Subtype-dependent \(N\)-Methyl-\(D\)-aspartate Receptor Amino-terminal Domain Conformations and Modulation by Spermine*

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**Background:** Amino-terminal domains (ATDs) of NMDA receptors dictate open probability and bind the modulator spermine.

**Results:** GluN2A ATD is more open than GluN2B; GluN1 ATD is more open in the presence of GluN2A, and spermine binding opens the ATDs of GluN1 and GluN2B.

**Conclusion:** The ATD conformations correlate to the open probability.

**Significance:** ATD conformations depend on the subunit composition and change upon modulator binding.

The \(N\)-methyl-\(D\)-aspartate (NMDA) subtype of the ionotropic glutamate receptors is the primary mediator of calcium-permeable excitatory neurotransmission in the central nervous system. Subunit composition and binding of allosteric modulators to the amino-terminal domain determine the open probability of the channel. By using luminescence resonance energy transfer with functional receptors expressed in CHO cells, we show that the cleft of the amino-terminal domain of the GluN2B subunit, which has a lower channel open probability, is on average more closed than the GluN2A subunit, which has a higher open probability. Furthermore, the GluN1 amino-terminal domain adopts a more open conformation when coassembled with GluN2A than with GluN2B. Binding of spermine, an allosteric potentiator, opens the amino-terminal domain cleft of both the GluN2B subunit and the adjacent GluN1 subunit. These studies provide direct structural evidence that the inherent conformations of the amino-terminal domains vary based on the subunit and match the reported open probabilities for the receptor.

Glutamate, the primary excitatory neurotransmitter in the mammalian central nervous system, activates the three subtypes of ionotropic glutamate receptors: AMPA receptors, kainate receptors, and NMDA receptors. NMDA receptors mediate the slower component of excitatory synaptic signaling that underlies learning and memory formation. NMDA receptors are obligate heterotetramers typically composed of GluN1 and GluN2 subunits, and they require both glutamate and glycine binding to activate the cation-selective pore. The GluN1 subunit can be one of eight splice variants, and the GluN2 subunit can be one of four subtypes, A to D. The splice variant and subtype composition of the receptor dictate gating kinetics and lend specificity to modulator binding. Each subunit of the receptor is organized into domains with an extracellular amino-terminal domain (ATD) and ligand-binding domain (LBD), the transmembrane, pore-forming region, and the intracellular carboxyl-terminal domain. The ATDs of glutamate receptors are known to be involved in receptor assembly and in controlling the open probability of the receptor (1–3). Specifically, receptors composed of GluN1-GluN2A subunits exhibit a higher channel open probability than those composed of GluN1-GluN2B subunits (4, 5). This inherent difference in the open probabilities arises to a large extent, from the ATDs, as switching the ATD of GluN2A with that of GluN2B results in a swap in the channel open probabilities albeit not completely (2). Additionally, the ATD of NMDA receptors can bind to allosteric modulators that can either inhibit or potentiate receptor activation (6–10). Both endogenous and exogenous modulators have been identified, and much interest has been focused on understanding the mechanisms of allosteric modulation of the receptor because these modulators provide a potential avenue for subtype-specific drug development.

The endogenous modulator spermine has several effects on NMDA receptor function depending on its interaction site. Spermine is a polycationic compound with its individual amine groups carrying a positive charge at physiological pH (11). Spermine is best known as an endogenous pore blocker of cation-selective channels (12). However, spermine can also be released into the synapse in an activity-dependent fashion (12) where it may modify NMDA receptor synaptic transmission (13–17). Extracellular spermine is thought to bind to the ATDs of GluN1-GluN2B NMDA receptors (1) and potentiates agonist-induced currents (13, 18). Spermine binding to the extracellular portion of the NMDA receptor also results in an increase in apparent glycine affinity (19). Based on mutational studies, spermine is thought to bind to acidic residues on the lower lobes of both GluN1 and GluN2B ATDs (blue residues in

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2 The abbreviations used are: ATD, amino-terminal domain; LBD, ligand-binding domain; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Interestingly, GluN1 subunits that include exon 5, a 21-residue stretch in the ATD lower lobe containing six basic residues, are no longer sensitive to spermine modulation. This suggests the positive charges introduced by exon 5 function similarly to spermine and obviate the spermine-binding site (18, 21).

