Characterization of a Soluble Ligand Binding Domain of the NMDA Receptor Regulatory Subunit NR3A

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NR3A is expressed widely in the developing CNS of mammals. Coassembly of NR3A with NR1 and NR2 modifies NMDA receptor-mediated responses, reducing calcium permeability and single-channel conductance. The ligand binding properties of NR3A are unknown but shape the role NR3A plays when incorporated into NMDA receptors. Here, a soluble NR3A ligand binding domain (NR3A S1S2) was constructed based on amino acid sequence alignments with other glutamate receptor ion channels and is expressed in Escherichia coli. After purification by affinity, gel filtration, and ion exchange chromatography, NR3A S1S2 behaves as a monomer even at a concentration of 20 mg/ml, as determined by size-exclusion chromatography and dynamic light scattering. NR3A S1S2 has very high affinity for glycine with an apparent dissociation constant (Kd) of 40 nM, 650-fold less than the Kd for NR1. Glutamate, which binds to NR2 subunits, also binds to NR3A, but with very low affinity (Kd = 9.6 mM); in contrast, binding of glutamate to NR1 was not detectable even at a 300 mM concentration. The antagonist binding profiles of NR3A and NR1 also show striking differences. 6-Cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX), and its analog CGP78608, bind to NR3A S1S2 with low micromolar affinity, whereas for NR1, the affinity of CGP78608 increases 1000-fold compared with CNQX. Other high-affinity NR1 antagonists also show very weak binding to NR3A. Protocols for purifying NR3A from mammalian cell lines and neurons, NR1 is obligatory for the trafficking of NR3A and NR3B to the plasma membrane, but ion channels formed by coassembly of NR1 and NR3A in the absence of NR2 are not functional (Perez-Otano et al., 2001; Matsuda et al., 2003). However, in oocytes, coexpression of NR1 with NR3A or NR3B forms a novel excitatory glycine receptor (Chatterton et al., 2002). Whether such an excitatory glycine receptor exists in vivo is controversial. Glycine triggers neuronal bursting in electrophysiological experiments on cerebrcortical neurons (Chatterton et al., 2002). However, a similar response occurs in cultures from NR1−/− mice, indicating that NR3A receptors and thus NR3 subunits are unlikely to be involved (Matsuda et al., 2003). Surprisingly, the ligand binding properties of the NR3 family are unknown, and it has been proposed that NR3A is a regulatory subunit that does not bind agonists (Villmann et al., 1999), that NR3A and NR3B are glycine-binding subunits (Chatterton et al., 2002), and that NR3B is likely to bind glutamate (Nishi et al., 2001). The ligand binding domain for many subtypes of glutamate receptor ion channels (iGluRs) can be isolated by genetic manipulation and expressed as soluble proteins in Escherichia coli; this has been achieved for the AMPA receptor GluR2 subunit (Chen and Gouaux, 1997), the kainate receptor GluR5 and GluR6 subunits (Mayer, 2005a; Nanao et al., 2005; Naur et al., 2005), and the NMDA receptor NR1 and NR2A subunits (Furukawa and Gouaux, 2003; Furukawa et al., 2005). These studies

Received Feb. 7, 2006; revised March 3, 2006; accepted March 23, 2006.

This work was supported by the intramural research program of the National Institute of Child Health and Human Development, National Institutes of Health (NICHD), Department of Health and Human Services. We thank Drs. Stuart Lipton and Dongxian Zhang for the gift of plasmids encoding the cDNAs for NR3A and NR3B, and Dr. H. Furukawa for sharing the purification protocol for NR1S2S2. Mass spectral analysis and Edman sequencing was performed by Howard Jaffe (Protein/Peptide Sequencing Facility, National Institute of Neurological Disorders and Stroke [NINDS], NIH). N-terminus sequencing was performed by the NINDS DNA sequencing facility.

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DOI:10.1523/JNEUROSCI.0560-06.2006

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DOI:10.1523/JNEUROSCI.0560-06.2006

Cellular/Molecular

Introduction

NMDA receptor ion channels have attracted substantial interest because of their unique roles in synaptic plasticity and memory formation (Nakanishi, 1992; Collingridge and Bliss, 1995). Conventional NMDA receptors are composed of obligate NR1 subunits that bind the coagonist glycine and of NR2A to NR2D subunits that bind glutamate (Monyer et al., 1992; Furukawa et al., 2005). Discrepancies between the properties of native NMDA receptors and those expressed from cloned NR1 and NR2 subunits led to the identification of NR3A and NR3B (Ciabarri et al., 1995; Sucher et al., 1995; Nishi et al., 2001; Chatterton et al., 2002).

