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Heteromeric NMDA Receptors: Molecular and Functional Distinction of Subtypes

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The *N*-methyl D-aspartate (NMDA) receptor subtype of glutamate-gated ion channels possesses high calcium permeability and unique voltage-dependent sensitivity to magnesium and is modulated by glycine. Molecular cloning identified three complementary DNA species of rat brain, encoding NMDA receptor subunits NMDAR2A (NR2A), NR2B, and NR2C, which are 55 to 70% identical in sequence. These are structurally related, with less than 20% sequence identity, to other excitatory amino acid receptor subunits, including the NMDA receptor subunit NMDAR1 (NR1). Upon expression in cultured cells, the new subunits yielded prominent, typical glutamate- and NMDA-activated currents only when they were in heteromeric configurations with NR1. NR1-NR2A and NR1-NR2C channels differed in gating behavior and magnesium sensitivity. Such heteromeric NMDA receptor subtypes may exist in neurons, since NR1 messenger RNA is synthesized throughout the mature rat brain, while NR2 messenger RNA show a differential distribution.

The excitatory neurotransmitter glutamate can evoke Ca^{2+} influx in neurons of the central nervous system (CNS). This Ca^{2+} influx is critical for activity-dependent synaptic plasticity (*1*) and, if excessive, can lead to neuronal death (*2*). Glutamate-activated Ca^{2+} currents are mediated by the NMDA receptor, a subtype of ionotropic excitatory amino acid (EAA) receptors with distinct pharmacological (*3*) and electrophysiological (*4–7*) features. A key step in characterizing the molecular makeup of this receptor has been achieved (*8*) by the expression cloning of one of its subunits, NMDAR1 (NR1). This study demonstrated that characteristic NMDA receptor properties can reside in homo-oligomeric structures. However, the current amplitudes obtained with NR1 in the *Xenopus* oocyte expression system were low (*8*), a result that predicts that natural NMDA receptors occur in hetero-oligomeric configurations, like other ligand-gated ion channels (*9*).

The primary structure of NR1 (*8*) revealed a family relation to the previously characterized ionotropic EAA receptor subunits (*10, 11*), with which NR1 shares several small sequence islands, particularly in regions around putative transmembrane (TM) segments. By polymerase chain reaction (PCR) amplification of rat brain cDNA with oligonucleotides constructed to detect such conserved sequences (*12*), we found three cDNAs encoding new glutamate receptor subunits, termed NMDAR2A (NR2A), NR2B, and NR2C (Fig. 1). The predicted proteins are between 55% (NR2A and NR2C) and 70% (NR2A and NR2B) identical but are only about 20% identical to homologous subunits (*10, 11*), including NR1 (*8*).

The new subunits, and NR1, carry an asparagine residue in the putative channel forming region TMII, whereas a glutamine or arginine residue resides in the homologous position of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)–selective glutamate receptor subunits (*10*). The exchange by site-directed mutagenesis of either of the latter amino acids for asparagine generates channels characterized by high Ca^{2+}/Cs^{+} and Ca^{2+}/Mg^{2+} permeability ratios and by near linear current-voltage (*I-V*) relations (*13*). Thus, this particular asparagine residue may constitute a distinctive functional determinant in subunits belonging to the NMDA receptor.

When compared to other subunits of the ionotropic glutamate receptor family, two of the three new subunits, NR2A and NR2B, are uniquely endowed with COOH-terminal extensions of greater than 600 residues that contain scattered regions of conserved sequence between the two forms (Fig. 1). The size of these COOH-termini is larger than the extracellular NH_2-terminal segment preceding the first TM region. This finding may cast doubt on the currently postulated topology for ionotropic EAA receptors (*10, 11*), which predicts an extracellular location for sequences distal to the last membrane-spanning region but has not been experimentally verified. If actually located inside the cell, the COOH-terminal sequences, particularly those of NR2A

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and NR2B, might provide additional target sites for cellular constituents (*14, 15*) regulating channel function, location, and assembly.