The structural mechanism of allosteric modulation of NMDA receptors has been thought to proceed by influencing the cleft of the bi-lobed ATD. Propping open the ATD of either the GluN1 or the GluN2 subunit increases the channel open probability (2, 22). Because spermine acts as potentiator, this mechanism suggests that spermine stabilizes an open conformation of the GluN2B ATD (9). Consistent with this, the extent of spermine potentiation is much less when the GluN2B ATD is already braced open by a thiol-reactive MTS (methanethiosulfonate) reagent (9). These studies provide indirect evidence that spermine potentiates the receptor by opening the GluN2B cleft; however, at present there is no direct study showing and quantifying the conformational changes in the ATDs upon spermine binding. To directly investigate the role of the ATD cleft in dictating open probabilities, we have used luminescence resonance energy transfer (LRET) to characterize the cleft distance in GluN2B and compare it to the distance between the same sites in GluN2A (23). In addition, we measured the openness of the GluN1 ATD when assembled with GluN2A or GluN2B. Finally, LRET directly revealed and quantified spermine-induced conformational changes in both the GluN1 and GluN2B ATDs. It is important to note that these measurements are made in a near physiological state, i.e. with receptors that have minimal changes relative to the wild-type proteins and studied in CHO cells without being purified or isolated using lipids and/or detergents.

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis—The GluN1-1a splice variant, GluN2A, and GluN2B of the rat NMDA receptors in the pcDNA 3.1 plasmid were generously provided by Shigetada Nakanishi. Mutations were introduced using traditional PCR methods, and the mutation and integrity of the plasmid were verified using DNA sequencing. The numbering system used includes the signal peptide. All LRET experiments were done in Cys-free backgrounds, where non-disulfide-bonded cysteines were mutared to serine. In GluN1, Cys-22 and Cys-459 were mutated to serine, and in GluN2B, Cys-232, Cys-399, and Cys-495 were mutated to serine. The Cys-free GluN2A subunit was made by introducing the mutations C231S, C395S, and C461S as described previously (23). To measure the distance between the upper and lower lobes of the GluN2A ATD, the Cys-free GluN1 was coexpressed with GluN2B with a His tag and the thrombin sequence inserted at residue 30 (H30 in Fig. 1B). The acceptor fluorophore used was Ni(NTA)3Cy3. The donor fluorophore for all LRET measurements was Alexa655. To measure between the upper lobes of the GluN1 and GluN2B ATDs, the GluN1 construct that was Cys-free except for Cys-22 was coexpressed with the Cys-free GluN2B with a His tag and the thrombin sequence inserted at residue 30 (H30 in Fig. 1B). The acceptor fluorophore used was Ni(NTA)3Cy3. The donor fluorophore for all LRET measurements was Alexa655. To measure between the upper upper lobe of GluN1 and GluN2B, the GluN1 construct that was Cys-free except for Cys-22, which was maintained as a cysteine, was coexpressed with GluN2B that was Cys-free except for Cys-232, which was also maintained as a cysteine. The acceptor fluorophore used was Alexa555. Analogous measurements in GluN2A involved coexpressing the Cys-free GluN1 with Cys-22 with GluN2A that was Cys-free except for the inherent Cys-231. The acceptor fluorophores for those measurements was Alexa555. To measure between the upper upper lobe of GluN1 and GluN2B ATDs, the GluN1 construct that was Cys-free except for Cys-22 was coexpressed with the Cys-free GluN2B with a His tag and the thrombin sequence inserted at residue 30 (H30 in Fig. 1B). The acceptor fluorophore used was Ni(NTA)3Cy3. The donor fluorophore for all LRET measurements was a thiol-reactive terbium chelate (Invitrogen). Measurement of the sensitized acceptor lifetime before and after thrombin cleavage allows for the direct quantification of background fluorescence (23–26, 36) and isolation of the specific signal from the NMDA receptor labeled at the given sites. A pictorial depiction of the constructs and associated mutations is shown in Fig. 1.

