Molecular Characterization of the Family of the N-Methyl-D-Aspartate Receptor Subunits*

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cDNA clones for four different N-methyl-D-aspartate (NMDA) receptor subunits (NMDAR2A–NMDAR2D) were isolated through polymerase chain reaction (PCR) screening of a rat brain cDNA library. These subunits are only about 15% identical with the key subunit of the NMDA receptor (NMDAR1) but are highly homologous (~50% homology) with one another. They also commonly possess large hydrophilic domains at both amino- and carboxyl-terminal sides of the four putative transmembrane segments. NMDAR2A and NMDAR2C expressed individually in Xenopus oocytes showed no electrophysiological response to agonists. However, these subunits in combined expression with NMDAR1 markedly potentiated the NMDAR1 activity and produced functional variability in the affinity of agonists, the effectiveness of antagonists, and the sensitivity to Mg2+ blockade. Thus, NMDAR1 is essential for the function of the NMDA receptor, and multiple NMDAR2 subunits potentiate and differentiate the function of the NMDA receptor by forming different heteromeric configurations with NMDAR1. Northern blotting and in situ hybridization analyses revealed that the expressions of individual mRNAs for the NMDAR2 subunits overlap in some brain regions but are also specialized in many other regions. This investigation demonstrates the anatomical and functional differences of the NMDAR2 subunits, which provide the molecular basis for the functional diversity of the NMDA receptor.

The diverse functions of glutamate neurotransmission in the mammalian central nervous system are mediated by a variety of glutamate receptors that are classified into two major groups termed ionotropic and metabotropic glutamate receptors (1). The ionotropic receptors can be subdivided into two distinct types of receptors, the receptors for N-methyl-D-aspartate (NMDA) and those for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate and kainate, both of which contain glutamate-gated, cation-specific ion channels (1–3). The metabotropic receptors are coupled to intracellular signal transduction through G proteins (4, 5).

The NMDA receptor plays a key role in many functions of glutamate transmission in the central nervous system. This receptor is essential for inducing long term potentiation, a long lasting change in neuronal responsiveness that is thought to underlie learning and memory (1, 6). It also plays a critical role in pathophysiological processes such as epilepsy and acute and delayed ischemic neuronal cell death, as well as some neurodegenerative diseases (7). The NMDA receptor is distinguished from other ionotropic receptors by the actions of a number of selective agonists and antagonists and also by its peculiar properties, including high Ca2+ permeability, modulation by glycine, voltage-dependent Mg2+ blockade, and inhibition by Zn2+ and several selective open channel blockers (1). The intracellular Ca2+ increase through the activation of the NMDA receptor is thought to be the key event in evoking both glutamate-mediated neuronal plasticity and neurotoxicity (6, 7). Voltage-dependent Mg2+ blockade is also postulated to be crucial for changing synaptic efficacy as occurs in long term potentiation (6).

Using a Xenopus oocyte expression system combined with electrophysiology, our group has cloned and characterized the key subunit of the NMDA receptor (NMDAR1), a 938-amino acid protein with four putative transmembrane (TM) segments following a large extracellular amino-terminal domain (3). The cloned receptor expressed in oocytes faithfully reproduces the pharmacological and electrophysiological properties characteristic of the NMDA receptor by forming a homomeric assembly (3). However, the existence of different subunits of the NMDA receptor was also suggested on the basis of the findings of not only the low efficacy in NMDA responses elicited by homomeric expression of NMDAR1 but also some disparities between the radioligand-binding sites and the NMDAR1 mRNA expression sites (3). Recently, the isolation of cDNA clones encoding three different subunits of the NMDA receptor in the rat and mouse has been reported from two laboratories (8–10). These subunits, termed NMDAR2A–NMDAR2C (also referred to as ε1–3, respectively), have been shown to exhibit no NMDA receptor activity in a homomeric configuration but to greatly potentiate the response to NMDA when co-expressed with NMDAR1 (8–10). In this investigation, we extended our molecular screening of the NMDA receptor subunits and identified four cDNA