When visualized by in situ hybridization (*16*), the three NR2 mRNAs exhibit overlapping, differential expression patterns in the rat brain (Fig. 2). In contrast with the almost ubiquitous and prominent expression of NR1 (*8*), the new subunits are more restricted in their expression. The distribution of NR2A mRNA bears the closest resemblance to that of NR1 and is present both in forebrain and in the cerebellum. The other two transcripts, NR2B and NR2C, have a more complementary distribution. NR2B is expressed in the forebrain, and NR2C shows the highest levels in the cerebellum where no NR2B mRNA is detected. All three NR2 transcripts are expressed in the thalamic nuclei, although there is less NR2A mRNA. The hypothalamus contains only the NR1 transcript, suggesting the existence of additional NMDA receptor subunits. The amygdaloid nuclei express mRNAs encoding NR2A and NR2B but not NR2C. In the caudate-putamen, there is no NR2C mRNA, but moderate signals are detected with the NR2A and NR2B probes. Differences in regional expression patterns are also apparent in the olfactory bulb. Whereas the NR1 and NR2A mRNAs appear to be expressed in most cell types (granule cells, mitral cells, tufted cells), the NR2B and NR2C mRNAs show a restricted distribution. NR2B-specific mRNA is expressed mainly in granule cells and NR2C mRNA in tufted and mitral cells.