Reagents and Chemicals—D,L-APV and DCKA were purchased from Abcam. Spermine and Alexa555 were purchased from Sigma. Thiol- reactive terbium chelate was purchased from Invitrogen. Ni(NTA)3Cy3 was prepared as described previously (23, 37) with bis-reactive Cy3 from GE Healthcare.

Transfection and Tissue Culture—CHO-K1 cells (ATCC) were maintained in Ham’s F-12 media (Invitrogen) and passed every 2 days. Cells were transfected when 50–80% confluent with Lipofectamine 2000 (Invitrogen). Four 10-cm dishes were transfected per LRET experiment. Each dish was washed twice with extracellular buffer, before being suspended in extracellular buffer and used for spectroscopic experiments. Cells were probed in a cuvette-based spectrofluorimeter using a QuantaMaster model QM3-SS with Fluorescan software (Photon Technology International). Data were analyzed with Origin 8.6 software (OriginLab Corp.). All samples were excited at 337 nm. Emission was detected at 565 nm for Ni(NTA)3Cy3. LRET samples were labeled with 200 nM each of donor and acceptor fluorophores. After labeling, cells were washed twice with extracellular buffer, before being suspended in extracellular buffer and used for spectroscopic experiments. Cells were probed in a cuvette-based spectrofluorimeter using a QuantaMaster model QM3-SS with Fluorescan software (Photon Technology International). Data were analyzed with Origin 8.6 software (OriginLab Corp.). All samples were excited at 337 nm. Emission was detected at 545 nm for donor-only measurements, two 10-cm dishes were transfected.

LRET Experiments—Cells for spectroscopic experiments were scraped up and isolated via centrifugation. Cells were washed in extracellular buffer containing (in mM) the following: 150 NaCl, 2.8 KCl, 1 CaCl2, 5 HEPES, pH 7.3 (HCl) and labeled in extracellular buffer for 1 h at room temperature. When GluN2A was coexpressed with GluN1, 10 mM Tricine was included in the buffer for apo measurements to chelate contaminating zinc (39). Donor-only samples were labeled with 200 mM terbium chelate. LRET samples were labeled with 200 mM each of the donor and acceptor fluorophores. After labeling, cells were washed twice with extracellular buffer, before being suspended in extracellular buffer and used for spectroscopic experiments. Cells were probed in a cuvette-based spectrofluorimeter using a QuantaMaster model QM3-SS with Fluorescan software (Photon Technology International). Data were analyzed with Origin 8.6 software (OriginLab Corp.). All samples were excited at 337 nm. Emission was detected at 545 nm for donor-only samples. The emission was detected at 565 nm for Alexa555 or 572 nm for Ni(NTA)3Cy3. The distance between the two fluorophores was calculated using Förster Equation 1,

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R = R_D \left( \frac{\tau_D}{\tau_D - \tau_{DA}} \right)^{1/6}
\]
NMDA Receptor Amino-terminal Domain Conformations

where $R$ is the distance between the fluorophores; $R_0$ is the distance at which energy transfer is half-maximal; $\tau_D$ is the lifetime of the donor fluorophore in the absence of the acceptor fluorophore; and $\tau_{DA}$ is the lifetime of the sensitized acceptor emission. LRET was measured before and after protease cleavage to allow for a direct quantification of the specific signal from the NMDA receptor and subtraction of the background fluorescence as described previously (23, 25, 26, 36). Cleavage with thrombin eliminates the LRET from the NMDA receptor by releasing a peptide fragment containing one of the fluorophores, so any resulting fluorescence after cleavage is due to labeling of cysteines on the surface of the CHO cells. Five units of high activity bovine thrombin (Calbiochem) were added to cleave the receptors at the introduced thrombin recognition sequence as described above. $n$ values given in Table 1 represent the number of biological repeats. Each sample ($n$) was scanned a minimum of three times, and each scan was an average of 99 sweeps. The LRET lifetimes have been normalized so that an easy comparison can be made between them. The error in the LRET lifetime (Table 1) is the S.E., and the error in the distances was calculated by propagating the errors in the lifetime using the Error Propagation Calculator developed by Thomas Huber in the Physics Department of Gustavus Adolphus College.