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The abbreviations used are: NMDA, N-methyl-D-aspartate; D-APV, d-(-)-2-amino-5-phosphonovalerate; TM, transmembrane; PCR, polymerase chain reaction; bp, base pairs.
clones encoding the rat NMDAR2 subunits. Two of them corresponded to NMDAR2A and NMDAR2B. The third one, although corresponding to NMDAR2C, differed from the reported NMDAR2C not only in the carboxyl-terminal sequence of the corresponding rat subunit but also at a certain limited region of the mouse counterpart. The fourth one represented a novel type of the NMDAR2 subunits. We here report the structures, properties, and expression patterns of the four rat NMDAR2 subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were purchased from the following sources: AZAPII and T7: RNA polymerase from Stratagene; AmpliTaq DNA polymerase from Perkin-Elmer Cetus Instruments; Superscript- RNase H′ reverse transcriptase and RNA ladder from BIBCO-BRL; NMDA, l-glutamate, glycine, and desipramine from Sigma; (+)-MK-801 and 7-chlorokynurenate from Research Biochemicals; α-(−)-2-amino-5-phosphonovaleate (α-APV) from Tocris Neuramin, Biodyne nylon membrane from Pall; and Sequenase DNA sequencing kit from U. S. Biochemical Corp.

**Polymerase Chain Reaction (PCR)**—The isolation of cDNA fragments encoding the NMDAR2 subunits was attempted by PCR techniques using two successive approaches. In the first attempt, a forebrain cDNA library (3) was divided into 24 fractions, each containing ~4,500 cDNA clones. Each fraction was used as DNA templates for PCR amplification. The sequence of the 5′ primer was the T7 promoter sequence (5′-AACGACTCTAGATG-3′) contained in the vector DNA of the forebrain cDNA library. The sequence of the 3′ primer was the 5′-CCGAATTCGGCAGN/GAAGT GCGCGCGGA/GTA-3′ sequence (NP-3) made according to the amino acid sequence YTANLAA of residues 647-653 of NMDAR1, which is conserved in all ionotropic glutamate receptors (2, 11, 12). PCR amplification was performed by using AmpliTaq DNA polymerase according to the following schedule: five cycles at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min, followed by 26 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. An aliquot of the PCR products was electrophoresed on an agarose gel and blotted to a nylon filter. The filter was hybridized to a mixture of α32P-labeled cDNA probes encoding nine ionotropic glutamate receptors (Glur1-6, KA-1, KA-2, and NMDAR1 (3, 11, 12)). Several PCR-amplified DNA fragments were used as probes in hybridization experiments. Prehybridization and hybridization were performed as described previously (3). Briefly, 32P-labeled sense riboprobes for the NMDAR2A-2D subunits were transcribed in vitro and hybridized with cryostat sections (10 μm) of adult rat brain. After washing and RNase A treatment, the sections were exposed to β-max film (Amersham Corp.) and developed after a 1-week exposure. Control hybridization experiments were carried out in adjacent sections by using the same probes in the presence of an excess amount of unlabeled probes.

**RESULTS**

cDNA Cloning of Four NMDAR2 Subunits—The two-step approach was used for the isolation of cDNA fragments encoding the NMDAR2 subunits by PCR amplification. The sequence comparison of NMDAR1 and other ionotropic glutamate receptors revealed an identical sequence, YTANLAA (residues 647-653 of NMDAR1), at the vicinity of the putative TM III segment of these receptors (3, 11, 12). We chose this amino acid sequence for the synthesis of a degenerative oligonucleotide used for the 5′ primer of PCR. However, there was no further noticeably conserved amino acid sequence to be used for the 3′ primer of PCR. Because the T7 promoter sequence is located upstream of cDNA inserts in the vector DNA of a rat forebrain cDNA library, we chose the T7 promoter sequence as the 3′ primer of PCR. The forebrain cDNA library was subdivided into 24 fractions, each containing ~3,000 cDNA clones. Each fraction was used as DNA templates for PCR amplification by using the above 5′ and 3′ primers. PCR-amplified products were analyzed by blot hybridization with probes consisting of a mixture of nine species of ionotropic glutamate receptors (Glur1-6, KA-1, KA-2, and NMDAR1). Many of the DNA fragments hybridized to these probes, but some did not. The unhybridized fragments were excised, subcloned, and sequenced. One of the cloned DNA fragments (psN2) did not correspond to any of the known ionotropic glutamate receptors but encoded a polypeptide that shared a similarity with a receptor family.
in both the hydrophobicity profile and amino acid sequence. In addition, the predicted amino acid sequence contained an asparagine residue characteristic of the NMDAR1 subunit at an equivalent position of the putative TM II segment.

