Contributions of the M1 Transmembrane Helix and Pre-M1 Region to Positive Allosteric Modulation and Gating of N-Methyl-D-Aspartate Receptors

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptors are glutamate-gated ion channels whose function is critical for normal excitatory synaptic transmission in the brain and whose dysfunction has been implicated in several neurologic conditions. NMDA receptor function is subject to extensive allosteric regulation by endogenous compounds and by exogenous small molecules. Elucidating the structural determinants and mechanism of action by which allosteric regulators control gating will enhance our understanding of NMDA receptor activation and facilitate the development of novel therapeutics. Here, we investigated the structural determinants for (3-chlorophenyl)(6,7-dimethoxy-1-((4-methoxyphenoxy)methyl)-3,4-dihydroisoquinolin-2(1H)-yl)methaneone (CIQ), a GluN2C/D-selective positive allosteric modulator. We show that CIQ does not bind to the amino-terminal domain of the NMDA receptor and does not share structural determinants with modulators acting at the agonist-binding domain dimer interface or ion channel pore. Rather, we identified critical determinants of CIQ modulation in the region near the first transmembrane helix of GluN2D, including in a putative pre-M1 cuff helix that may influence channel gating. We also show that mutations within the GluN2D pre-M1 region alter open probability of the NMDA receptor. These results suggest a novel site of action for potentiation of NMDA receptors by small molecules and implicate the pre-M1 region in NMDA receptor gating.

Introduction

N-methyl-D-aspartate (NMDA) receptors are ligand-gated ion channels that mediate excitatory synaptic transmission in the central nervous system (Traynelis et al., 2010). These non-selective cation channels are tetrameric complexes comprising GluN1, GluN2, and GluN3 subunits with typical NMDA receptors containing two GluN1 subunits and two GluN2 subunits (Ulbrich and Isacoff, 2008). The GluN1 subunit binds the coagonist glycine or D-serine and the GluN2 subunit binds the coagonist glutamate. There are four GluN2 subunits, GluN2A, 2B, 2C, and 2D, each of which is encoded by a separate gene (Hollmann and Heinemann, 1994). NMDA receptor composed of different GluN2 subunits exhibit markedly different biophysical and pharmacological properties (Vicini et al., 1998), which enables NMDA receptor subtypes to play distinct roles in brain physiology and development (Cull-Candy and Leszkiewicz, 2004).

NMDA receptor subunits are composed of three semi-autonomous domains: an amino-terminal domain (ATD), an agonist-binding domain (ABD), and a transmembrane domain. In addition, they contain a large intracellular region consisting of 100–600 amino acids. The transmembrane domain consists of three transmembrane helices—M1, M3, and M4—and a re-entrant pore loop, called M2. NMDA receptors have several allosteric sites, including the side-to-side GluN1/GluN2 dimer interface of the ATD (Karacas et al., 2011; Mony et al., 2011), the back-to-back GluN1/GluN2 dimer interface of the ATD (Karakas et al., 2011; Mony et al., 2011), and the ion channel pore (Antonov and Johnson, 1996; Kashiwagi et al., 2011), the back-to-back GluN1/GluN2 dimer interface of the ATD (Karakas et al., 2011; Mony et al., 2011), and the ion channel pore (Antonov and Johnson, 1996; Kashiwagi et al., 2011; Blanpied et al., 2005).

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ABBREVIATIONS: ABD, agonist-binding domain; AMPA, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATD, amino-terminal domain; BAPTA, 1,2-bis(2-aminoethoxy)ethane-N,N,N,N’-tetraacetic acid; CIQ, (3-chlorophenyl)(6,7-dimethoxy-1-((4-methoxyphenoxy)methyl)-3,4-dihydroisoquinolin-2(1H)-yl)methaneone; CP-465022, 3-(2-chloro-phenyl)-2-[2-(6-diethylaminomethyl-pyridin-2-yl)-vinyl]-6-fluoro-3H-quinazolin-4-one; DOP-1105, 5-(4-Bromophenyl)-3-(1,2-dihydro-6-methyl-2-oxo-4-phenyl-3-quinolinyl)-4,5-dihydro-1H-pyrazole-1-carboxylic acid; GYKI-53655, 1-(4-Aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydroisoquinolin-2(1H)-yl)methaneone; MTS, methanethiosulfonate; NMDA, N-methyl-D-aspartate; OGN46, (E)-4-(6-methoxy-2-(3-nitrostyryl)-4-oxoquinazolin-3(4H)-yl)-benzoic acid; TCN-201, 3-chloro-4-fluoro-N-[(2-phenylcarbonyl)hydratino]carbonyl[phenyl]methyl]-benzenesulfonamide; TCN-213, N-(cyclohexylmethyl)-2-[[5-[phenylmethyl]amino]-1,3,4-thiadiazol-2-y]thio)acetamide.

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NMDA receptors are potentiated by several endogenous molecules, including arachidonic acid (Miller et al., 1992), dynorphin A (Zhang et al., 1997), sulfated neurosteroids (Wu et al., 1991), and polynucleotides (Ransom and Stec, 1988; McGurk et al., 1990; Reynolds, 1990; Williams et al., 1990). In addition, aminoglycosides potentiate NMDA receptors in a manner similar to potentiation by polynucleotides (Masuko et al., 1999). These compounds show varying subunit-selectivity and structural determinants of action. Additionally, the first class of positive allosteric modulators selective for GluN2C- and GluN2D-containing NMDA receptors was recently reported (Mullasseril et al., 2010). This class of potentiators doubles the current response to maximally effective concentrations of agonist for NMDA receptors containing GluN2C or GluN2D. These modulators are not agonists and do not affect the potency of either glutamate or glycine. Moreover, two regions of the GluN2 subunit were previously found to be critical for the selectivity of this class of potentiators: a 16-amino acid stretch linking the ATD with the ABD and a point mutation in the M1 transmembrane helix.