Electrophysiology—CHO cells were transfected using Lipofectamine 2000 with wild-type or mutant GluN1, GluN2B, and enhanced GFP at a microgram ratio of 1.5:4.5:1, respectively, expected based on the crystal structures (27, 28), where there is no cross-talk across the subunits for these sites. Importantly, the same positions and fluorophores were also used to measure across the GluN2A ATD cleft (23). The LRET lifetime measured between these sites in GluN2A was 312 ± 5 µs (Fig. 2A), corresponding to a distance of 48.8 ± 0.4 Å between the two fluorophores (Table 1). The single lifetime suggests that the measurements correspond to distances within the subunit, as expected based on the crystal structures (27, 28), where there is no cross-talk across the subunits for these sites. Importantly, the same positions and fluorophores were also used to measure across the GluN2A ATD cleft (23). The LRET lifetime measured between these sites in GluN2A was 312 ± 5 µs (Fig. 2A), corresponding to a distance of 48.8 ± 0.4 Å between the two fluorophores (Table 1). The single lifetime suggests that the measurements correspond to distances within the subunit, as expected based on the crystal structures (27, 28), where there is no cross-talk across the subunits for these sites.

RESULTS

Conformation of the GluN2 ATD, a Comparison between GluN2A and GluN2B—To measure the conformations of GluN2A and GluN2B ATDs in intact functional receptors, we combined specific labeling sites and enzymatic cleavage positions to isolate specific signals from within an ATD (Fig. 1B) (23–26). First, the apo or resting conformation of the GluN2B ATD was probed by measuring between His-30 and Cys-232 in GluN2B (Fig. 1B and 2A). The LRET lifetime was determined by measuring the lifetime of sensitized acceptor emission upon donor excitation. The LRET lifetime in the apo-state after background fluorescence subtraction could be well represented by a single exponential with a lifetime of 252 ± 12 µs (Fig. 2A), which corresponds to a distance of 48.8 ± 0.4 Å between the two fluorophores (Table 1). The single lifetime suggests that the measurements correspond to distances within the subunit, as expected based on the crystal structures (27, 28), where there is no cross-talk across the subunits for these sites. Importantly, the same positions and fluorophores were also used to measure across the GluN2A ATD cleft (23). The LRET lifetime measured between these sites in GluN2A was 312 ± 5 µs (Fig. 2A), corresponding to a distance of 51.2 ± 0.1 Å (23). These measurements show that the GluN2B cleft is inherently in a more closed conformation than the GluN2A cleft in the apo-receptor.

GluN1 ATD Adopts Different Conformations with Different GluN2 Subunits—Having identified intrinsic differences in GluN2A compared with GluN2B, we performed the same experiment with GluN1. LRET measurements were made between Cys-22 and S224C in GluN1 either when coexpressed

FIGURE 1. ATD structure with suggested spermine-binding site and mutation sites for LRET experiments. A, crystal structure of the GluN1-GluN2B ATD dimer structure is shown highlighting the suggested spermine-binding residues in blue (PDB code 4PE5) (9, 10). B, schematics of individual subunits are shown indicating the locations of mutations and the resulting amino acid sequence. The Cys-free constructs involved the mutation to serine of Cys-22 and Cys-459, unless Cys-22 was retained to be labeled, and Cys-232, Cys-399, and Cys-495 in GluN2B, unless Cys-232 was retained to be labeled.
with GluN2A or GluN2B. The sensitized emission of the acceptor again yielded a single exponential decay regardless of the coexpressed GluN2 subunit. The measurement of the GluN1 ATD cleft in the presence of GluN2A yielded an LRET lifetime of 324 ± 13 μs (Fig. 2B), a distance of 51.2 ± 0.3 Å. Repeating this experiment revealed, surprisingly, that the distance across the GluN1 cleft is decreased when placed in a GluN1/GluN2B heteromer. Specifically, the LRET lifetime of the apo-GluN1 ATD is 275 ± 4 μs, a distance of 49.2 ± 0.1 Å (Fig. 2B and Table 1). Importantly, these measurements were made on the same instrument using identical GluN1 plasmids, fluorophores, and solutions. There is clearly a difference in the conformation adopted by the GluN1 subunit that is dependent on the GluN2 subunit completing the receptor.