The amino acid sequence deduced from clone pSN2 showed an additional sequence conservation (WNGM1/EGE) with NMDAR1 at the preceding region of the TM I segment. As a second step of cDNA cloning of the NMDAR2 subunits, a degenerative oligonucleotide primer corresponding to this conserved sequence was synthesized, and this primer in combination with the above degenerative oligonucleotide primer was used for the second PCR amplification experiments. The homologous sequences were enriched by PCR amplification from a mixture of oligo(dT)-primed cDNAs of the rat forebrain mRNA, and amplified DNA products were electrophoresed, excised, and subcloned for sequencing. Through this procedure, we identified three additional cDNA clones (pSN3, pSN4, and pSN5), which closely resembled pSN2 in their deduced amino acid sequences. To obtain full sizes of cDNA inserts for individual receptor subunits, we screened a rat forebrain cDNA library by hybridization with the cDNA probes derived from pSN2-pSN5. More than one clone was isolated for each receptor subunit, and the nucleotide sequences of representative clones containing the largest cDNA inserts (pNR2A, pNR2B, pNR2C, and pNR2D) were determined by the chain termination method. In addition, the nucleotide sequence of a different cDNA clone pNR2D-2 of the pNR2D group was determined at the region where restriction patterns were different between pNR2D-1 and pNR2D-2.

Deduced Amino Acid Sequences of Four NMDAR Subunits—The nucleotide sequences determined for the four cDNA clones indicated that they contained large open reading frames with an overall sequence similarity with one another. The reading frame of pNR2B, however, was smaller in its 5'-terminal portion than those of the other cDNA clones. The cDNA inserts in the remaining two clones of this group were analyzed by restriction enzyme and were found to similarly lack the 5'-terminal portion. Around the time we completed the nucleotide sequence determination and characterization of four different species of cDNA clones (pNR2A-pNR2D), two other laboratories reported the molecular cloning of three subunits of the NMDA receptor family. There are some differences between the amino acid sequences revealed in this study and those reported from the two laboratories as discussed below. However, because three of the four amino acid sequences reported in this study principally corresponded to those of the rat NMDAR2 subunits reported by Monyer et al. (9), we adopted the terms of NMDAR2A-NMDAR2C in their report (9) for the corresponding NMDA receptor subunits revealed in this study; these subunits were also referred to as r1-r3 for the mouse counterparts, respectively (10).