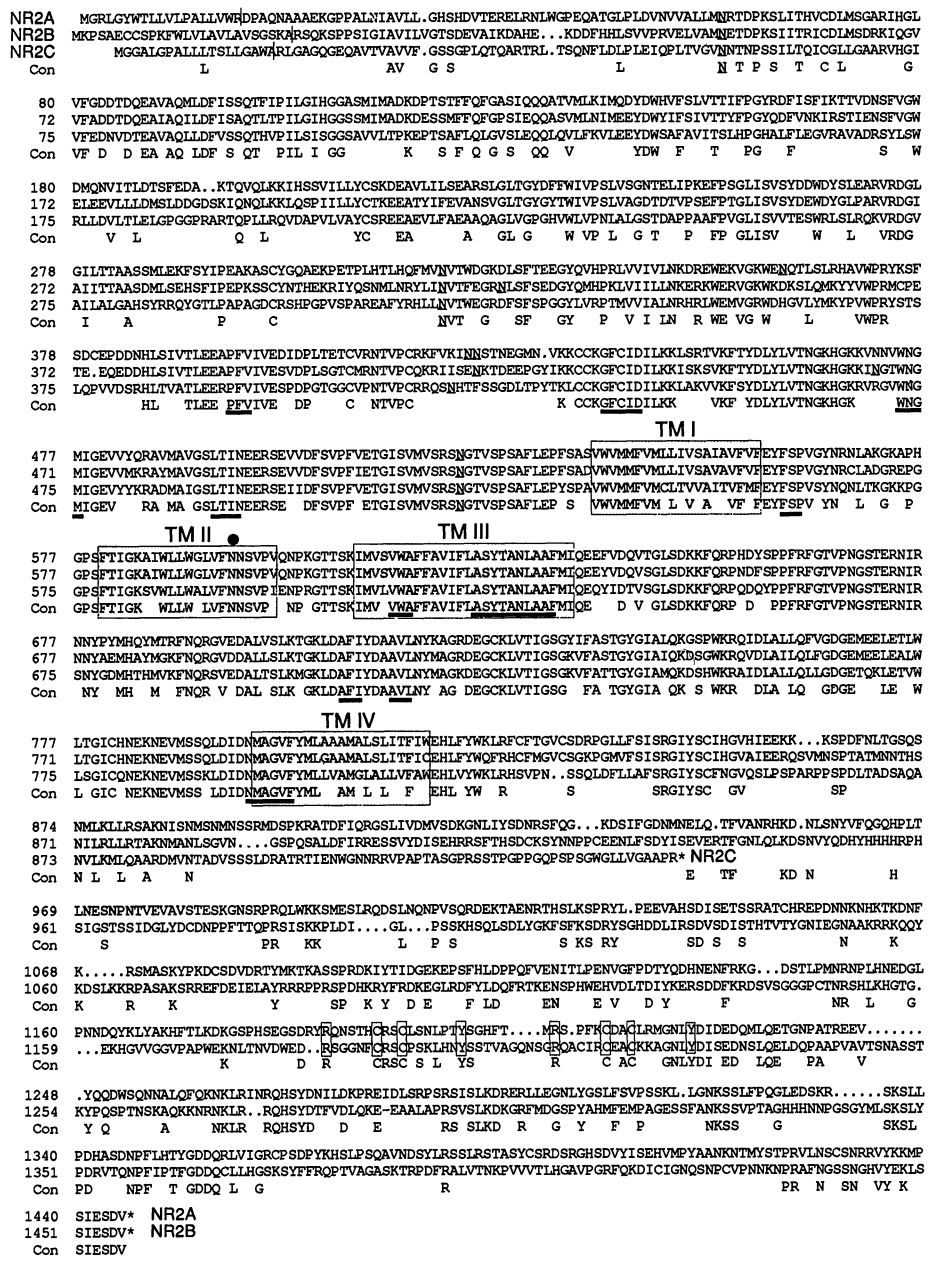

Fig. 1. Comparison of polypeptide sequences encoded by a family of three NMDA receptor subunit cDNAs (*23*). Sequences were derived as in (*12*) and are numbered on the left in the order of NR2A, NR2B, and NR2C. Positive numbers start with the predicted mature NH_2-termini. An additional line (Con) lists amino acids conserved in all three subunits and continues to show conserved residues between NR2A and NR2B in their extended COOH-termini. In these latter sequences, a conserved sequence motif ($RX_{4,5}CX_2CX_6Y$) distantly related to a zinc finger element (*24*) is indicated by boxed amino acids. Consensus N-linked glycosylation sites in regions preceding TMI are underlined, and putative transmembrane regions TMI to TMIV are boxed. A filled circle highlights the asparagine residue postulated to be a functional determinant of NMDA receptors. Stretches of three or more consecutive amino acids conserved between NMDAR1 (*9*) and the three NR2 subunits are underlined in bold.

The *Xenopus* oocyte system was employed (*17*) to study the functional properties of NR2 subunit expression. No detectable currents were recorded after bath application of glutamate or NMDA to oocytes expressing one or two NR2 subunits, indicating that NR2 subunits may not form functional homomeric or heteromeric channels. However, large currents were measured in oocytes coexpressing NR1 and any one of the newly identified subunits. The coexpression of NR1 and NR2A leads to NMDA (100 μM)-activated currents, which when measured during the current plateau ranged from 680 to 3000 nA ($n = 5$). The NMDA-induced currents in oocytes expressing NR1 and NR2B or NR1 and NR2C subunits were smaller, ranging from 120 to 290 nA ($n = 8$). On average, current amplitudes were 100 times larger than they were in oocytes expressing homomeric NR1 channels, indicating that heteromeric configurations are likely to form from NR1 subunits and members of the NR2 subunit family. Consistent with the notion of heteromultimer formation is the observation that responses of cultured 293 cells to 100 μM NMDA were observed only upon cotransfection with vectors for the NR1 and either NR2A or NR2C subunits (*18*).

Native NMDA receptors are distinguished from other transmitter-gated, cationic channels by their voltage-dependent block by extracellular Mg^{2+} (*5*), their high Ca^{2+}/Na^+ permeability ratio (*4*), and by gating kinetics that are characterized by slow onset and offset time courses to pulses of high concentrations of agonist (*19*). These functional properties were measured for heteromeric NR1-NR2A and NR1-NR2C receptor channels, transiently expressed in 293 cells (*18*). Figure 3A illustrates whole-cell currents mediated by the NR1-NR2A channel in response to application of 100 μM NMDA at −60 mV in