Spermine-induced Conformational Changes—To determine the effect of spermine, the LRET lifetimes were measured across the GluN2B cleft (again between His-30 and Cys-232, Fig. 1B) before and after the addition of the GluN2B-specific potentiator spermine. The LRET lifetime of the equilibrium state increases from 252 ± 12 to 369 ± 34 μs (Fig. 3A), corresponding to a distance of 52.8 ± 0.8 Å, an increase of 4 Å (Table 1). The increase in the distance upon addition of spermine is consistent with an opening of the cleft of the GluN2B ATD. Importantly, this construct was potentiated by spermine to the same extent as the wild-type receptor (9, 19), with spermine increasing equilibrium responses by 66 ± 10% (n = 3) and 41 ± 5% (n = 5), respectively (Fig. 4). Therefore spermine binding stabilizes an open conformation of the GluN2B ATD. Because spermine likely binds at the lower lobe interface between the GluN1 and GluN2B ATDs (Fig. 1) (9, 10), we reasoned that it may also alter the conformation of the GluN1 ATD.

The GluN1 cleft can regulate channel function as its deletion or cleavage affects allosteric inhibition and potentiation (9, 29), and propping open the GluN1 ATD cleft with a thiol-reactive reagent potentiates the receptor (22). However, aside from potentially contributing amino acids to the spermine-binding site (9, 10), the structural influence of the GluN1 ATD in spermine modulation is unexamined. The GluN1 ATD can be passive, acting as a scaffold to support the GluN2 ATD and allow efficient propagation of GluN2 conformational changes to the LBDs and the pore. Alternatively, the GluN1 ATD can be directly impacted by and undergo conformational changes upon modulator binding, particularly a modulator-like spermine that likely binds at the interface between the GluN1 and GluN2B ATDs. To determine the role of the GluN1 ATD in spermine potentiation, we measured conformational changes in the GluN1 cleft by introducing two cysteines, one each in the upper and lower lobes of the ATD (Fig. 3). These cysteines, the inherent one at position 22 and the second introduced by the S224C mutation in the GluN1 ATD, allow for direct monitoring across the cleft of the bi-lobed ATD. This GluN1 construct was coexpressed with a Cys-free GluN2B. The LRET lifetime of the GluN1 cleft for the apo-receptor could be well represented by a single exponential decay corresponding to 275 ± 4 μs (Figs. 2C and 3B), giving a distance of 49.2 ± 0.1 Å (Table 1). Again, based on the crystal structure, this distance is consistent with the measurement being within a single ATD and not across the subunits. Additionally, this construct was normally potentiated with equilibrium responses increasing by 79 ± 7% (n = 9) in the presence of spermine (Fig. 4). Addition of spermine led to an increase in the LRET lifetime to 340 ± 29 μs (Fig. 3), corresponding to a distance of 51.5 ± 0.7 Å (Table 1). These data suggest that spermine binding stabilizes the GluN1 ATD in an open conformation.