Fig. 1 shows the amino acid sequences of the NMDAR2 subunits on the basis of the sequence determination of our cDNA clones, pNR2A-pNR2D. The amino acid sequence deduced from clone pNR2A was identical with the NMDAR2A sequence reported by Monyer et al. (9) throughout the protein sequence except for 2 amino acid substitutions at residues 246 (Phe/Leu) and 758 (Thr/Ser). As expected, the sequence predicted from clone pNR2B lacked 255 amino acid residues at the amino-terminal portion of the NMDAR2B sequence reported (9). The sequence deduced from pNR2C agreed with the NMDAR2C sequence (9) at the amino-terminal portion, but there were noticeable differences between our sequence and those reported by Monyer et al. (9) and Kutsuwada et al. (10). 1) When the translation initiation codon was taken as the first ATG in the large open reading frame of our cDNA sequence, there was a 13-amino acid extension beyond the ATG codon that was assigned as the initiation codon by the two groups. 2) Our NMDAR2C sequence agreed with that reported by Monyer et al. (9) up to residue 967, except for 4 amino acid substitutions at residues 149 (Gly/Thr), 201 (Ala/Arg), 227 (Arg/Pro), and 710 (Arg/Gly). The following sequences, however, completely diverged from each other. The sequence reported by Monyer et al. (9) contained 8 amino acids following the common sequence and terminated at residue 975, whereas our sequence extended up to residue 1250. This sequence difference seems to result from the presence or absence of 1 nucleotide at 5 consecutive G residues in the open reading frame of the cloned cDNAs, because an alternative reading frame in our NMDAR2C cDNA sequence results in the appearance of the 8-amino acid sequence and its following termination codon reported by Monyer et al. (9). 3) The mouse NMDAR2C sequence reported by Kutsuwada et al. (10) had a similar large carboxyl-terminal extension and was highly homologous to our sequence up to the carboxyl end (~95% amino acid homology). However, a significant sequence divergence was noted at a limited portion of the two sequences (from residue 988 to residue 1024 of our NMDAR2C sequence), and this sequence difference can again be explained by the frame shift of the nucleotide sequence in this region of our cloned cDNA. We determined the nucleotide sequences of four independent cDNA clones of the pNR2C group and confirmed that the four clones all possess an identical sequence at the above regions. Thus, the observed sequence differences may reflect DNA polymorphism in rats of different laboratories, species difference, or cloning artifacts during cDNA synthesis, cDNA cloning, or sequence determination. The amino acid sequence deduced from pNR2D cloned showed an overall homology with the NMDAR2A-NMDAR2C but differed from any of these NMDAR2 subunits. We thus named this subunit NMDAR2D. Two independent pNR2D cDNA clones (pNR2D-1 and pNR2D-2), however, had 82-nucleotide deletions/additions at the region corresponding to the carboxyl terminus of NMDAR2D and thus encoded two different carboxyl-terminal sequences, as indicated in Fig. 1. On the basis of the sequence determination of our cDNA clones, we concluded that NMDAR2A, R2B, R2C, R2D-1, and R2D-2 consist of 1464, 1482, 1250, 1356, and 1323 amino acid residues, respectively, all of which are considerably larger than NMDAR1 (988 amino acid residues).

Structural Features of Four NMDAR2 Subunits—The hydrophobicity analysis revealed an overall structural similarity between the NMDAR2 subunits and other ligand-gated ion channels (14). The NMDAR2 subunits possess five hydrophobic segments consisting of more than 20 uncharged amino acid residues, one at the amino terminus probably serving as a signal peptide and the others forming four transmembrane segments at the middle portion of the protein sequences. The NMDAR2 subunits, like NMDAR1, are thought to comprise four membrane-spanning domains preceded by a large extracellular sequence. These subunits, however, show a peculiar structural feature, as compared with other ligand-gated ion channels in that they all contain the large carboxyl-terminal extension following the TM IV segment.

The amino acid sequence homology is unexpectedly low (~15%) between each member of the NMDAR2 subunits and the NMDAR1 subunit, but considerably higher (~60%) among four members of the NMDAR2 subunits. This sequence homology is differently distributed according to the structural domains, and the homology is extremely high.
The amino acid sequences of NMDAR2, NMDAR2C, and NMDAR2D were deduced from the largest open reading frames of cDNA inserts of clones pNR2A, pNR2C, and pNR2D-1, respectively. The sequences of NMDAR1 and NMDAR2B were taken from those reported by Moriyoshi et al. (8) and Monyer et al. (9), respectively. The amino acid sequence deduced from clone pNR2B starts from residue 256 of the NMDAR2B sequence indicated. An alternative form of NMDAR2D that differs at the carboxyl terminus was revealed by the sequence determination of clone pNR2D-2, and two alternative sequences beginning with residue 1265 at the carboxyl terminus of the NMDAR2D sequence indicated. An alternative form of NMDARLD that differs at the carboxyl terminus was revealed by the sequence determination of clone pNR2D-2, and two alternative sequences beginning with residue 1265 at the carboxyl terminus of the NMDAR2D sequence indicated. An alternative form of NMDAR2D that differs at the carboxyl terminus was revealed by the sequence determination of clone pNR2D-2, and two alternative sequences beginning with residue 1265 at the carboxyl terminus of the NMDAR2D sequence indicated.