To gain a better understanding of allosteric potentiation of NMDA receptors, which could lead to therapeutics with novel selectivity and mechanisms of action, we sought to determine which regions of the receptor might contribute to the binding site and thereby control the actions of tetrahydroisoquinoline allosteric potentiators typified by CIQ.

Materials and Methods

DNA Constructs and Mutagenesis. cDNAs for rat GluN1-1a (GenBank accession numbers U11418 and U08261; hereafter GluN1) and rat GluN2D (L31611) were used. The GluN2D amino-terminal domain deletion construct (2DΔATD) was generated by removing approximately 400 bp from the 5’ untranslated region of a previously described GluN2D ATD deletion construct (Yuan et al., 2009). The GluN1 ATD deletion construct was generated using a modified QuikChange reaction as described (Makarova et al., 2000). Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s protocol. All mutations were verified by dideoxy DNA sequencing (Eurofins MWG Operon, Huntsville, AL). The amino acids are numbered according to the full-length protein, including the signal peptide, with the initiating methionine as 1. For expression in Xenopus laevis oocytes, cDNA constructs were linearized by restriction enzymes and used as a template to produce cRNAs using the mMessage mMachine kit (Ambion (Life Technologies), Grand Island, NY) according to the manufacturer’s protocol.

Two-Electrode Voltage-Clamp Recordings. Defolliculated stage VI Xenopus laevis oocytes (EcoCyte Bioscience, Austin, TX) were injected with cRNA encoding GluN1 and GluN2D. After cRNA injection, the oocytes were stored at 15–20°C in culture media containing (in mM): 88 NaCl, 2.4 NaHCO3, 1 KCl, 0.33 Ca(NO3)2, 0.41 CaCl2, 0.82 MgSO4, 5 Tris-HCl (pH 7.4 with NaOH), 1 U/ml penicillin, 0.1 mg/ml gentamicin sulfate, and 1 μg/ml streptomycin. Two-electrode voltage-clamp recordings were performed 2–7 days postinjection at room temperature (23°C). The extracellular oocyte recording solution contained (in mM) 90 NaCl, 1 KCl, 10 HEPES, 0.5 BaCl2, and 0.01 EDTA (pH 7.4 with NaOH). Solutions were applied by gravity and solution exchange was controlled by custom software operating an 8-port modular valve positioner (Hamilton Company, Reno, NV). Voltage and current electrodes were filled with 3.0 M KCl, and currents were recorded at a holding potential of −40 mV. Voltage control was accomplished with a two-electrode voltage-clamp amplifier (OC-725; Warner Instruments, Hamden, CT).

For (3-chlorophenyl)6,7-dimethoxy-1-((4-methoxyphenoxy)methyl)-3,4-dihydroisoquinolin-2(1H)-yl)methanone (CIQ) concentration-response curves, the recording solutions also included 5 mM 2-(hydroxypropyl)-β-cyclodextrin, which was found to improve the solubility of CIQ, and receptors were activated by 100 μM glutamate and 30 μM glycine. For glutamate concentration-response curves, 30 μM glycine was present in all solutions. For glycine concentration-response curves, 100 μM glutamate was present in all solutions.

Whole-Cell and Outside-Out Patch-Clamp Electrophysiology. Human embryonic kidney 293 cells (CR1 1573, sex unknown; American Type Culture Collection, Rockville, MD) were seeded on glass coverslips coated with poly(γ-lysine) (0.1 mg/ml) approximately 48 h before the experiments. The culture medium was Dulbecco’s modified Eagle’s medium with Glutamax-I (catalog #10569; Gibco (Life Technologies), Grand Island, NY) supplemented with 10% dialyzed fetal bovine serum (catalog #26400; Gibco). The electrodes were filled with internal solution containing (in mM) 110 D-gluconic acid, 110 CsCl, 2 HEPES, 2 NaATP, 2 MgCl2, 5 D,L-2-amino-5-phosphonovalerate and 200 μM 7-chlorokynurenic acid were added to the culture medium. The cells were used for experiments approximately 18–24 h after transfection.

Whole-cell voltage-clamp recordings were performed at ~60 mV using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) at room temperature (23°C). Recording electrodes (3–4 MΩ) were made from thin-wall glass micropipettes (World Precision Instruments, Sarasota, FL) using a Flaming-Brown puller (Model P-1000; Sutter Instrument Company, Novato, CA). The electrodes were filled with internal solution containing (in mM) 110 Na-glucoside, 110 CsOH, 30 CaCl2, 5 HEPES, 4 NaCl, 0.5 CaCl2, 2 MgCl2, 5 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA), 2 NaATP, and 0.3 NaGTP (pH 7.35 with CsOH). The extracellular recording solution was composed of (in mM) 150 NaCl, 10 HEPES, 3 KCl, 0.5 CaCl2, and 0.01 EDTA (pH 7.4 with NaOH). Currents were low-pass filtered at 8 kHz (<3 dB, 8-pole Bessel filter; Frequency Devices, Ottawa, IL) and digitized at 20 kHz using Clampex (Molecular Devices). Outside-out channel recordings were performed at ~80 mV using recording electrodes (5–9 MΩ) made from thick-wall borosilicate glass (Warner Instruments). Internal and extracellular solutions were the same as above. Currents were filtered at 60 kHz as above and digitized at 40 kHz.

Data Analysis. CIQ concentration-response curves were individually fit to the Hill equation using a variable slope:

\[ \text{Response (\%) = Max + (100 - Max) \times \left(1 + \left(\frac{x}{EC_{50}}\right)^{-nH}\right)} \]

where Max is the maximum CIQ current as a percentage of the glutamate/glycine current, EC50 is the half-maximally effective concentration of CIQ, x is the experimental concentration of CIQ, and nH is the Hill slope. Glutamate and glycine concentration-response curves from individual oocytes were also fit by the Hill equation:

\[ \text{Current = Max + (Min - Max) \times \left(1 + \left(\frac{x}{EC_{50}}\right)^{-nH}\right)} \]

where Max is the maximum current elicited by agonist, Min is the current elicited by the lowest concentration of agonist and except for 2D(Y578A) was not significantly different from baseline current, x is the experimental concentration of agonist, and nH is the Hill slope. Glutamate and glycine curves from individual oocytes were then normalized to the fitted minimum and maximum current and averaged for display.

