNN/AA and N1 Q/A/N2A NNN/AA receptors (Fig. 5A). Kinetic analyses indicated that indeed these two latter mutants had lower open probabilities, and this was largely because their closed durations were longer (Fig. 5B, Table 2). State models derived from fits to these one-channel data predicted a minimally changed mechanism for N1 Q/A/N2A receptors but more pervasive changes for N1/N2A NNN/AA and N1 Q/A/N2A NNN/AA receptors (Fig. 5C). Overall, the mutations reduced rate constants for forward transitions and in some instances also accelerated rate constants for backward transitions. These gating models, appended with glutamate binding equilibria for which we assumed wild-type rate constants, matched well the desensitization time course, τD, however, the N1/N2A NNN/AA macroscopic deactivation time course was not as fast as measured in excised patches. This result suggests that aside from gating deficits the N1/N2A NNN/AA mutant may also have substantially faster glutamate dissociation kinetics (Fig. 5D, Table 1).

Discussion

At most central excitatory synapses the excitatory postsynaptic potential represents the summation of AMPA and NMDA receptor-mediated currents. The rise and fall of the excitatory post-synaptic current is controlled by the faster rising AMPA receptors and slower deactivating NMDA receptors, respectively (49, 50). The structural basis of this essential functional difference between the two synaptic receptors is unclear. The first atomic structure of the N1/N2A LBD dimer was identified as a series of intersubunit interactions in NMDA receptors that are absent in AMPA receptors; these may contribute to the kinetic differences between the two receptor classes (15). In support of this hypothesis, contacts mediated by one of these unique residues, N1 Tyr-535, were identified as critical for maintaining the characteristically slow NMDA receptor deactivation (15). The mechanism by which class-specific intersubunit contacts, including those mediated by N1 Tyr-535, contribute to NMDA receptor kinetics is unknown. Generally, the rise and deactivation time constant of macroscopic currents represent the combined expression of a receptor’s reaction mechanism, which for ligand-gated channels consists of agonist binding and channel gating (51). In this study the goal was to assign changes in macroscopic kinetics to specific changes in binding and/or gating.

To this end we used electrophysiology and statistical modeling of one-channel currents to estimate microscopic binding and gating rate constants for NMDA receptors carrying substitutions at positions responsible for class-specific intersubunit interactions. Our results show for the first time that stronger intersubunit interactions within the NMDA receptor LBD, afforded by unique contacts, promote NMDA receptor opening by reducing energy barriers to activation and/or by stabilizing pre-open states at the expense of resting states, with no effect on open state stabilities. These results support the current view that these supplementary interactions endow NMDA receptor macroscopic currents with slower deactivation.

For the N1 Y/W mutant, which has a stronger hydrophobic interface between N1 and N2A subunits, we determined slight
changes in both glutamate dissociation and channel gating that were each consistent with slower deactivation; however, simulations with the kinetic model deduced from one-channel data suggested that the slower deactivation reflected primarily the change in microscopic gating. Overall, the mutation caused receptors to populate pre-open states at the expense of resting states, and this increased occupancy appeared to be independent of agonist presence. For example, although the glutamate $K_d$ increased slightly, from $3.5 \mu M$ for WT to $4.5 \mu M$ for N1/Y/W, this reflected slower glutamate association, from $17 \mu M^{-1}s^{-1}$ to $10 \mu M^{-1}s^{-1}$, and also slower dissociation, from $60 s^{-1}$ to $45 s^{-1}$ (Fig. 3); these kinetic changes are consistent with a LBD structure that is more closed even in the absence of glutamate. Similarly, the gating transition, which consisted of a slower $C_2 \rightarrow C_1$ transition, from $670 s^{-1}$ to $810 s^{-1}$, shifted the equilibrium constant for this transition, from 0.3 to 0.5, in effect further draining the occupancy of resting states in favor of pre-open states. Notably, these kinetic changes were relatively small and were not reflected in equilibrium parameters such as open probability, mean open and closed durations, or the predicted macroscopic EC$_{50}$ (Table 2).

In contrast, side-chain truncations that eliminated NMDA receptor-specific intersubunit interactions, whether N1/Y/S or N2A/N/NAA, caused receptors to gate with substantially lower $P_o$, 0.06 and 0.13, respectively, versus 0.5 for WT, and as for the N1/Y/W mutant, open durations were not affected for either N1/Y/S or N2A/N/NAA. The kinetic model deduced from one-channel data suggested that the additional interactions afforded by N1 Tyr-535, N2A Asn-693, and N2A Asn-697 reduced the energetic barriers that resting receptors must traverse to access pre-open states. In these models, for both N1/Y/S and N2A/N/NAA, the $C_3 \leftrightarrow C_2$ and $C_2 \leftrightarrow C_1$ equilibria were shifted substantially toward resting states, from 3.3 to 0.14 and 1.6 and from 0.31 to 0.07 and 0.09, respectively, thus resulting in a substantial drainage of open state occupancies (Table 2). In addition, these mutants may have increased glutamate dissociation rates. For example, although the gating we measured for N2A/N/NAA matched well the macroscopic desensitization kinetics, which should not be affected by changes in glutamate affinity, it accounted only partially for the measured change in macroscopic deactivation kinetics, thus implying that an increase in glutamate dissociation predominates in this receptor’s deactivation kinetics. This observation is consistent with a scenario where the current deactivates faster when these particular intersubunit interactions are absent because glutamate-
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bound LBDs require more energy to close, and glutamate has many more chances to dissociate.

Our novel observations that N1 Y/S and N1 Y/A mutants are sensitive to aniracetam, an AMPA receptor modulator, demonstrates that exactly the side chain of N1 Tyr-535 prevents aniracetam binding to NMDA receptors; in addition, our results that aniracetam reverses the specific gating deficits induced by Y535S and that its effects on macroscopic N1 Y/S currents were similar to those reported for AMPA currents indicate a conserved sequence of events in NMDA and AMPA receptor activation reaction and provide mechanistic evidence for the essential role played by the side chain of Tyr-535 in biologically relevant NMDA receptor behaviors.

Author Contributions—W. F. B. and K. A. C. recorded whole-cell currents and most single-channel traces and analyzed all data. W. F. B. performed molecular docking simulations. K. A. C. recorded and analyzed fast responses from excised patches. L. K. T. contributed single-channel data. W. F. B., K. A. C., and G. K. P. designed the experiments, interpreted the results, and wrote the manuscript.

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