Measurements between Upper Lobes of ATDs—The spermine-induced conformational change of the GluN1 ATD does not preclude its possible role as a scaffold supporting the GluN2B ATD. Indeed, we would expect that interactions between the subunits are critical to the mechanism of allosteric modulation; if the ATDs were completely free to move independent of each other, how could such conformational rearrangements propagate downward to impact channel gating? The ATDs require some anchor point to efficiently conduct conformational changes. In the case of zinc inhibition, the upper lobes of the ATDs do not undergo any motions upon binding of zinc or of the agonists glutamate and glycine (23). These data suggest that the upper lobes may provide such a
rigid scaffold, allowing spermine-induced, or other modulator, motions to influence conformational equilibria of the LBD and the pore. To test this and probe the upper lobes of the ATDs during spermine potentiation, measurements were next made between His-30 in GluN2B and Cys-22 in GluN1 (Fig. 1B) and labeling the receptor with terbium chelate and Ni(NTA)$_2$Cy3. These constructs allowed for the specific introduction of the donor fluorophore at the upper lobe (N terminus) of the GluN1 ATD and the acceptor fluorophore at the upper lobe (N terminus) of the GluN2B ATD (Fig. 1). Spermine potentiation was unaltered, and spermine increased the equilibrium response by 76 ± 8% (n = 7) (Fig. 4). The LRET lifetime for the apo-receptor was well represented by a single lifetime of 362 ± 26 µs (Fig. 5), corresponding to a distance of 52.1 ± 0.6 Å (Table 1). The binding of spermine to the receptor resulted in no change in the distance, as the lifetime was 311 ± 43 µs (Fig. 5), giving a distance of 50.7 ± 1.2 Å (Table 1). Therefore, the upper lobes of the ATDs are not moving relative to each other when spermine binds. However, spermine opens up both the GluN1 and GluN2B ATDs (Fig. 3). Comparing the measurements of the equilibrium states in the presence and absence of spermine suggests that spermine binding causes the movement of the lower lobes of the ATDs.

**Lower Lobes of ATDs Are Segments Moving Because of Spermine Binding**—To evaluate the movement of just the lower lobe of the GluN2B ATD, we measured the LRET between the lower lobe of the GluN2B ATD (Cys-232) and the upper lobe of the GluN1 ATD (Cys-22). Spermine potentiation was preserved in this construct, with a 65 ± 8% (n = 9) increase in equilibrium response (Fig. 4). The sensitized acceptor lifetime of the apo-receptor was 228 ± 16 µs (Fig. 6), a distance of 47.6 ± 0.6 Å (Table 1). The lifetime decreased to 161 ± 14 µs (Fig. 6) when the receptor was bound by spermine, corresponding to a distance of 44.6 ± 0.6 Å (Table 1). These data suggest that although spermine binding stabilizes an open GluN2B ATD cleft, the lower lobe of the ATD additionally rotates bringing

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### TABLE 1

Lifetimes and distances from LRET measurements

The n value is shown in parentheses after the ligated state for each construct. Listed next to the constructs is the acceptor fluorophore used. The donor fluorophore for all measurements was terbium chelate. The error in the LRET lifetime is the S.E., and the error in the distances was calculated by propagating the errors in the lifetimes. The cartoons represent the sites between which measurements were made. Orange ATDs are of the GluN2B subunit, and teal ATDs are from the GluN1 subunit.