The time constants describing the onset of 5H-dibenzo[a,d]cyclohepten-5,10-imine (dizocilpine maleate; MK-801) inhibition (τMK-801 block) were measured by fitting a single exponential function to the current decay from 90 to 10% of the peak current after MK-801 application.
where $I$ is the current at time $t$, $\tau$ is the time constant of MK-801 inhibition, $I_{\text{peak}}$ is the peak agonist-evoked current, and $C$ is an offset. Relative open probabilities ($P_{\text{open}}$) were calculated as $1/\tau_{\text{MK-801}}$ block and normalized to the average value for GluN1/GluN2D (Blanke and VanDongen, 2008; Gielen et al., 2009; Vance et al., 2011).

For the alanine scanning mutagenesis, statistical significance was assessed using a one-way analysis of variance with Dunnett’s post hoc test versus GluN1/GluN2D with $a$ set to 0.05. For statistical testing of $EC_{50}$ values, a one-way analysis of variance with Dunnett’s post hoc test was calculated using the logarithm of the $EC_{50}$ values with $a$ set to 0.05.

**Reagents.** CIQ was purchased from Life Chemicals (catalog number F0635-0139; Burlington, ON, Canada). (+)-MK-801 was purchased from Tocris Bioscience (Bristol, UK). Ketamine was a gift from Dr. Stephen Holtzman (Emory University). Glutamate and glycine were purchased from Sigma-Aldrich (St. Louis, MO). 2-(hydroxypropyl)-β-cyclodextrin was purchased from Acros Organics (West Chester, PA). All other chemicals were purchased from Fisher Scientific.

**Results**

**CIQ Does Not Act at Known Modulatory Sites.** Previous work identified two distinct regions of GluN1/GluN2D that were critical for potentiation by CIQ and analogs: the ATD-ABD linker and the first transmembrane helix, M1 (Mullasseril et al., 2010). These regions are seemingly independent of each other based on homology to the GluN2 tetrameric crystal structure, with the ATD-ABD linker being about 65–70 Å extracellular to the M1 helix (Sobolevsky et al., 2009). Thus, it is unlikely that positive allosteric modulators could directly interact with both regions simultaneously.

Moreover, it is unclear whether these regions might be allosterically coupled, as is the case for the ATD and the ABD in which ATD ligands such as $Zn^{2+}$ and ifenprodil allosterically regulate glutamate binding (Kew et al., 1996; Paolletti et al., 1997; Zheng et al., 2001; Erreger and Traynelis, 2005). Therefore, we sought to reconcile the contribution of these apparently discrete regions to potentiation by CIQ. To do so, we systematically explored the importance of both well-established and emerging regulatory sites on the NMDA receptor (Fig. 1) for positive allosteric modulation of GluN1/GluN2D receptors. These sites, shown in Fig. 1, are the interface between the GluN1 and GluN2 ATDs (site I), the ion channel pore (site II), the lower lobe of the ABD (site III), and the agonist-binding domain dimer interface (site IV). These four modulatory sites are critical for the actions of GluN2B-selective antagonists (e.g., ifenprodil) and positive modulators (e.g., spermine), NMDA receptor channel blockers (e.g., ketamine), GluN2C/2D-selective antagonists (e.g., (E)-4-(6-methoxy-2-(3-nitrostyryl)-4-oxoazol-3(4H)-yl)-benzoic acid (QNZ46)), and GluN2A-selective antagonists [e.g., 3-chloro-4-fluoro-N-[(4-(2-phenylcarbonyl)hydrazinocarbonyl)phenyl] methyl]benzenesulfonylamide (TCN-201), respectively.

To evaluate the importance of site I in positive allosteric modulation of GluN1/GluN2D receptors, we recorded CIQ potentiation of receptors lacking the GluN2D ABD and the ATD-ABD linker (GluN2DΔATD) and found that CIQ potentiation was unaffected when then ATD was deleted from GluN2D (Fig. 2B). These results suggest that CIQ does not bind solely to the ATD of GluN2D. However, the GluN2B-selective antagonist ifenprodil was recently shown to bind NMDA receptors at the interface of the GluN1/GluN2 ATD dimer (Karakas et al., 2011), raising the possibility that residues from the GluN1 ATD could contribute to the CIQ binding site. Contrary to this hypothesis, removal of the ATD and ATD-ABD linker from both GluN1 and GluN2D (GluN1ΔATD/GluN2DΔATD) did not reduce potentiation by CIQ (Fig. 2C). These data eliminate the possibility that CIQ potentiates GluN2B and GluN2D-containing NMDA receptors by binding to the ATD (site I).

We next assessed whether CIQ acts at the channel blocker site (site II; Fig. 3A) on GluN2C- and GluN2D-containing receptors by measuring concentration-response curves for two structurally distinct channel blockers, $Mg^{2+}$ (Fig. 3B) and ketamine (Fig. 3C), at a holding potential of −80 mV in the absence or presence of 10 μM CIQ. We predicted that if CIQ binds to or in some way perturbs the channel blocker site, then the potency of channel blockers, the extent of inhibition, or both would be altered in the presence of CIQ. Yet, CIQ affected neither the degree of inhibition nor the potency for both $Mg^{2+}$ and ketamine at GluN1/GluN2D (Table 1). These data suggest that CIQ does not compete for binding with channel blockers or dramatically alter the nature of the conduction pathway. These data are consistent with no effect of CIQ on the stability of the open channel, that is, mean open time (Mullasseril et al., 2010), which is a key determinant of channel block.