The physiological roles of NR3A and NR3B, which share only 24–29% amino acid sequence identity with NR1 and NR2, have yet to be fully established. Coexpressed with NR1 and NR2, NR3A and NR3B act as modulators of ion flux, reducing single-channel conductance and permeability to Ca2+. (Das et al., 1998; Sasaki et al., 2002; Matsuda et al., 2003). In NR3A−/− mice, spine density, and the amplitude of NMDA receptor currents, increase compared with wild type (Das et al., 1998). In mammalian cell lines and neurons, NR1 is obligatory for the trafficking of NR3A and NR3B to the plasma membrane, but ion channels formed by coassembly of NR1 and NR3A in the absence of NR2 are not functional (Perez-Otano et al., 2001; Matsuda et al., 2003). However, in oocytes, coexpression of NR1 with NR3A or NR3B forms a novel excitatory glycine receptor (Chatterton et al., 2002). Whether such an excitatory glycine receptor exists in vivo is controversial. Glycine triggers neuronal bursting in electrophysiological experiments on cerebrocortical neurons (Chatterton et al., 2002). However, a similar response occurs in cultures from NR1−/− mice, indicating that NMDA receptors and thus NR3 subunits are unlikely to be involved (Matsuda et al., 2003).

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established that selectivity and affinity of the isolated ligand binding cores faithfully replicates that of intact membrane-bound receptors. Here, we report synthesis of the NR3A subunit ligand binding domain based on amino acid sequence alignments between NR3A, NR1, and NR2A. We find that NR3A is a high-affinity glycine binding subunit, with a unique selectivity profile strikingly different from that for NR1. Glutamate also binds to NR3A, but at concentrations too low to be of physiological significance.

Materials and expression and purification. Based on amino acid sequence alignments with the NR1 and NR2A ligand binding domains, the crystal structures of which have been solved (Furukawa and Gouaux, 2003; Furukawa et al., 2005), two peptides, N511-R660 and E776-K915, were isolated by PCR from the cDNA for full-length NR3A and joined by a GT dipeptide linker. The resulting NR3A S1S2 construct was then inserted into a G7 dipetide linker. The resulting NR3A S1S2 construct was then inserted into a G7 dipetide linker.

Trypsin digestion of apo- and ligand-bound NR3A S1S2 Proteolysis protection assays provide a convenient method to rapidly screen proteins for ligand-induced conformational changes and to identify candidate agonists and antagonists for the ligand binding domains of neurotransmitter receptors (Chen et al., 1998). As shown in Figure 2A, glycine (1 mM) protected NR3A S1S2 from digestion by trypsin, whereas the apo protein was rapidly hydrolyzed. Similar protection against proteolysis occurs when the AMPA receptor GluR2 S1S2 construct is incubated with trypsin in the presence of 1 mM glutamate (Chen et al., 1998). The result of this experiment suggests that NR3A binds glycine with high affinity. Subsequent proteolysis protection experiments were used to screen a broad range of agonists and antagonists that are known to bind to the glycine recognition site on the NMDA receptor NR1 subunit. These experiments revealed that, in addition to glycine and D-serine, the NR1 partial agonists L-glutamate and NMDA also show no protective activity, indicating that if NR3 does not form stable dimers at a protein concentration of ~500 μM.

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The most intriguing results came from two quinoxalinediones, 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX) and [(1S)-1-[[7-bromo-1,2,3,4-tetrahydro-2,3-dioxo-5-quinoxalinyl]methyl]amino]ethylphosphonic acid hydrochloride (CGP78608), both of which increased the extent of digestion of full-length NR3A S1S2 compared with the result for apo protein, but which, in addition, caused the appearance of a prominent peptide fragment of an apparent MW of 16.6 kDa on SDS-PAGE (Fig. 2B). A second pattern was observed for the quinolines 7-chlorokynurenic acid (7-CKA), 5,7-dichlorokynurenic acid (5,7-DCKA), and trans-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline (L689560), which also enhanced proteolysis, but without stabilizing the 16.6 kDa MW peptide. The third pattern was observed for kynurenic acid, cycloleucine,
and 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinoline (L701324), for which the proteolysis profile was similar to that for apo protein. Densitometric analysis gave the following order of protection, calculated as the ratio of the density of a 32.9 kDa band compared with the value when trypsin was omitted from the reaction (in %): 81 glycine, 76 d-serine, 35 ACPC, 33 ACBC, 22 DCS, 22 t-serine, 20 NMDA, 19 cycloleucine, 18 apo protein, 18 l-glutamate, and 7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinoline.