| Ligated State (n) | Donor Only Lifetime (µs) | Donor-Acceptor Lifetime (µs) | Distance (Å) |
|-------------------|--------------------------|-----------------------------|--------------|
| **GluN2B Cleft — Between H30 and C232 in GluN2B** |
| Apo (3)           | 1649 ± 1                 | 252 ± 12                    | 48.8 ± 0.4   |
| Spermine (4)      | 1659 ± 1                 | 369 ± 34                    | 52.8 ± 0.8   |
| **GluN1 Cleft — Between C22 and S224C in GluN1, with GluN2B** |
| Apo (2)           | 1740 ± 1                 | 275 ± 4                     | 49.2 ± 0.1   |
| Spermine (2)      | 1714 ± 1                 | 340 ± 29                    | 51.5 ± 0.7   |
| **GluN1 Cleft — Between C22 and S224C in GluN1, with GluN2A** |
| Apo (2)           | 1685 ± 1                 | 324 ± 13                    | 51.2 ± 0.3   |
| Zinc (2)          | 1744 ± 1                 | 336 ± 12                    | 51.2 ± 0.3   |
| **GluN1-GluN2B Upper Lobes — Between C22 in GluN1 and H30 in GluN2B** |
| Apo (5)           | 1724 ± 1                 | 362 ± 26                    | 52.1 ± 0.6   |
| Spermine (2)      | 1693 ± 1                 | 311 ± 43                    | 50.7 ± 1.2   |
| **GluN1 Upper Lobe-GluN2B Lower Lobe — Between C22 in GluN1 and C232 in GluN2B** |
| Apo (3)           | 1706 ± 1                 | 228 ± 16                    | 47.6 ± 0.6   |
| Spermine (3)      | 1682 ± 1                 | 161 ± 14                    | 44.6 ± 0.6   |
Cys-232 closer to the upper lobe of the GluN1 ATD. Therefore, spermine causes the lower lobe of GluN2B to be rotated inward, toward the upper lobe of the GluN1 ATD. Studies attempting to identify the binding site of spermine implicated several acidic residues at the lower lobes of both GluN1 and GluN2B subunits, which alter spermine affinity. Residues including Asp-170, Glu-181, Glu-185, and Glu-186 in GluN1 and Glu-191, Glu-198, Glu-200, and Glu-201 in GluN2B were identified (residues highlighted in blue in Fig. 1) (9, 10). Based on the crystal structures of the full-length NMDA receptor (27, 28), these residues are too far apart to bind spermine. In further support of the closer proximity of these domains at the lower lobes is that cross-linkers less than 8 Å in length can be used to cross-link the lower lobes of the GluN1-GluN2B ATDs (9). The crystal structures were obtained in the presence of the allosteric inhibitors ifenprodil in one case and Ro25-6981 in the other (27, 28). These structures thus would correspond to an inhibited state of the receptor. Consequently, spermine binding to these residues should be expected to move the lower lobes of the ATDs closer.

DISCUSSION

This work provides insight into the conformational changes occurring upon spermine binding to the ATDs of the NMDA receptor. The LRET measurements were made in whole receptors expressed in mammalian cells, and the protein sequence, and consequently structure, was minimally perturbed by the mutations necessary for the experiments. Functional characterization of the constructs used for the spectroscopy confirms that these receptors behave like the wild-type receptor. We can conclude that the upper lobes of the ATDs are stable and do not undergo any large conformational changes on average when spermine binds (Fig. 5). However, spermine binding does cause an opening of the ATDs of both the GluN1 and GluN2B ATDs (Fig. 3). The measured distances of the GluN1 and GluN2B ATDs match the distances in the ATDs in the full-length structure (27, 28). The lack of movement of the upper lobes of the ATDs suggests that the movements taking place are due to movement of the lower lobes of the ATDs. When looking at the crystal structures of the full-length NMDA receptor (27, 28), the residues are too far apart in that, presumably inhibited, state to bind spermine. Distances measured between spermine-binding residues highlighted in Fig. 1 range from 18 to 23 Å in the crystal structure, but thiol reactive reagents less than 8 Å in length are able to cross-link the lower lobes of the GluN1-GluN2B ATDs (9). For binding to occur, these residues must be brought closer, requiring a rotation of the lower lobes of the ATDs to bridge the distance. We infer this rotation from the combined opening of the GluN1 and GluN2B ATDs and the movement of the lower lobe of the GluN2B ATD toward the upper lobe of the GluN1 ATD. Moreover, these findings are consistent with cross-linking studies that suggested that mobility of the lower lobes of the ATDs are critical to allosteric modulation (31). Overall, the data suggest that spermine potentiates the NMDA receptor by stabilizing open conformations of both the GluN1 and GluN2B ATDs.