The ABD harbors several ligand binding sites; thus, we explored the potential interaction of CIQ with each of these sites. Agonists bind in the cleft of the clambshaped ABD; however, CIQ is unlikely to bind within the agonist-binding pockets for two reasons. First, CIQ neither activates nor inhibits the receptor, in contrast to other molecules binding at
these sites, which are either agonists or competitive antagonists. Second, CIQ does not detectably alter glutamate or glycine potency (Mullasseril et al., 2010), suggesting that the nature of the agonist-binding pockets is unchanged by CIQ. In addition to the agonist-binding sites, the ABD contains two regulatory sites that are critical for the actions of subunit-selective inhibitors. One site is located at the membrane-proximal lower lobe of the ABD (site III in Fig. 1) and is important for noncompetitive inhibition by quinazoline-4-ones (Hansen and Traynelis, 2011) and dihydroquinoline-pyrazolines (Acker et al., 2011). We tested whether this region may also be critical for positive allosteric modulation of GluN1/GluN2D by measuring CIQ potentiation of GluN2D receptors containing point mutations that markedly affected inhibition by quinazoline-4-ones (Hansen and Traynelis, 2011), and dihydroquinoline-pyrazolines (Acker et al., 2011). CIQ potentiation, however, was unaffected by mutations in this region that alter inhibition by both quinazoline-4-ones and dihydroquinoline-pyrazolines (Fig. 3D), suggesting that the lower portion of the ABD clamshell proximal to the membrane helices of the receptor does not contribute to the molecular determinants for positive allosteric modulation of GluN1/GluN2D receptors.

A second modulatory site for subunit-selective inhibitors resides at the dimer interface between GluN1 and GluN2 ABDs (site IV in Fig. 1) and is critical for glycine-dependent inhibition by TCN-201 and N-(cyclohexylmethyl)-2-((5-[[phenylmethyl]amino]-1,3,4-thiadiazol-2-yl)thio)acetamide (TCN-213) (Hansen et al., 2012; McKay et al., 2012). Given the role of the ABD dimer interface in mediating allosteric coupling between the ATD and the ion channel gate (Gielen et al., 2008, 2009), and the importance of the ABD dimer interface of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors for the activity of positive modulators such as cyclothiazide (Sun et al., 2002), we asked whether the ABD dimer interface of NMDA receptors might contribute to potentiation by CIQ. Two residues in GluN1 contributing to the dimer interface, Phe754 and Arg755, were critical for inhibition of GluN1/GluN2A receptors by TCN-201 (Hansen et al., 2012), and mutation of these residues to alanine altered the binding of TCN-201. By contrast, CIQ potentiation was

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**Fig. 2.** GluN1/GluN2D (A), GluN1/GluN2D ΔATD (B), or GluN1ΔATD/GluN2D ΔATD (C) receptors were expressed in Xenopus laevis oocytes and current responses recorded using a two-electrode voltage-clamp. Currents were activated by 100 μM glutamate and 30 μM glycine and CIQ (1, 3, 10, 30, and 100 μM) was subsequently coapplied with agonists. (D) CIQ potentiated GluN1/GluN2D, GluN1/GluN2D ΔATD (2D ΔATD), and GluN1ΔATD/GluN2D ΔATD (N1 ΔATD/2D ΔATD) with EC50 values of 8.9, 8.4, and 7.0 μM, respectively. Currents were normalized to the glutamate- and glycine-elicited currents. Data are shown as mean ± S.E.M. and are from 3–32 oocytes.

**Fig. 3.** (A) Modulatory sites II, III, and IV are depicted on a homology model of GluN1/GluN2D (Acker et al., 2011). The GluN2 subunits are highlighted in yellow and the predicted location of the plasma membrane is represented by gray lines with the orientation indicated. For clarity, the ATD is not shown. (B and C) Interaction of CIQ with site II was assessed by measuring the potencies of two channel blockers, Mg2+ and ketamine, in the absence (control) or presence of 10 μM CIQ. Concentration-response curves were evaluated from current responses of GluN1/GluN2D receptors expressed in oocytes. Currents were normalized to a percentage of the initial glutamate (100 μM)- and glycine (30 μM)-activated currents in the absence of inhibitor. Data are depicted as mean ± S.E.M. and are from 6 oocytes for each condition. (D) CIQ positive modulation was assessed at GluN1/GluN2D receptors containing point mutations at site III that critically affected inhibition of GluN2D by QNZ46. (E) CIQ potentiation was not affected by point mutations or chimeric NMDA receptors that significantly affected inhibition by TCN-201 at site IV in GluN1/GluN2A receptors.
not altered at GluN1(F754A)/GluN2D and GluN1(R755A)/GluN2D receptors (Fig. 3E). The S2 segment of the GluN2 ABD also contributes residues to the ABD dimer interface and introducing this region of GluN2A into GluN2D conferred inhibition by TCN-201 to GluN2D (Hansen et al., 2012). Yet, CIQ potentiation was not diminished in GluN2D chimeric receptors containing the entire ABD from GluN2A [i.e., 2D(2A S1S2)] or just the S2 segment, as in the chimera 2D(2A S2) (Fig. 3E). These data suggest that positive allosteric modulation of GluN2C- or GluN2D-containing receptors is not mediated through the ABD dimer interface. These results are consistent with the molecular determinants for CIQ residing outside the ATD and being more membrane-proximal than the ABD dimer interface.