Under the assumed membrane topology, there are six, six, five, and six canonical Asp-X-Ser/Thr sequences for potential N-glycosylation sites (15) at the extracellular amino-terminal regions of NMDAR2A, R2B, R2C, and R2D, respectively. Similarly, a large number of possible N-glycosylation sites are present at the extracellular carboxyl-terminal regions of NMDAR2A (12 sites) and R2B (10 sites), but only one and no such sites are observed at the carboxyl-terminal regions of NMDAR2C and R2D, respectively. At the carboxyl termini of these polypeptides, all but NMDAR2D-1 share a common sequence, Ser/Pro-Ser-Leu/Ile-Glu-Ser-Glu/Asp-Val, which
may play a certain role in the functions of these subunits. Another interesting feature is that the NMDAR2 subunits, like NMDAR1, all possess many possible phosphorylation sites for Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type II and protein kinase C (16). These protein kinases have been reported to play a crucial role in the induction and maintenance of long term potentiation (17).

The TM II segments of the ligand-gated ion channels are thought to line the channel pore and determine ionic conductance and ionic selectivity (14). Several structural characteristics of the TM II segment of NMDAR1 have been pointed out in our previous report (3). The TM II segment of NMDAR1 is flanked by a glutamic acid at the extracellular side and a stretch of these amino acids at the cytoplasmic side. These negatively charged amino acids are not present at either side of TM II segments of the NMDAR2 subunits. Instead, all subunits contain a positively charged lysine residue within the TM II segments. This segment of NMDAR1 comprises a threonine residue (residue 602) at the position where the threonine residue controls ion permeation of the nicotinic acetylcholine receptor (18). The threonine residue is conserved at the equivalent position of all four NMDAR2 subunits. NMDAR1 possesses an asparagine residue (residue 616) at the corresponding position where the glutamine/arginine substitution determines the Ca\textsuperscript{2+} permeability and channel conductance of the non-NMDA receptor (19–22). This asparagine residue is present at the corresponding position of all NMDAR2 subunits, suggesting that an asparagine ring that controls the Ca\textsuperscript{2+} permeability and channel conductance is formed in a channel pore constituted by a heteromeric assembly of the NMDAR1 and NMDAR2 subunits.

Functional Properties of Heteromeric Assemblies of the NMDA Receptor—We examined current responses to application of 100 μM NMDA and 10 μM glycine in *Xenopus* oocytes injected with *in vitro* synthesized mRNAs for individual NMDAR2 subunits. NMDAR1 possesses an asparagine residue (residue 616) at the corresponding position of all four NMDAR2 subunits, suggesting that an asparagine ring that controls the Ca\textsuperscript{2+} permeability and channel conductance is formed in a channel pore constituted by a heteromeric assembly of the NMDAR1 and NMDAR2 subunits.

When 100 μM NMDA, together with 10 μM glycine, was applied in a Mg\textsuperscript{2+}-free solution, both NMDAR1/R2A and NMDAR1/R2C heteromeric receptors evoked a response with a rapid initial spike followed by a steady-state current (Fig. 2, a and d). The initial spike evoked by NMDA in oocytes.

![Fig. 2. Electrophysiological responses of the NMDAR1/ R2A and NMDAR1/R2C heteromeric receptor channels in *Xenopus* oocytes. Experimental details are described under "Experimental Procedure." NMDA-induced current responses were analyzed in *Xenopus* oocytes injected with a mixture of *in vitro* synthesized mRNAs for NMDAR1 (0.5 ng) and NMDAR2A (5 ng) in a-c and those for NMDAR1 (0.5 ng) and NMDAR2C (5 ng) in d-f.](image-url)
ence in the sensitivity to Mg\(^{2+}\) blockade was also examined by ramping voltage slowly from −100 to 40 mV in Ba\(^{2+}\)-Ringer solution in the absence and presence of Mg\(^{2+}\) (100 µM or 1 mM). In both heteromeric formations, current-voltage curves were almost linear at negative potentials in the absence of Mg\(^{2+}\), but the currents were reduced by the addition of Mg\(^{2+}\) under hyperpolarized potentials (Fig. 3). Thus, these two heteromeric receptors are blocked by Mg\(^{2+}\) in a voltage-dependent manner. However, the NMDAR1/R2A receptor was more markedly inhibited than the NMDAR1/R2C receptor by Mg\(^{2+}\) at the two different concentrations.