CNQX and CGP78608 protect domain 1 from hydrolysis

The proteolysis results for CNQX and CGP78608 suggest that when these NR1 antagonists bind to the ligand recognition site of NR3A they induce a conformational change distinct from both the apo state and that produced by glycine and d-serine. The antagonist-induced conformation exposes proteolysis sites that are either occluded or less frequently accessible in the apo- and glycine-bound states, increasing sensitivity to proteolysis by trypsin. At the same time, these antagonists induce strong protection of a 16.6 kDa peptide, named fragment A, that is almost 50% of the mass of the NR3A S1S2 construct, together with a 6 kDa peptide named fragment B. Fragment A is too small to be domain 1 and too large to be domain 2, for which the mass values are 20.2 and 12.7 kDa, respectively, calculated by assigning Phe649 and Tyr873 as the boundary of domain 2, with domain 1 constituting the remainder of the NR3A S1S2 construct, based on alignment with the crystal structure of the NR1 ligand binding core (Furukawa and Gouaux, 2003). Likewise, the composition of fragment B is not immediately obvious from its mass alone.

Surprisingly, when the CNQX- or CGP78608-protected proteolysis reactions were analyzed by gel filtration chromatography under oxidizing conditions, we observed a complex elution profile with two high molecular peaks, at 13.0 and 13.9 ml, corresponding to apparent MWs of 42.5 and 26.7 kDa, respectively, followed by multiple small peaks corresponding to low molecular proteolytic fragments (Fig. 3A). When intact NR3A S1S2 was run on the same column, it eluted at 13.5 ml, corresponding to an apparent MW of 33.5 kDa. The eluate from the gel filtration column was analyzed by reducing SDS-PAGE, which revealed that the fractions eluting at 13.0 and 13.9 ml both contained fragments A and B, in approximately stoichiometric quantities (Fig. 3B). This is a surprising result given the nearly threefold difference in mass of fragments A and B and suggests they are part of a stable complex that is resistant to further proteolysis in the presence of CNQX and CGP78608. When the same fractions from the gel filtration column were analyzed by nonreducing SDS-PAGE, we observed an additional peptide of intermediate MW named fragment C, which coeluted at 13.0 ml with fragment A, whereas the composition of the peak eluting at 13.9 ml was unchanged (Fig. 3B). We then ran the gel filtration column under reducing conditions, with 10 mM DTT added to the elution buffer, for a sample treated with 10 mM DTT after the digestion reaction had been stopped with PEFa and EDTA (dashed line). B, SDS-PAGE analysis of a silver-stained gel for fractions from the SEC run under oxidizing conditions (chromatogram drawn with a solid line). T, Samples with trypsin but no NR3A S1S2; pre, NR3A S1S2 digested under identical conditions but without CNQX; pre, NR3A S1S2 digested in the presence of CNQX, before gel filtration; lanes 6 –11, fractions 6 –11 from the SEC column that were either treated with BME before SDS-PAGE (Reducing) or run without BME (Non-reducing); Frag, fragment. C, Stereoview of a homology model of the NR3A S1S2 construct colored to indicate the location of fragments (FRAG) A, B, and C; the remainder of the protein is drawn as a transparent object to indicate cleavage of domain 2 into small peptides that were not resolved by SDS-PAGE.

Figure 3. CNQX protects domain 1 from proteolysis. A, Superose 12 HR 10/30 SEC profile of 1.4 mg of NR3A S1S2 at 7 mg/ml digested with trypsin at a ratio of 20:1 for 30 min in the presence of 2 mM CNQX (solid line); the column was run again, with 10 mM DTT added to the elution buffer, for a sample treated with 10 mM DTT after the digestion reaction had been stopped with PEFa and EDTA (dashed line). B, SDS-PAGE analysis of a silver-stained gel for fractions from the SEC run under oxidizing conditions (chromatogram drawn with a solid line). T, Samples with trypsin but no NR3A S1S2; pre, NR3A S1S2 digested under identical conditions but without CNQX; pre, NR3A S1S2 digested in the presence of CNQX, before gel filtration; lanes 6 –11, fractions 6 –11 from the SEC column that were either treated with BME before SDS-PAGE (Reducing) or run without BME (Non-reducing); Frag, fragment. C, Stereoview of a homology model of the NR3A S1S2 construct colored to indicate the location of fragments (FRAG) A, B, and C; the remainder of the protein is drawn as a transparent object to indicate cleavage of domain 2 into small peptides that were not resolved by SDS-PAGE.