**Residues in the M1 Helix Affect CIQ Potentiation.** Previous studies identified a single amino acid residue in the M1 transmembrane helix of GluN2D, Thr592, that when mutated to the corresponding GluN2A residue (isoleucine) eliminated potentiation by CIQ (Mullasseril et al., 2010). To further explore the importance of the M1 helix in mediating potentiation by CIQ, we used alanine scanning mutagenesis of this region. A sequence alignment of the GluN1 and GluN2 NMDA receptor subunits together with the GluA2 AMPA receptor (Fig. 4A) was used as a guide to individually mutate 23 residues in GluN2D to alanine (or cysteine if the wild-type residue was alanine). These residues correspond to the residues comprising the M1 transmembrane helix in the GluA2 membrane-spanning crystal structure (Sobolevsky et al., 2009) and are shown in a homology model of GluN1/GluN2D in Fig. 4B. We assessed the effects of 10 μM CIQ on these mutants and found six residues in GluN2D that when mutated to alanine reduced potentiation by CIQ: Val582, Trp583, Phe587, Val588, Leu591, and Thr592 (Fig. 4C). In addition, we found two residues that increased the potentiation by CIQ: Val584 and Met586 (Fig. 4C). CIQ concentration-response curves (Fig. 5, A and B) revealed varying effects of these mutations on CIQ potency and efficacy. CIQ potency, but not efficacy, was reduced at 2D(L591A) and 2D(T592A). By contrast, the efficacy of CIQ at 2D(V582A) and 2D(V588A) was significantly attenuated. CIQ caused no detectable potentiation at 2D(F587A). CIQ efficacy increased at 2D(V584A) and 2D(M586A) with little to no decrease in CIQ potency (Table 2). Currents elicited from 2D(W583A), however, were small (<50 nA) and showed linear rundown with agonist application, preventing reliable estimation of CIQ potency, consistent with previous studies (Kashiwagi et al., 2002; Thomas et al., 2006). In addition, mutation of this tryptophan to phenylalanine, 2D(W583F), did not affect CIQ modulation [10 μM CIQ response (% glu/gly) was

### Table 1: CIQ does not interact with site II modulators

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<thead>
<tr>
<th>Modulator</th>
<th>IC50 Control</th>
<th>IC50 CIQ</th>
<th>Minimum Response</th>
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<tr>
<td>Mg2+</td>
<td>80 ± 10 μM</td>
<td>66 ± 6 μM</td>
<td>1.8 ± 0.8% Glutamate</td>
</tr>
<tr>
<td>Ketamine</td>
<td>1.58 ± 0.08 μM</td>
<td>1.41 ± 0.12 μM</td>
<td>5.4 ± 0.8% Glutamate</td>
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Fig. 4. (A) A sequence alignment of NMDA receptor subunits with GluA2 (Sobolevsky et al., 2009) is indicated as a cylinder above the alignment. (B) The position of the GluN2D M1 helix is highlighted in a side-on view of the transmembrane region of a homology model of GluN1/GluN2D (Acker et al., 2011). (C) GluN2D subunits with point mutations in the M1 transmembrane helix were coexpressed with GluN1 in *Xenopus laevis* oocytes and current responses recorded using two-electrode voltage clamp. Currents were first activated by 100 μM glutamate and 30 μM glycine and then 10 μM CIQ was coapplied with glutamate and glycine. *Statistically significant change from wild-type GluN1/GluN2D (P < 0.05, one-way analysis of variance with Dunnett’s post-test) and those mutants are highlighted in gray. Responses were normalized to the glutamate- and glycine-induced current. Data are from 4–52 oocytes.
affected glutamate or glycine potency. Although agonist-reduced at 2D(M586A) (Table 2). No other M1 mutations at 2D(V582A) (Table 2). By contrast, glutamate potency was glutamate and glycine potencies were significantly increased function, we recorded glutamate and glycine concentration-
residue is a critical structural element for the M1 helix and not
interpret the change in glutamate potency at 2D(V582A) and 2D(M586A) to reflect a change in the efficacy of glutamate (i.e., the ability of glutamate binding to cause the ion channel pore to open) and suggest that these mutations alter gating of the receptor.

We next asked whether the residues in the M1 helix that when mutated affect CIQ potentiation, but not glutamate
potency, clustered in three-dimensional space. We plotted the residues comprising the M1 helix as spheres on a generic protein α helix, having 3.6 amino acids per turn and a 5.4 Å translation per turn, and highlighted the residues affecting CIQ potentiation (Fig. 5D). Strikingly, the residues seemed to reside on only one side of the helix, suggesting that these residues could all potentially interact directly with CIQ. It is also noteworthy that the equivalent residues in the GluA2 tetrameric crystal structure line “gaps” between the transmembrane domains that were hypothesized to be occupied by amino acid residues from transmembrane AMPA receptor regulatory proteins (Sobolevsky et al., 2009).

If CIQ interacts with residues in the M1 transmembrane helix, there may be other residues in this region of the receptor on either GluN1 or GluN2 that mediate potentiation by CIQ. To explore this idea, we used a homology model of GluN1/GluN2D (Acker et al., 2011) to identify residues located within 5 Å of the GluN2 M1 helix. The residues we identified resided in the M4 transmembrane helix of GluN1 and a short stretch of amino acids immediately extracellular to the GluN2 M1 helix. We mutated these residues and assessed potentiation by 10 µM CIQ. Only two residues in the M4 helix of GluN1 affected CIQ potentiation: GluN1(M813A) and GluN1(F817A) (Fig. 6). Although these residues do not cluster in three-dimensional space with the amino acids from the GluN2 M1 helix that affected CIQ modulation, they do occupy a provocative location in a homology model of GluN1/GluN2D. They are positioned on the GluN1 M4 helix such that their side chains protrude into a region between the M1 helix and the GluN2 M3 gate helix. For example, Met813 of GluN1 is about 4.5 Å from the serine in the SYTANLAAF motif of GluN2D. Thus, Met813 and Phe817 of the GluN1 M4 helix may be positioned to mediate interactions between the GluN2D M1 helix and the GluN2D M3 gate helix.