Further pharmacological properties of the NMDAR1/R2A and NMDAR1/R2C heteromeric receptors were investigated by determining dose-response curves of various agonists and antagonists. The serial application of agonists was found to cause desensitization of the oocyte Ca\(^{2+}\)-dependent Cl\(^{-}\) channel. We thus used Ba\(^{2+}\)-Ringer to minimize the effect of secondarily activated Cl\(^{-}\) conductance and determined amplitudes of steady-state currents elicited by serial application of agonists or antagonists under voltage clamp at −80 mV. Dose-response analysis of L-glutamate and NMDA indicated that the effective concentrations for half-maximal response (EC\(_{50}\)) of L-glutamate were 3.7 and 1.0 µM for the NMDAR1/R2A and NMDAR1/R2C receptors, respectively, whereas those of NMDA were 57 and 32 µM for the respective receptors (Fig. 4a). Thus, the effectiveness of these agonists is slightly different between these two heteromeric receptors. D-APV is a competitive antagonist for the NMDA receptor by acting at the NMDA-binding site (24), whereas 7-chlorokynurenate is a noncompetitive antagonist that inhibits glycine binding (25). The inhibitory effects of these two antagonists were analyzed by increasing concentrations of the respective compound together with 100 µM NMDA and 10 µM glycine. Effective concentrations for half-maximal inhibition (IC\(_{50}\)) of D-APV were determined to be 2.5 µM for the NMDAR1/R2A and 13 µM for the NMDAR1/R2C (Fig. 4b). The IC\(_{50}\) values of 7-chlorokynurenate were 0.79 µM for the NMDAR1/R2A and 3.2 µM for the NMDAR1/R2C (Fig. 4c). Thus, both antagonists more efficiently act on the former receptor than on the latter.

The electrophysiological characterization described above indicated that the two heteromeric receptors are considerably different in their sensitivities to Mg\(^{2+}\) blockade. This difference was confirmed by dose-response analysis of Mg\(^{2+}\) inhibition for the two heteromeric receptors (Fig. 5a). This analysis indicated that the IC\(_{50}\) values for Mg\(^{2+}\) were 10 µM for NMDAR1/R2A and 130 µM for NMDAR1/R2C. Because the inhibition of (+)-MK-801 was recovered very slowly, it was difficult to determine the IC\(_{50}\) value of (+)-MK-801 by serial application of this compound. In contrast, the recovery of inhibition by a similar channel blocker, desipramine, occurred more rapidly, probably due to fast dissociation from its binding to the channel pore (26). We thus determined dose-response curves of desipramine for the two heteromeric receptors. The IC\(_{50}\) values of desipramine were very similar between the two receptors (Fig. 5b), and this was in contrast to the different sensitivity to Mg\(^{2+}\) blockade between the two receptors.

Expression Patterns of the NMDAR2 mRNAs—Northern blotting and in situ hybridization analyses were performed to examine the expressions of four NMDAR2 mRNAs. On Northern analysis, the sizes of the mRNAs for NMDAR2A, R2B, R2C, and R2D were estimated to be about 12, 15, 6, and 7 kilonucleotides, respectively (Fig. 6). This analysis also showed that both mRNAs for NMDAR2A and R2B are abundant in the cerebral cortex and hippocampus. The NMDAR2C mRNA is prominently expressed in the cerebellum, whereas the NMDAR2D mRNA is expressed highly in the subcortical regions.