RVT and LLTVGKFAI, respectively. Analysis by MALDI-TOF of CNQX-protected proteolytic fragments under reducing conditions gave m/z values of 16,625 Da for fragment A and 6047 Da for fragment B. In combination with the results of N-terminal sequencing, we deduced that fragment A corresponds to the N terminus of the NR3A S1S2 construct, from N511 to R658, with the first two amino acid vector-encoded residues that precede the start of domain 1. In a homology model based on the crystal structure of the NR1 S1S2 construct, fragment A terminates in domain 2 immediately before helix I (Fig. 3C). Fragment B starts at L861, immediately after the end of helix I, and ends at K915, the C terminus of the NR3A S1S2 construct. Mass spectral analysis of the CNQX-protected proteolysis reactions prepared using oxidizing conditions showed the appearance of a peak of m/z value 7601, whereas the 6047 Da peak was reduced in amplitude. This matches results obtained by SDS-PAGE under nonreducing conditions. We explain the formation of fragment C as a result of a disulfide bond between Cys859 and Cys913, which connects fragment B to a small peptide starting at A847 and ending at K860, for
which the theoretical MW is 1555 (Fig. 3C). The small peptide corresponds to helix I in domain 2. Under oxidizing conditions, the predicted mass of fragment B, linked via a disulfide bond to helix I, is 7600 Da, nearly identical to the experimentally observed peak of m/z 7601. The coelution of fragments A and B or fragments A and C, during gel filtration, indicates that when CNQX and CGP76808 bind to NR3A, they form a complex that protects the whole of domain 1, the interdomain β strands that link domains 1 and 2, and helix I in domain 2 from proteolysis by trypsin. This is the first biochemical demonstration that domain 1 of the glutamate ligand binding core forms a stable entity in solution that can be isolated from domain 2. This reinforces the interpretation of crystallographic and kinetic experiments on AMPA receptor ligand binding cores, which suggested that ligands first bind to domain 1, in which the ligand binding site exists as a preorganized assembly, and then trigger allosteric transitions to a conformational state supporting interactions with domain 2 (Abele et al., 2000; Armstrong and Gouaux, 2000). The higher apparent MW for the CNQX-protected proteolytic fragments observed during gel filtration, under either oxidizing or reducing conditions (Fig. 3A), compared with the results obtained by SDS-PAGE and mass spectrometry, most likely represents a change in the shape of the peptide fragments compared with native NR3A S1S2, probably as a result of unwinding of helix I.

**Agonist binding properties of NR3A S1S2**

To determine the affinity of the NR3A subunit ligand binding core for glycine, a saturation binding assay was performed using \(^{3}H\)-glycine over the concentration range 4.7–300 nM (Fig. 4A). Analysis of this experiment gave a \(K_d\) value of 40.4 nM, approximately 650-fold less than for the published value for the NR1 subunit (Table 1).

We then performed displacement assays using \(^{3}H\)-glycine to measure the affinity of additional ligands for NR3A. D-Serine, which acts as an agonist at recombinant NMDA receptors generated by coexpression of NR1 and NR2 subunits (Wafford et al., 1995), inhibits the activation by glycine of NMDA receptors generated by coexpression of NR1 and NR3 subunits in Xenopus oocytes (Chatterton et al., 2002). Like glycine, D-serine also binds to NR3A S1S2 with high affinity, \(K_d = 643\) nM, a value only 16-fold greater than the \(K_d\) of NR3A for glycine and 11-fold less than the \(K_d\) of D-serine for NR1. The binding of serine is stereo selective: the 158 \(\mu\)M \(K_d\) for the L-isomer is 240-fold greater than the \(K_d\) for D-serine and nearly 4000-fold larger than the value for glycine. The ratio of the affinity of the isomers of serine for NR3A is quantitatively similar to that for NR1 (Furukawa and Gouaux, 2003). The high affinity of glycine and D-serine but not of L-serine for NR3A is consistent with the proteolysis data shown in Figure 2, which reveals that both ligands produce an agonist-like profile distinct from that for NR1 antagonists.