We also identified four residues in the GluN2D S1–M1 linker that influence positive modulation by CIQ: Phe574, Leu575, Pro577, and Tyr578 (Fig. 7). These residues, which are immediately extracellular to the GluN2 M1 helix, are of interest because they form a purportedly crucial gating element for glutamate receptor ion channels. The corresponding amino acids in the tetrameric crystal structure of a GluA2 AMPA receptor form a cuff helix that is parallel to the membrane and makes van der Waals contacts with the M3 helix that forms the gate (Sobolevsky et al., 2009). Moreover, the pre-M1 cuff helices in AMPA receptors have been proposed to be key determinants of receptor gating by restricting movement of the M3 helices in the closed state of the ion channel, but mediating channel opening upon agonist binding (Sobolevsky et al., 2009). The proposed role of the pre-M1 region in receptor gating is quite interesting given that CIQ increases the channel opening frequency in a gating step that precedes channel opening (Mullasseril et al., 2010). Consistent with the proposed role of these pre-M1 residues in receptor gating, we observed profound changes in both glutamate (Fig. 7; Table 2) and glycine (Table 2; Supplemental Fig. 1) potencies for 2D(F574A) and 2D(Y578A).

**Pre-M1 Residues Control Channel Open Probability.** We further explored the potential of these GluN2D pre-M1 residues to contribute to channel gating by estimating the open probability of the 2D pre-M1 alanine mutants using the onset of MK-801 inhibition (Blank and VanDongen, 2008; Gielen et al., 2009; Vance et al., 2011). We expected that
mutations at residues influencing gating would accelerate or decelerate MK-801 binding depending on whether the mutations increased or decreased gating efficiency, respectively. The time course of MK-801 inhibition was dramatically slowed for 2D(F574A), 2D(L575A), 2D(E576A), and 2D(Y578A) (Fig. 8), suggesting that these residues are involved in mediating ion channel opening after agonist binding. Surprisingly, mutation of Pro577, which corresponds to an "elbow" in the pre-M1 helix of GluA2 and is highly conserved across glutamate receptor ion channels but absent from K−

### Table 2

<table>
<thead>
<tr>
<th>Mutant Region</th>
<th>CIQ EC_{50} (\mu M)</th>
<th>100 (\mu M) CIQ Response</th>
<th>Glutamate EC_{50} (\mu M)</th>
<th>Glycine EC_{50} (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluN2D</td>
<td>–</td>
<td>9.3 ± 0.3</td>
<td>253 ± 5</td>
<td>0.82 ± 0.07</td>
</tr>
<tr>
<td>2D(F574A) Pre-M1</td>
<td>13.0 ± 1.5*</td>
<td>150 ± 1</td>
<td>0.057 ± 0.003*</td>
<td>0.060 ± 0.001*</td>
</tr>
<tr>
<td>2D(L575A) Pre-M1</td>
<td>38 ± 18</td>
<td>53 ± 4</td>
<td>0.24 ± 0.03*</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>2D(E576A) Pre-M1</td>
<td>10.7 ± 0.4</td>
<td>321 ± 12</td>
<td>0.178 ± 0.009*</td>
<td>0.106 ± 0.002</td>
</tr>
<tr>
<td>2D(P577A) Pre-M1</td>
<td>7.2 ± 0.6*</td>
<td>127 ± 2</td>
<td>0.398 ± 0.014</td>
<td>0.147 ± 0.002</td>
</tr>
<tr>
<td>2D(Y578A) Pre-M1</td>
<td>4.5 ± 0.4*</td>
<td>320 ± 30</td>
<td>0.0027 ± 0.0002**</td>
<td>0.0011 ± 0.0001**</td>
</tr>
<tr>
<td>2D(V582A) M1</td>
<td>6.6 ± 0.6*</td>
<td>143 ± 3</td>
<td>0.17 ± 0.01*</td>
<td>0.04 ± 0.01*</td>
</tr>
<tr>
<td>2D(L584A) M1</td>
<td>8.7 ± 0.7</td>
<td>311 ± 12</td>
<td>0.78 ± 0.04</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>2D(M586A) M1</td>
<td>13.3 ± 1.1*</td>
<td>510 ± 40</td>
<td>1.80 ± 0.12*</td>
<td>0.26 ± 0.01*</td>
</tr>
<tr>
<td>2D(F587A) M1</td>
<td>NE</td>
<td>86 ± 3</td>
<td>0.80 ± 0.05</td>
<td>0.25 ± 0.02*</td>
</tr>
<tr>
<td>2D(V588A) M1</td>
<td>11.3 ± 1.2</td>
<td>123 ± 3</td>
<td>0.93 ± 0.09</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>2D(L591A) M1</td>
<td>34.6 ± 5.9*</td>
<td>218 ± 9</td>
<td>0.99 ± 0.06</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>2D(T592A) M1</td>
<td>32.5 ± 2.9*</td>
<td>210 ± 4</td>
<td>1.06 ± 0.08</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>N1(M813A) M4</td>
<td>11.0 ± 0.7</td>
<td>337 ± 19</td>
<td>0.168 ± 0.008*</td>
<td>0.082 ± 0.003</td>
</tr>
<tr>
<td>N1(F817A) M4</td>
<td>12.1 ± 0.6</td>
<td>430 ± 30</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined; NE, no effect.

*The lowest concentration of glutamate tested (3 nM) elicited currents greater than 100 nA from GluN1/GluN2D(Y578A) receptors. Therefore, the EC_{50} of glutamate was calculated by fixing the minimum current to be 0 pA.

*P < 0.05 versus GluN2D, one-way analysis of variance with Dunnett’s post hoc test.