In situ hybridization revealed overlapping but distinct distribution of each NMDAR2 mRNA in the central nervous system. The NMDAR2A mRNA is widely expressed in many brain regions, and this expression is prominent in the cerebral cortex, hippocampus, internal granular layer of the olfactory bulb, anterior olfactory nuclei, olfactory tubercle, some of thalamic nuclei, inferior colliculus, pontine nuclei, inferior olivary nuclei, and cerebellar cortex (Fig. 7a). The NMDAR2B

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**Fig. 3. Voltage-dependent Mg\(^{2+}\) blockade of NMDA responses of the NMDAR1/R2A receptor (a) and the NMDAR1/ R2C receptor (b).** Current-voltage curves were determined by ramping voltage slowly (50 mV/s) from −100 to 40 mV during the application of 100 µM NMDA and 10 µM glycine in the absence and presence of Mg\(^{2+}\) (100 µM or 1 mM) as described under "Experimental Procedures".

**Fig. 4. Dose-response analysis of activation by L-glutamate and NMDA (a) and inhibition by D-APV (b) and 7-chlorokynurenate (c).** Various concentrations of agonists or antagonists were serially applied, and the steady-state currents were measured in oocytes injected with the mRNAs for NMDAR1 and NMDAR2A (open circles and squares) or those for NMDAR1 and NMDAR2C (open and closed circles) were determined by the application of the indicated concentrations of the respective agonist together with 10 µM glycine, and those for D-APV and 7-chlorokynurenate were obtained by the application of the indicated concentrations of antagonists together with 100 µM NMDA and 10 µM glycine. Each point represents the mean ± S.E. of the responses obtained from 3-5 oocytes. Theoretical curves were drawn according to the equation I = IC\(_{50}\)[1 + (EC\(_{50}\)/A)] for a and I = IC\(_{50}\)[1 + (IC\(_{50}\)/A)] for b and c, where I represents the current response; IC\(_{50}\), the maximum response; A, the concentration of agonist or antagonist; and n, the Hill coefficient. IC\(_{50}\) was taken as 100% response in a, whereas NMDA responses without addition of antagonists were taken as 100% response in b and c.

**Fig. 5. Dose-response curves for inhibition by Mg\(^{2+}\) (a) and desipramine (b).** Steady-state responses were determined by application of 100 µM NMDA plus 10 µM glycine in the presence of the indicated concentrations of Mg\(^{2+}\) and desipramine. For other explanations, see the legend to Fig. 4.

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mRNA showed a wide but more restricted distribution in its expression pattern. Its expression is present in most of the telencephalic and thalamic regions but low in the hypothalamus, cerebellum, and lower brain stem regions (Fig. 7b). The distribution of the NMDAR2C mRNA is more localized, and its expression is extremely high in the granular layer of the cerebellum. Moderate expression of this mRNA is observed in the glomerular and mitral cell layers of the main olfactory bulb, some of the thalamic nuclei, pontine nuclei, and vestibular nuclei (Fig. 7c). The NMDAR2D mRNA is mainly expressed in the diencephalic and lower brain stem regions (Fig. 7d).

Strong signals were observed in the glomerular layer of the main olfactory bulb, ventral pallidium, most of the thalamic nuclei, hypothalamus, superior colliculus, substantia nigra, vestibular nuclei, pontine nuclei, and deep cerebellar nuclei. Weaker signals were found in the cerebral cortical regions and the granular layer of the cerebellum.

**DISCUSSION**

We report here the isolation of cDNA clones encoding four different NMDA receptor subunits through PCR-mediated DNA amplification followed by molecular screening of a rat brain cDNA library. These subunits, termed NMDAR2A-NMDAR2D, share a structural similarity with other ligand-gated ion channels and possess four putative transmembrane segments (TM I-IV). They are only 15% identical with NMDAR1 but highly homologous (~50%) within this receptor subunit family. The peculiar structural feature of this receptor family is the presence of large extensions of the putative extracellular domains at both amino- and carboxyl-terminal regions that may play a certain role in the diverse function and regulation of the NMDA receptor. NMDAR1 in a homomeric configuration exhibits all electrophysiological and pharmacological properties characteristic of the NMDA receptor (3). In contrast, the NMDAR2 subunits alone show no ability to respond to glutamate or NMDA. However, when co-expressed with NMDAR1, NMDAR2A and NMDAR2C, as well as NMDAR2B (9, 10), markedly potentiate the NMDAR1 activity and confer the functional variability in the electrophysiological and pharmacological properties. In this investigation, we have failed to indicate the ability of NMDAR2D to potentiate the NMDAR1 activity. However, this receptor is highly homologous to NMDAR2C throughout the protein sequence. It is thus conceivable that NMDAR2D could also have an activity that is similar to the other members of this subunit family under appropriate expression conditions. Thus, NMDAR1 serves as a key subunit necessary for the NMDA receptor-channel complex, and the individual NMDAR2 subunits produce the functional diversity by forming a heteromeric configuration with NMDAR1.