In the absence of crystal structures of the GluN2A ATD, the conformational state of GluN2A ATD is unclear. Here, we find that the distance between identical labels between the upper

FIGURE 3. Spermine stabilizes an open cleft of both GluN1 and GluN2B ATDs. A, LRET measurements across the GluN2B clefts are shown. The measurements were made between His-30 and Cys-232 in GluN2B. B, LRET measurements between the upper and lower lobe of the GluN1 ATD. Measurements were made between Cys-22 and S224C in GluN1. C, donor-only measurements from the GluN2B subunit. D, donor-only measurements from the GluN1 subunit. In all panels, the black curve is from the apo-receptor, and the blue curve is from the spermine-bound receptor.
and lower lobes of the GluN2B ATD is 48.8 Å (Fig. 2 and Table 1) but 51.2 Å in the apo-GluN2A ATD (23). Therefore, our LRET measurements show that the GluN2B ATD is on average more closed than the GluN2A ATD. Interestingly, this correlates with the open probability ($P_{\text{open}}$) of the receptor (2, 3). The GluN2A subunit has a more open ATD conformation and a higher $P_{\text{open}}$ (0.61 ± 0.05 (32)), whereas the GluN2B subtype has a more closed ATD conformation and a lower $P_{\text{open}}$ (0.20 ± 0.03 (33)). These findings are also in good agreement with previous work, which used MTS reagents to support the open GluN2 ATD, resulting in a greater degree of potentiation in GluN1/GluN2B than GluN1/GluN2A receptors (2). However, if you try to draw a correlation between receptor $P_{\text{open}}$ and the conformation of the GluN2 ATD, the correlation is incomplete. Although ATD domain swapping can convert GluN2A and GluN2B chimeras into lower or higher $P_{\text{open}}$ subunits, respectively, the switch is not complete. This indicates there are other factors involved in determining $P_{\text{open}}$ (2). Although it is possible these factors lie outside of the ATDs, our data suggest that the conformation of the GluN1 ATD can also influence open probability of the receptor, and part of the reason GluN2A-containing receptors have a higher open probability than GluN2B-containing receptors is because the GluN1 ATD adopts a different conformation when coexpressed with these different GluN2 subunits. The difference in GluN1 conformation, although likely a result of its partner subunit, could be a factor in dictating $P_{\text{open}}$, because altering the GluN1 ATD conformation in an assembled receptor alters the $P_{\text{open}}$ (9). Our data suggest that the factors governing $P_{\text{open}}$ are complex and can be influenced by different factors. Furthermore, the difference in the GluN1 cleft with either GluN2A or GluN2B provides structural evidence that the interface between the GluN1/GluN2 ATDs varies. A difference in the interface helps explain why subtype-specific modulators exist that bind at the interface between the ATDs, despite the almost 60% sequence identity between the GluN2A and GluN2B ATDs.

Previously, we found that zinc inhibition of GluN2A receptors occurs by stabilizing a closed conformation of the GluN2A ATD, inducing an ~4 Å decrease between sites in the upper and lower lobes (23). However, zinc binding does not influence the overall conformation of the GluN1 ATD as does spermine binding (Fig. 7 and Table 1). Interestingly, the conformation of
the zinc-bound GluN2A ATD (i.e. low $P_{\text{open}}$) is similar to the apo-GluN2B ATD (48.8 Å and low $P_{\text{open}}$). Furthermore, the state of the spermine-bound GluN2B ATD (52.4 Å and high $P_{\text{open}}$) is comparable with that of the apo-GluN2A ATD (51.4 Å and high $P_{\text{open}}$). Taken together, such correlations explain how zinc and spermine inhibit and potentiate the receptor, respectively, by influencing the average conformation of the ATD. An inhibitor closes the ATD cleft, whereas a potentiator opens the GluN2 ATD cleft (Fig. 8). If zinc binding causes a cleft closure in the GluN2B ATD as expected, then the inherent difference in the cleft conformation of GluN2A and GluN2B explains why zinc completely inhibits GluN1-GluN2B receptors but only partially inhibits GluN1-GluN2A receptors (34, 35). Our results also suggest why spermine is not able to potentiate GluN2A-containing receptors as the resting GluN2A ATD is in an open conformation. Future experiments should be targeted at understanding how these conformational changes at the ATDs are influencing the LBD layer and ultimately the pore of the receptor.

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