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**Fig. 6.** Residues comprising the M4 helix of GluN1 were individually mutated to alanine and coexpressed with GluN2D in oocytes. CIQ potentiation of glutamate (100 \(\mu M\)-) and glycine (30 \(\mu M\))-stimulated currents was then measured using two-electrode voltage-clamp recordings. *Residues displaying significantly altered CIQ potentiation, which are also highlighted in gray (P < 0.05, one-way analysis of variance with Dunnnett’s post hoc test). NR signifies that oocytes expressing the corresponding GluN1 point mutant failed to generate agonist-evoked currents larger than 5 nA in at least three separate injections of cRNA. Shown above the graph is an amino acid sequence alignment of the M4 region of GluN1 and GluA2; the M4 helix from the GluA2 crystal structure (Sobolevsky et al., 2009) is depicted as a cylinder on top of the alignment. Amino acids are numbered with the initiating methionine as 1.
channels (Sobolevsky et al., 2009), caused only a modest increase in the rate of onset of MK-801 inhibition. These results suggest that molecular determinants of CIQ potentiation converge on key determinants of receptor gating and imply an interesting mechanism of action whereby CIQ binding to the M1 helix of GluN2D increases the efficiency by which the pre-M1 region can promote channel opening.

CIQ Cannot Reach Its Modulatory Site by Diffusion through the Membrane. The location of multiple residues that affect the action of CIQ clustered in the transmembrane region raised the possibility that CIQ was required to partition into the plasma membrane to exert its effects. To determine whether this was the case, we recorded GluN1/GluN2D currents in the whole-cell configuration and included 10 μM CIQ in the recording pipette. We waited for 10 min after achieving the whole-cell configuration to allow for dialysis of the cell and then coapplied 10 μM CIQ with glutamate and glycine to the exterior of the cell. We reasoned that if CIQ must partition into the membrane, then it could do so equally well from the intracellular or extracellular face. If CIQ included in the pipette solution entered the plasma membrane to access its site, then the receptors should prebind CIQ and no further potentiation would be observed when CIQ was applied extracellularly. However, when CIQ was included in the pipette solution, GluN1/GluN2D receptors were still potentiated by extracellular CIQ to the same extent as control cells with normal internal solution (Fig. 9; \( P < 0.05 \), versus control pipette solution).

One possible caveat to this experiment is that CIQ could be a substrate for transporters in the cell, degradative enzymes, or otherwise be moved into organelles with a consequent decrease in its effective intracellular concentration. We therefore repeated this experiment in excised outside-out patches that lack all organelles and contain only about 1 μm² of membrane and associated cytoskeletal components. We

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**Fig. 7.** (A) An amino acid sequence alignment of the pre-M1 region of NMDA receptor subunits and the GluA2 subunit is shown with the pre-M1 helix of the GluA2 crystal structure depicted above as a cylinder. (B) The position of the GluN2D pre-M1 helix is illustrated in a homology model of GluN1/GluN2D viewed parallel to the membrane. The ATD and ABD are omitted for clarity. (C) Residues comprising the GluN2D pre-M1 region were mutated to alanine and potentiation by 10 μM CIQ was measured using two-electrode voltage-clamp recordings of oocytes. Residues highlighted in gray and marked with an asterisk exhibited CIQ potentiation that was significantly different from GluN2D (\( P < 0.05 \), one-way analysis of variance with Dunnnett’s post hoc test). (D) CIQ concentration-response curves were evaluated on point mutants from (C) with altered CIQ modulation. CIQ EC₅₀ values are given in Table 2. The data from GluN2D are replicated from Fig. 5A for comparison. Data are depicted as mean ± S.E.M. and are from 3–4 oocytes. (E and F) Glutamate and glycine concentration-response curves for the GluN2D mutants in (D) were measured using two-electrode voltage-clamp recordings (see Table 2 for EC₅₀ values). Individual curves were normalized to fitted minimum and maximum currents for that curve except for 2D(Y578A). The lowest concentration of glutamate (3 nM) or glycine (1 nM) tested at 2D(Y578A) produced currents that were larger than 100 nA and were at least half of the maximal agonist-evoked response. Hence, curves for 2D(Y578A) were fixed to a minimum of zero. Data are presented as mean ± S.E.M. and are from 3–6 oocytes.

**Fig. 8.** (A) Shown are two-electrode voltage-clamp recordings illustrating the rate of block by MK-801 of GluN1/GluN2D receptors containing point mutations in the GluN2D pre-M1 helix. Currents were activated by 100 μM glutamate and 30 μM glycine at a holding potential of −60 mV followed by coapplication of 200 nM (+)-MK-801. Traces were normalized to the glutamate/glycine current. (B) Open probability (\( P_{\text{open}} \)) of GluN2D point mutations, calculated as the reciprocal of the time constant of onset of MK-801 block (\( t_{\text{MK-801 block}} \)) and normalized to the values for GluN2D, are shown. Bars represent mean ± S.E.M. for 3–10 oocytes.
selected patches containing multiple GluN1/GluN2D channels so as to avoid potential complications of variable activity of a single channel throughout the duration of the experiment and to maximize our ability to measure an increase in the average current response of the patch. The potentiation of GluN1/GluN2D receptors by CIQ applied to the exterior of the patch was comparable when the internal pipette solution contained no CIQ (control) or contained 10 μM CIQ (Fig. 9; \( P < 0.05 \), CIQ pipette solution versus control). These results are similar to those obtained in the whole-cell configuration, and together suggest that CIQ cannot access its modulatory site from the intracellular side of the receptor nor by diffusion into the plasma membrane. Rather, direct extracellular aqueous access to the receptor appears necessary for positive modulation by CIQ.

**Discussion**

The most important conclusion of this study is that CIQ, a positive allosteric modulator of GluN2C- and GluN2D-containing NMDA receptors, does not bind the ATD. Rather, our data suggest that CIQ interacts with residues in the M1 transmembrane helix, and that CIQ potentiation is mediated by residues in the GluN2 pre-M1 region and the GluN1 M4 transmembrane helix. Moreover, we show for the first time that the GluN2 pre-M1 region may be a critical determinant of NMDA receptor gating. Mutations in this region not only influence allosteric regulation by CIQ, but also alter receptor open probability, assessed by the rate of onset of MK-801 channel block.