Analysis of the affinity of NR1 subunit partial agonists for NR3A reveals substantial differences in the ligand binding properties of the two receptor subtypes despite their common activation by glycine. The rank order of affinity for NR3A is ACBC (\(K_d = 14.4\) \(\mu\)M) > ACPC (\(K_d = 97.7\) \(\mu\)M) > DCS (\(K_d = 277\) \(\mu\)M), whereas for NR1, the rank order is ACPC (\(K_d = 4.8\) \(\mu\)M), > DCS (\(K_d = 241\) \(\mu\)M) > ACBC (\(K_d = 830\) \(\mu\)M). NR1 shows absolute selectivity for glycine over glutamate, which is the crystal structure of the NR1 ligand binding core reveals to result from steric occlusion of the glutamate ligand \(γ\) carbonyl group by the side chain of Trp731 (Furukawa and Gouaux, 2003). In NR3A and NR3B, the smaller Met residue replaces Trp731, and thus it was possible that NR3A and NR3B could bind glutamate as has been proposed previously (Nishi et al., 2001). To test this, displacement assays were performed using L-glutamate and NMDA. NR3A was observed to show low affinity binding for L-glutamate, with a \(K_d\) of 9.55 mM; for NMDA, no displacement was observed at a concentration of 1 mM. Binding with such low affinity is unusual but not without precedent, and for the NR1 subunit cyclolucine (\(K_d = 15.3\) mM) binds with comparably low affinity (Inanobe et al., 2005). As a control to exclude an artifact resulting from contamination by glycine of the glutamate solutions prepared for this assay, we repeated the experiment using NR1 S1S2 and \(^{3}H\)MDL105519 to measure ligand binding to NR1 subunits as described previously (Furukawa and Gouaux, 2003). At a concentration of 300 mM, L-glutamate did not produce any detectable displacement of \(^{3}H\)MDL105519 for binding assays with NR1, whereas for NR3A, the agonist binding site was nearly saturated at this concentration of glutamate (Fig. 5A).
Agonist binding properties of NR3A

Electrophysiological assays suggest that glycine binds to both the NR1 and NR3 subunits when these are coexpressed in Xenopus oocytes (Chatterton et al., 2002). For both NR3A and NR3B, desensitization precluded the accurate measurement of concentration response curves, but it is clear that when coexpressed with NR1, the receptors generated by coassembly with NR3A are more sensitive to glycine than those generated by coassembly of NR1 with NR2 (Chatterton et al., 2002). Although these experiments are consistent with binding of glycine to NR3, they do not exclude an allosteric influence of NR3 on NR1, which could occur even if NR3 did not bind glycine. Our experiments with the isolated ligand binding cores of NR3A and NR1 reveal that the affinity of NR3A for glycine, $K_d = 40 \text{ nM}$, is 650 times that for NR1, suggesting that in vivo NR3A subunits are likely to be saturated by glycine, or, as discussed below, $\alpha$-serine. In electrophysiological experiments with Xenopus oocytes, $\alpha$-serine acts as an antagonist of responses to glycine when NR3 subunits are coexpressed with NR1 (Chatterton et al., 2002). This was a surprising result given that $\alpha$-serine is an established glycine site agonist for native NMDA receptors (Kleckner and Dingledine, 1988), and indeed $\alpha$-serine may even be the main endogenous ligand for NR1 subunits (Woloserk et al., 1999; Shieper et al., 2005). In proteolysis protection assays, we find that $\alpha$-serine produces a digestion profile identical to that produced by glycine and distinct from that produced by the NR1 glycine site competitive antagonists CNQX and CGP78608. Because $\alpha$-serine, CNQX, and CGP78608 all bind to NR3A with approximately similar affinities, with $K_d$ values ranging from 0.6 to 5 $\mu$M, it seems likely that $\alpha$-serine acts as an agonist at NR3A and perhaps produces functional antagonism as a result of rapid desensitization. Such a mechanism would not be without precedent and was described for the high-affinity kainate receptor agonist (2S,4R)-4-methyl glutamate (Zhou et al., 1997). The results of proteolysis protection assays for other NR1 subunit agonists and partial agonists, which are active in binding assays with NR3A, were not informative with respect to their digestion profile, because of the weak protection observed for $\alpha$-serine, DCS, ACPC, and ACBC at the concentrations tested.
Antagonist binding properties of NR3A