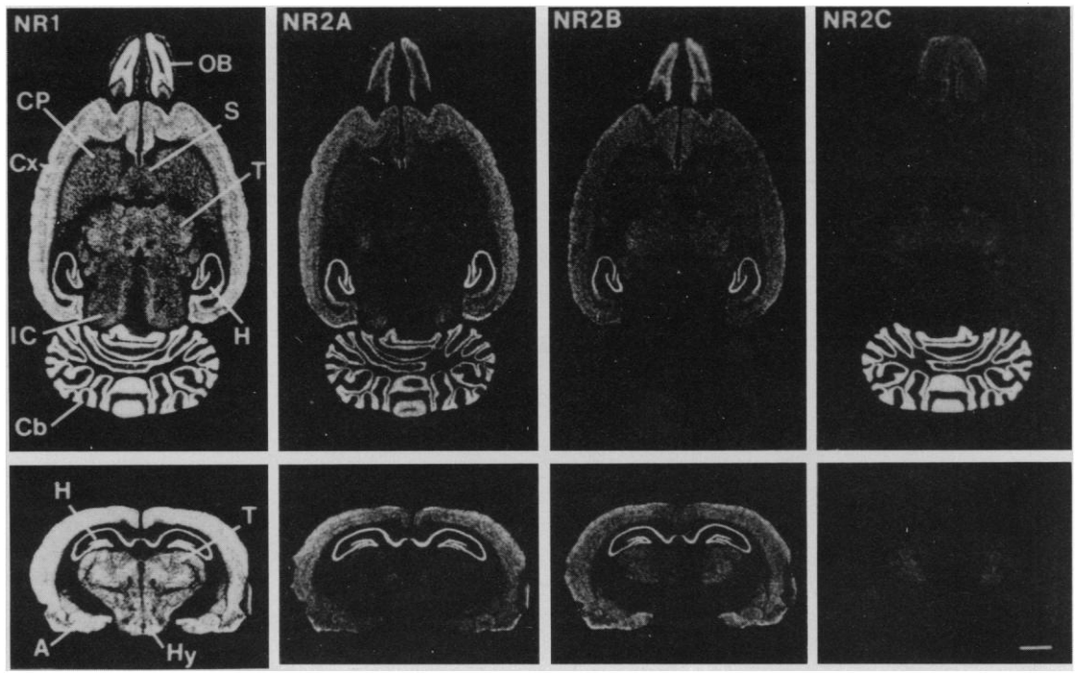

Fig. 2. Regional distribution of NMDA receptor mRNAs in horizontal and coronal sections of the rat brain. A, amygdaloid nuclei; Cb, cerebellum; CP, caudate putamen; Cx, cortex; H, hippocampus; Hy, hypothalamic area; IC, inferior colliculi; OB, olfactory bulb; S, septal nuclei; T, thalamic nuclei. Scale bar, 16 mm.