**Structural Determinants of CIQ Potentiation Reside in the Transmembrane Region.** Although the linker between the ATD and ABD was previously identified as a molecular determinant of CIQ action (Mullasseril et al., 2010), CIQ does not bind this region of the receptor because removal of the ATD and the ATD-ABD linker from both GluN1 and GluN2D did not affect CIQ potentiation. The actions of CIQ are in contrast to positive modulation of GluN1/GluN2B receptors by polyamines such as spermine, which seems to involve positive charges located on the lower lobe of the GluN2B ATD (Mony et al., 2011) and alternatively spliced GluN1 ATD (Traynelis et al., 1995). Hence, the structural
determinants of positive allosteric modulation by CIQ are distinct from those of polyamines. It remains to be determined whether the ATDs of GluN2C and GluN2D harbor binding sites for allosteric modulators and whether the downstream mechanisms of GluN2B potentiation by polyamines are conserved at GluN2C and GluN2D receptors.

The majority of residues in the GluN2D M1 helix critical for potentiation by CIQ cluster on one side of the helix (Fig. 5D). However, two of those residues (Val582 and Met586) are located on the opposite side of the helix. Mutation of both of these residues to alanine also changes glutamate potency, which likely reflects changes in gating of these mutants because these residues are situated far outside the agonist-binding pocket. Perhaps mutation of these residues alters the conformation of the M1 helix, thereby preventing CIQ from binding. Alternatively, these mutations change the manner in which the M1 helix moves upon agonist binding and by extension disrupt the changes that occur in gating when CIQ is present. In this context, it is interesting to note that in a homology model of GluN1/GluN2D, Val582 and Met586 of GluN2D are positioned within approximately 3 Å of Met813 and Phe817 in the GluN1 M4 helix, raising the possibility that these residues interact during channel gating. Moreover, these residues are also located adjacent to the M3 gate helix, in particular the serine in the conserved SYTANLAAF gating motif and two phenylalanine that are one and two helical turns below SYTANLAAF (Fig. 10). Although further experiments would be needed to confirm interaction of these residues, it is tempting to speculate that Val582 and Met586 of GluN2D together with Met813 and Phe817 of GluN1 couple movement of the M1 helix upon agonist binding to movement of the M3 gate helix.

Of the five residues we identified in the GluN2 M1 helix that appear critical specifically to potentiation by CIQ (Val584, Phe587, Val588, Leu591, and Thr592; Fig. 5D), only one of those residues, Thr592, differs between GluN2A/2B and GluN2C/2D. Indeed, CIQ did not potentiate GluN2D (T592I) receptors, in which this residue had been mutated to the homologous residue in GluN2A/2B (Mullasseril et al., 2010). We further observed that mutation of this residue to valine also eliminates CIQ potentiation, whereas mutation to serine has no effect on CIQ potentiation (unpublished data). Hence, hydrogen bond capabilities at this residue might be critical for the actions of CIQ and may help explain the selectivity of CIQ for GluN2C/2D over 2A/2B. For example, CIQ may directly interact with the side chain of Thr592 and loss of the hydroxyl group, which occurs with the isoleucine residue at this position in GluN2A/2B, may prevent CIQ from binding. Alternatively, the side chain of Thr592 may be critical for conformational changes that occur downstream of CIQ binding and lead to increased channel openings.

Selectivity of CIQ for GluN2C/2D over GluN2A/2B could also arise from differences in the arrangement of the transmembrane helices in GluN2A/2B versus GluN2C/2D. Perhaps the M1 helix of 2A/2B is rotated compared with the M1 helix in 2C/2D and thus the residues on the outside of the transmembrane region that could potentially interact with modulators are different. It is also likely that movements of the transmembrane helices upon agonist binding differ between 2A/2B and 2C/2D given the markedly different open and closed times of these receptors (Traynelis et al., 2010). Hence, functional rearrangements of the transmembrane helices may be differentially sensitive to modulation by CIQ.

**Role of Pre-M1 Region in Gating.** Several lines of evidence implicate the pre-M1 region in gating of glutamate receptors. In AMPA receptors, changes in receptor leak currents occur when amino acids on the M3 helix facing the pre-M1 helix are mutated to cysteine and reacted with methanethiosulfonate (MTS) reagents, suggesting these residues are important for gating of AMPA receptors (Sobolevsky et al., 2003). Additionally, residues at the interface of the pre-M1 and M4 helices were critical for noncompetitive inhibition of AMPA receptors by 1-(4-Aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI-53655) and 3-(2-chloro-phenyl)-2-[6-(diethylaminomethyl-pyridin-2-yl)vinyl]-6-fluoro-3H-quinazolin-4-one (CP-465022) (Balannik et al., 2005). In NMDA receptors, mutations in the pre-M1
region of GluN1 (Gln556 and Pro557; Kashiwagi et al., 2002) and GluN2C (Glu530 and Ser533; Sobolevsky et al., 2007) result in either spontaneously active channels or channels that become spontaneously active upon modification by MTS reagents. Moreover, introduction of cysteines at several residues in the pre-M1 region of GluN2A resulted in channels with small or abnormal glutamate-activated currents (Thomas et al., 2006). Mutations in the pre-M1 region giving rise to spontaneously active channels (either alone or after reaction with MTS reagents) may reflect a shift in the gating equilibrium toward the open state, that is, an increase in the gating efficiency, which has been shown for several residues in the S1-M1 linker (Talukder et al., 2010). In a complementary way, mutations yielding receptors with small glutamate-activated currents may be due to uncoupling of the ion channel pore from agonist binding.

In this study, mutations in the pre-M1 region of GluN2D at Phe574, Leu575, and Pro577, disrupted positive modulation by CIQ. CIQ potentiates the receptor by accelerating a pregating step, thereby increasing the opening frequency of the receptor (Mullasseril et al., 2010). Hence, mutations yielding receptors with small glutamate-in-the S1-M1 linker (Talukder et al., 2010). In a complementary way, mutations yielding receptors with small glutamate-activated currents may be due to uncoupling of the ion channel pore from agonist binding. 


Giesen K-D,