Of the eight NR1 subunit antagonists tested for binding to NR3A, only CNQX and CGP78608 showed activity, with a low micromolar $K_d$. CNQX shows very little discrimination between NR1 and NR3, with $K_d$ values differing only 2.5-fold. All other NR1 antagonists exhibit strong selectivity for NR1, with $K_d$ values $10^3$ to $10^4$ times larger for NR3A. This suggests that when used at appropriate concentrations, it should be possible to selectively block the activation of NR1 subunits in heteromeric assemblies containing both NR1 and NR3 subunits; the best ligands for this purpose would be L689560 and 5,7-DCKA. A complication of such experiments arises from the 650-fold higher affinity of glycine for NR3A compared with NR1, which will offset the difference in affinity for antagonists for responses measured at equilibrium (Neyton and Paoletti, 2006). However, for brief applications of agonists or during synaptic activation of NMDA receptors, this complication is largely avoided because of the slow rate of dissociation that is typical for high-affinity NMDA receptor competitive antagonists (Benveniste and Mayer, 1991). An interesting result that arises from comparison of competitive antagonist affinity for NR1 versus NR3A subunits is that the second- and third-generation NR1 subunit antagonists have substantially increased affinity for NR1 but little change in affinity for NR3. Thus, compared with CNQX, the quinoxalinedione derivative CGP78608 binds with 1000-fold higher affinity to NR1 but 2-fold lower affinity to NR3A. Likewise, 5,7-DCKA binds to NR1 with a fivefold higher affinity than 5-chlorokynurenic acid, whereas for NR3A, these compounds have equal affinity. These results suggest that the ligand binding profiles of NR1 and NR3A are substantially different and that it should be possible to develop antagonists that bind selectively to NR3A and perhaps to NR3B.

Mechanisms underlying the different binding properties of NR3A and NR1

The isolation of the ligand binding core of NR3A paves the way for a structural analysis by x-ray crystallography. Although we have obtained crystals of a glycine complex, these are small, suffer radiation damage, and have low crystallographic symmetry. However, amino acid sequence alignments with the NR1 subunit, for which a crystal structure has been solved, and homology models based on this already point to some key differences between NR1 and NR3 subunits. The ligand binding cavity of the NR1 subunit glycine complex (volume, 56 Å$^3$) is four to six times smaller than that of the glutamate-bound complexes of other iGluR subunits (Mayer, 2005b). Conformational rearrangements involving the Val689 and Trp731 side chains increase the volume of the NR1 cavity to 72 and 116 Å$^3$ in the ACPC and ACBC complexes (Inanobe et al., 2005). In NR3A, these side chains are replaced by alanine and methionine, respectively. The substitution of these smaller residues will increase the volume of the ligand binding cavity in the NR3 subunits, and it is likely that this removes steric constraints that prevent binding of glutamate to NR1 but not NR3A.

The partial agonists ACPC and ACBC make important con-
tacts with the side chain of Val689 in NR1 (Inanobe et al., 2005), and the substitution of Val689 by Ala in NR3A as well as the replacement of Trp731 by Met are likely to play a key role in the different affinities of NR1 and NR3A for ACPC, ACBC, and cycloleucine. Strikingly, the affinity of ACBC for NR3A is nearly 60-fold greater than for NR1, and the rank order of affinity for these ligands differs: ACBC > ACPC > cycloleucine for NR3 versus ACPC > ACBC > cycloleucine for NR1. This suggests that in NR3A, the ligand binding cavity has been altered so that the four carbon atom carbocyclic ring of ACBC fits the binding cavity of NR3A more precisely than the smaller and larger carbocyclic rings of ACPC and cycloleucine. Likewise, the van der Waals contact with the side chain of Trp731 made by the 5-halogen atom of the NR1 antagonist 5,7-DCKA is likely to be lost in NR3 subunits because of the substitution of Met at the equivalent position. Finally, we note that replacement of Phe754 by Ala in NR3A removes an important interaction between the hinge region linking domains 1 and 2 in NR1 with helix F (Inanobe et al., 2005).

In the absence of structural data, it is less easy to explain why NR3A binds glycine with 650-fold higher affinity than NR1, because the key residues that bind glycine are conserved in the two subunits. Likely mechanisms include differences in contacts between domains 1 and 2 that stabilize the closed-cleft glycine-bound conformation more effectively in the NR3 complex. Solvent-mediated hydrogen bond networks in the expanded cavity of the NR3 ligand binding core also likely play a role.

In summary, we show that the agonist and antagonist binding properties of NR3A have a unique profile distinct from that for NR1. The NR3A subunit is widely expressed in the embryonic and early postnatal nervous system. Its high affinity for glycine and D-serine suggests that it will be saturated at ambient amino acid concentrations in vivo. The functional consequences of this for NMDA receptor function in the triheteromeric NMDA receptor assemblies that likely exist in vivo have yet to be established, but the information provided here will be useful in resolving this issue.

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