The detailed characterization of the heteromeric receptors of NMDAR1/R2A and NMDAR1/R2C has shown that these heteromeric receptors bear all of the basic properties characteristic of the NMDA receptor: Ca^{2+} permeability, glycine modulation, voltage-dependent Mg^{2+} blockade, and selective inhibition by competitive and noncompetitive antagonists and open channel blockers. However, different combinations of the NMDAR2 subunits in a heteromeric configuration with NMDAR1 confer functional variability in the affinity for agonists, the effectiveness of antagonists, and the sensitivity to blockade of Mg^{2+}. Some of these results are consistent with those reported by Monyer et al. (9) and Kutsuwada et al. (10). It is feasible that the structural heterogeneity in the extracellular domains of the NMDAR2 subunits is responsible for governing different affinities of agonists and antagonists that act at a glutamate-binding site and a glycine-modulatory site. Among various properties of the heteromeric receptors examined, the sensitivity to Mg^{2+} blockade is notably different between the NMDAR1/R2A and NMDAR1/R2C receptors. Our recent mutational analysis of the NMDAR1 has indicated that an asparagine at the putative channel-forming TM II segment plays a critical role in determining the Ca^{2+} permeability and sensitivity to Mg^{2+} blockade (27). Furthermore, mutation of this asparagine by replacing it with glutamine or arginine reduced both Mg^{2+} and desipramine blockades to similar extents, indicating that a putative asparagine ring formed by the assembly of the TM II segments is critical in determining binding of both Mg^{2+} and an open channel blocker. However, all NMDAR2 subunits contain an asparagine residue at equivalent positions and also show a high sequence conservation at the putative channel-forming TM II segments. Thus, it seems unlikely that the putative asparagine ring in the middle of a channel is responsible for differentiating the sensitivity of Mg^{2+} blockade. Instead, as
pointed out by Ascher and Nowak (28), there may exist a negatively charged surface potential that controls the accumulation of divalent cations at an extracellular vestibule of the NMDA receptor-channel complex. It is thus tempting to speculate that there is a heterogeneity in negative surface charges associated with the channel vestibule between different NMDAR2 subunits and that once these cations reach the channel vestibule, permeation and blockade of these ions are determined by the asparagine ring at the channel pore formed by the heteromeric NMDA receptor subunits.

The NMDAR1 mRNA is expressed ubiquitously in almost all neuronal cells throughout the brain regions (3). In contrast, the mRNAs for different NMDAR2 subunits show overlapping, but differential expression patterns in the rat brain. For example, the NMDAR2A mRNA is prominently expressed in the cerebral cortex and hippocampus, whereas the NMDAR2B mRNA is distributed in the forebrain. The NMDAR2C and NMDAR2D mRNAs predominate in the cerebellum and in the diencephalic/lower brain stem regions, respectively. Thus, the diversity of the physiological and pharmacological properties of the NMDA receptors at different brain regions is generated by anatomical and functional differences of multiple NMDAR2 subunits. Such functional diversity of the NMDA receptors in different brain regions has been reported by ligand-binding experiments, as well as biochemical and electrophysiological studies (29–33). Further molecular dissection of the NMDA receptors will provide much insight into the complex mechanisms of glutamate neurotransmission, synaptic plasticity, and neurotoxicity, as well as the molecular mechanisms of ion permeation and functional modulation characteristic of the NMDA receptor.

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