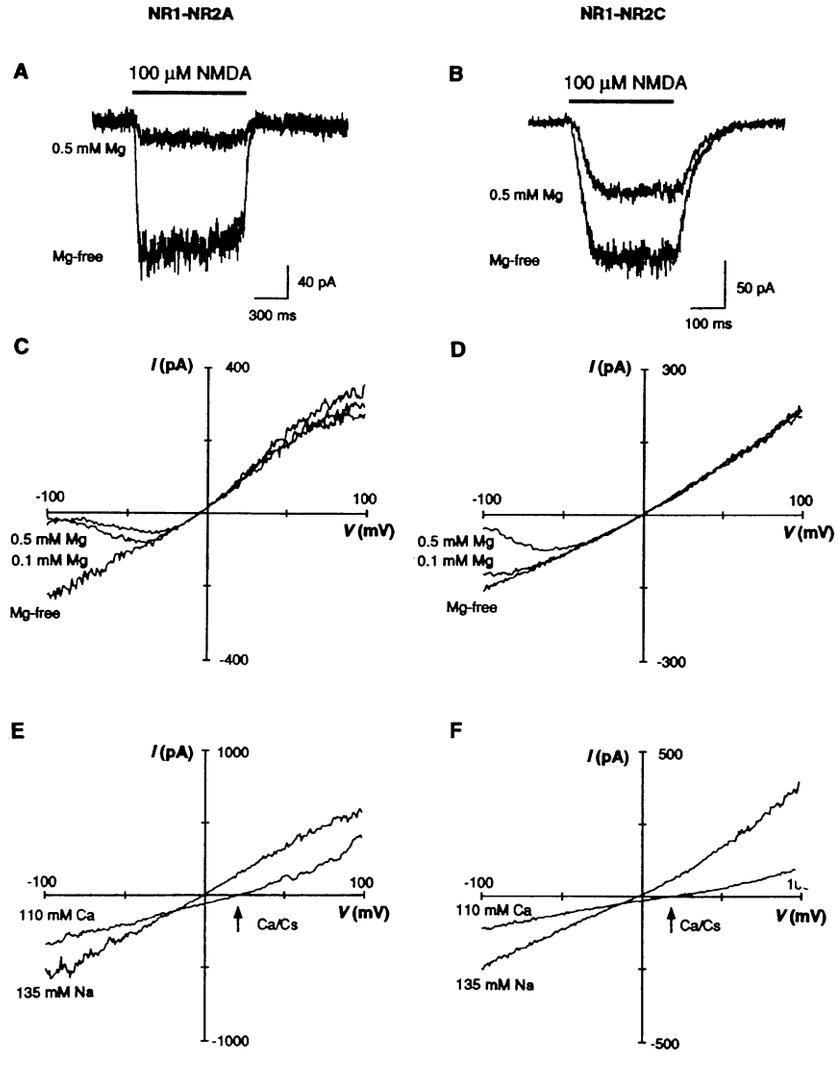

Fig. 3. Conductance and ion permeability properties of recombinant NMDA receptor channels expressed in 293 cells after transfection with NR1 and either NR2A- or NR2C-subunit–specific vectors. All experiments were performed in the presence of 10 μM glycine in the extracellular solution. (**A** and **B**) Effect of extracellular Mg^{2+} on NMDA-activated whole-cell currents in cells expressing two NMDA receptor channel subtypes. The current responses of a cell expressing NR1-NR2A channels (A) and NR1-NR2C channels (B), respectively, were measured in the absence and in the presence of 0.5 mM extracellular Mg^{2+} at −60 mV membrane potential. (**C** and **D**) Voltage and concentration dependence of block by extracellular Mg^{2+} on glutamate (100 μM)–activated steady-state *I-V* relations in cells expressing two different subunit combinations, NR1-NR2A (C) and NR1-NR2C (D), respectively. The three traces in each graph represent steady-state whole-cell *I-V* relations measured during voltage ramps, in the absence of extracellular Mg^{2+} and in the presence of 0.1 mM and 0.5 mM Mg^{2+}. (**E** and **F**) High divalent permeability of recombinant NMDA receptor subtypes. Shift to positive reversal potentials of glutamate (E)– or NMDA (F)–activated whole-cell current when the extracellular solution is changed from high Na^+ (Mg^{2+}-free rat Ringer) to high Ca^{2+} extracellular solution. In both NMDA receptor subtypes, the reversal potential shifts from a control value close to 0 mV to a positive membrane potential: 21.8 mV for the NR1-NR2A subtype and 19.5 mV for the NR1-NR2C subtype as indicated by the arrows. The average values of shifts in reversal potentials were 22.5 ± 1 mV (n = 3) for cells expressing NR1-NR2A channels and 19.0 ± 0.4 mV (n = 5) for cells with NR1-NR2C channels.

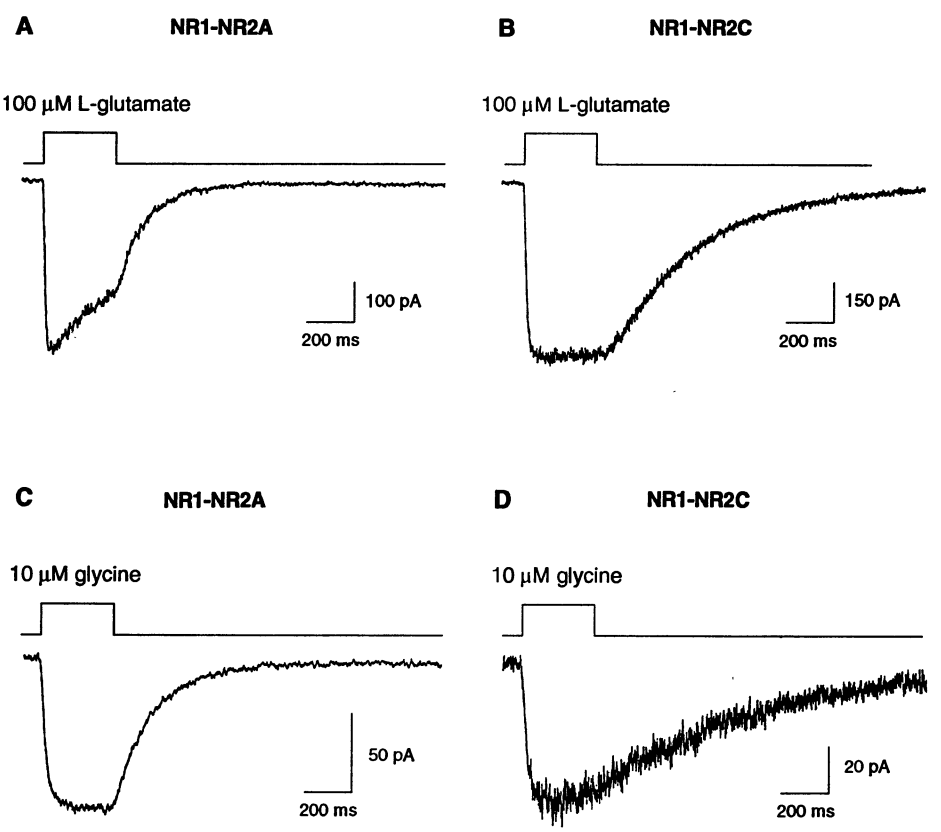

Fig. 4. (**A** and **B**) Slow onset and offset time course of glutamate (100 μM)-activated whole-cell currents in transfected 293 cells. Cells were exposed to control solution containing 10 μM glycine, followed by a 300-ms pulse of a solution containing both 10 μM glycine and 100 μM glutamate. The 20 to 80% rise times of the response were very similar for the two receptor subtypes [12.1 ± 2.5 ms (n = 4) for NR1-NR2A channels; 13.8 ± 0.8 ms (n = 3) for NR1-NR2C channels]. The offset time constants differed considerably between the two subtypes and were 118 ± 11 ms (n = 4) for NR1-NR2A (A) and 382 ± 45 ms (n = 3) for NR1-NR2C (B). The offset decay time constants after coapplication of 100 μM NMDA and 10 μM glycine (not shown) were 30.9 ± 2.2 ms (n = 5) for NR1-NR2A and 50.8 ± 13 (n = 4) for NR1-NR2C. These values compare to 84 ± 9 ms (n = 5) and 319 ± 36 ms (n = 4) for the two receptor subtypes when measured during coapplication of 100 μM glutamate and 10 μM glycine. (**C** and **D**) Effect of glycine on glutamate-activated whole-cell currents. Cells were first exposed to 100 μM glutamate in the nominal absence of glycine and no current response was detected. A 300-ms pulse of 10 μM glycine elicited a current response with subtype-specific offset decay time constants [147 ± 15 ms (n = 3), NR1-NR2A; 683 ± 93 ms (n = 4), NR1-NR2C]. However, the 20 to 80% rise times were comparable NR1-NR2A; NR1-NR2C. Offset decay constants were obtained by fitting single exponential functions to digitized records [33 ± 2 ms (n = 3)/32 ± 1 ms (n = 4)]. Membrane potential, −60 mV.

nominally Mg^{2+}-free rat Ringer solution and after addition of 0.5 mM Mg^{2+}. The response consists of a rapid rise to a steady-state current, which is turned off quickly after removal of agonist. In the presence of Mg^{2+}, the response amplitude is reduced to about 20% of the control value. The block of the response is concentration and voltage dependent. The steady-state I-V relation (Fig. 3C) illustrates the effect of extracellular Mg^{2+} on inward currents. In the absence of Mg^{2+}, the I-V relation is almost linear but is J-shaped in the presence of submillimolar concentrations of Mg^{2+}, as described for native NMDA receptor channels (5). The strength of this block depends on the particular subunit combination, since for the channel assembled from NR1 and NR2C subunits the blocking action of Mg^{2+} is considerably weaker (Fig. 3, B and D). This result indicates that NMDA receptor subtypes may differ in their sensitivity to Mg^{2+}, and hence, as seen with other transmitter-gated channels (9), functional diversity of native NMDA receptors may be expected from assembly of different subunit combinations. Figure 3, E and F, illustrates the high Ca^{2+}/Cs^{+} permeability ratio of heteromeric NMDA receptor channels, a functional property that is shared among the subtypes. The reversal potential for the glutamate-activated whole-cell current shifted from close to 0 mV in normal, high Na^{+} extracellular solution to around 20 mV in high (110 mM) Ca^{2+} extracellular solution in cells expressing the NR1-NR2A or the NR1-NR2C receptor subtypes. The reversal potentials in high Ca^{2+} extracellular solution are close to those seen in these conditions in native NMDA receptor channels (20) and are identical to those determined for homomeric AMPA-selective glutamate receptor channels engineered to carry an asparagine in the putative TMII segment (13).

Another hallmark of native NMDA receptor channels is the relatively slow onset and offset time course of the current response after application of short pulses of high glutamate concentrations (19). Figure 4 illustrates whole-cell current responses of cells expressing heteromeric NMDA receptor channels to brief application (300 ms) of 100 μM glutamate, a saturating concentration, in the presence of 10 μM glycine. The rise time course of the current is relatively slow when compared, for example, to the rise time course of currents recorded under the same conditions from AMPA-selective GluR channels and is comparable to that of currents mediated by NMDA receptors in native membranes (19). The decay time constant of the offset current after the glumamate pulse is also slow. It depends on the applied agonist, since the offset decay after application of NMDA at saturation concentration (100 μM) is considerably faster (legend to Fig. 4), as also seen in native NMDA receptors (19). The offset decay depends on the subunit composition and is considerably faster in cells expressing NR1-NR2A channels (Fig. 4A) than in cells expressing NR1-NR2C channels (Fig. 4B). Both the slow onset and slow offset of NMDA receptor–mediated currents in native membranes (19) are comparable to the slow gating behavior of recombinant NMDA channels and thus reflect properties of the NMDA receptors that depend on their molecular composition. The effect of glycine on cells expressing the two receptor subtypes was also different (Fig. 4, C and D). Application of 100 μM glutamate in the nominal absence of glycine did not activate detectable currents, as in native NMDA receptors (19). A brief addition of 10 μM glycine increased the currents as long as glycine was present. After removal of glycine, NR1-NR2A– and NR1-NR2C–specific currents decayed with very different offset time courses.

The existence in brain of pharmacologically different NMDA receptor subtypes has been reported (21, 22). Our results provide molecular, anatomical, and functional evidence for subtypes generated by heteromeric assembly of the NR1 subunit with different members of the NR2 subunit family. These NMDA receptor subtypes with their distinct physiological properties may subserve the induction of activity-dependent synaptic plasticity in a cell-specific manner.

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12. Two degenerate oligonucleotide primers designed after largely conserved peptide sequences in ionotropic EAA receptor subunits (*10, 11*) were used to PCR amplify [K. B. Mullis and F. A. Faloona, *Methods Enzymol.* **155**, 335 (1987)] homologous sequences from rat brain cDNA. The primers [5′-GCGAATTCTGGAA(C, T)GG(C, A)TGATGGG(G, A, T,C)GA-3′; 5′-GCGGTACCAA(A, G)GC(A, T, G)CCA(A, G)(A, G)TT(A, T, G)GC(A, T)GT(A, G)T-3′] were constructed to peptides WNGMMGEI (60 residues NH_2-terminal to TMI) and YTANLAAF (end of TMIII). Reactions (10 mM tris-HCl, pH 8.7, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each of dGTP, dATP, dCTP, dTTP) were contained in 50 μl, 20 ng of rat brain cDNA, 50 pmol of each primer, and 1 U of *Thermus aquaticus* DNA polymerase (Perkin-Elmer/Cetus). Thirty-five cycles (94°C, 0.6 min; 55°C, 0.2 min; 72°C, 0.7 min) were performed with a programmable thermocycler (Perkin-Elmer). The amplified DNA (≈500 bp) was excised from the gel and subcloned into M13 vectors for sequence analysis [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)] by restriction endonuclease sites in the 5′ ends of the primers. Cloned full-length cDNAs having sequences identified from the PCR products were obtained by screening rat brain cDNA libraries constructed in the λgt10 vector, with ^{32}P-labeled PCR fragments as probes. The complete nucleotide sequences are in the GenBank-EMBL database under accession numbers M91561 (NR2A), M91562 (NR2B), and M91563 (NR2C).
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16. The following antisense oligonucleotides targeted at subtype-specific regions between TMI and TMII were used as probes: TTCCTCCTCCTCCTCACTGTTCACCTTGAATCGGCCAAAGGGACT (oligo NR1, complementary to sequences encoding amino acid residues 566 to 580 of the mature NR1 polypeptide); AGAAGGCCCGTGGGAGCTTTCCCTTTGGCTAAGTTTC (oligo NR2A, complementary to codons 567 to 579 of NR2A); GGGCCTCCTGGCTCTCTGCCATCGGCTAG GCACCTGTTGTAACCC (oligo NR2B, complementary to codons 557 to 572 of NR2B); TGGTCCACCTTTCTTGCCCTTGGTGAGGTTCTGGTTGTAGCT (oligo NR2C, complementary to codons 562 to 576 of NR2C). In situ hybridization experiments were as described [W. Wisden, B. J. Morris, S. P. Hunt, *Molecular Neurobiology: A Practical Approach*, J. Chad and H. Wheal, Eds. (IRL Press, Oxford, 1991), pp. 205–225]. Briefly, oligonucleotides were 3′ end-labeled with terminal deoxynucleotidyl transferase and α[^{35}S]dATP, and sections were hybridized overnight at 42°C in 50% formamide, 0.6 M NaCl, 0.06 M sodium citrate (4× SSC), and 10% dextran sulfate with 1 pg/μl of probe. The sections were washed in 1× SSC at 60°C for 20 min. Exposure time to Kodak XAR-5 film was 17 days.
17. For expression of receptors [C. Methfessel *et al.*, *Pfluegers Arch.* **407**, 577 (1986)] *Xenopus laevis* oocytes were isolated manually, injected with 10 ng of cRNA [D. A. Melton *et al.*, *Nucleic Acids Res.* **12**, 7035 (1984); P. A. Krieg and D. A. Melton, *ibid.*, p. 7057] and incubated in OR-2 [82.5 mM NaCl, 2.5 mM KCl, 1.0 mM Na_2HPO_4, 5.0 mM HEPES, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, polyvinylpyrolidone (0.5 g/liter), pH 7.2, supplemented with antibiotics] at 19°C. Collagenase treatment (Typ II, Sigma, 1 mg/ml in OR-2 for 1 hour) was 1 day after injection. Electrophysiological recordings (2 to 6 days after injection) were in the two-electrode voltage-clamp configuration with a TEC 01C amplifier (NPI Electronic, Tamm, Germany), while oocytes were perfused with normal frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 7.2).
18. The full-length cDNAs for NR1, NR2A, and NR2C were subcloned from the recombinant phagemid pBluescript SK(–) (Stratagene) into a eukaryotic expression vector [C. M. Gorman, D. R. Gies, G. McCray, *DNA Protein Engin. Technol.* **2**, 3 (1990)]. The NR2B cDNA could not be stably integrated into the expression vector. Cloned NR1 cDNA (*8*) was isolated from a rat brain cDNA library. Transient single or combinatorial subunit expression was performed in 293 cells (ATCC CRL 1573) [C. Chen and H. Okayama, *Mol. Cell. Biol.* **7**, 2745 (1987); D. B. Pritchett, H. Lüddens, P. H. Seeburg, *Science* **245**, 1389 (1989)]. Cells on cover slips were transferred 48 hours after transfection to the stage of an inverted microscope and bathed in a chamber continuously perfused with normal rat Ringer solution (NRR) containing 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Hepes (pH 7.2, NaOH). Patch-clamp recordings (List EPC-7 amplifier, Darmstadt, Germany) at room temperature were in the whole-cell configuration [O. P. Hamill *et al.*, *Pfluegers Arch.* **391**, 85 (1981)] on single cells. Pipette solution contained 140 mM CsCl, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM Hepes (pH 7.2, CsOH). Mg^{2+}-free NRR was used as an extracellular solution in most of the experiments. $MgCl_2$ (0.1 or 0.5 mM) was added to this solution when Mg^{2+} blockade was studied. High Ca^{2+} solution contained 110 mM $CaCl_2$, 5 mM Hepes [pH 7.2, $Ca(OH)_2$]. L-Glutamate (0.1 or 1.0 mM) or NMDA (100 μM) and 10 μM glycine dissolved in one of the tested extracellular solutions were applied rapidly to the cells by means of a Piezo-driven double-barreled system [B. Sommer *et al.*, *Science* **249**, 1580 (1990)]. Before application of agonists, cells were equilibrated for at least 30 s with control solution. Data collection and analysis were as in (*13*).
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23. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
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