Biochemical Evidence for the Co-association of Three N-Methyl-D-aspartate (NMDA) R2 Subunits in Recombinant NMDA Receptors

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Lynda M. Hawkins, Paul L. Chazot‡, and F. Anne Stephenson§

From the Department of Pharmaceutical and Biological Chemistry, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX, United Kingdom

The molecular size of native NMDA receptors, as determined by both gel filtration and native polyacrylamide gel electrophoresis, is in the range of 605–850 kDa (8–10), which is consistent with the co-assembly of between four and six subunits. Immunopurification studies of native NMDA receptors have identified a population of receptors containing at least two different NR1 splice variants (10, 11). Furthermore, several groups have demonstrated the co-immunoprecipitation of anti-NR2A and anti-NR2B antibody immunoreactivities from rat forebrain, indicating the co-association of at least two NR2 subunits within an NMDA receptor complex (12–14). The determination of the subunit copy number has also been addressed by the coexpression of wild-type and mutant forms of either the NR1 or NR2 subunit. These results have been inconclusive, with reports of two or three NR1 subunits and two or three NR2 subunits/functional NMDA receptor complex (15–17).

In this study, human embryonic kidney (HEK) 293 cells were transfected with the NR1-1a and NR2A NMDA receptor subunits in combination with both FLAG- and c-Myc epitope-tagged NR2B subunits. The expressed receptors were detergent-extracted and subjected to double immunopurification using anti-NR2A and anti-FLAG antibody immunoblot columns in series. Immunoblotting of the double immunopurified NR2A/NR2BFLAG-containing material demonstrated the presence of anti-NR1, anti-NR2A, anti-FLAG, and, more importantly, anti-c-Myc antibody immunoreactivities.

The presence of anti-c-Myc antibody immunoreactivity in the double immunopurification material showed the co-assembly of three NR2 subunits, i.e. NR2A/NR2BFLAG/NR2Bc-Myc, within the same NMDA receptor complex. Control experiments excluded the possibility that the co-immunopurification of the three NR2 subunits was an artifact of the solubilization procedure. These results, taken together with those previously described that showed two NR1 subunits/oligomer, suggest that the NMDA receptor is at least pentameric.

The N-methyl-D-aspartate (NMDA) subtype of the excitatory glutamate receptor is a hetero-oligomeric protein composed of the two classes of NMDA receptor subunits, namely, NR1 and NR2. The NR1 subunit is encoded by a single gene, which undergoes extensive alternative splicing to generate eight different splice forms. The NR2 subunit class consists of four genes encoding the subunits NR2A–NR2D. The subunits are thought to co-assemble in different combinations to form functionally distinct NMDA receptor subtypes (1). Biochemical and functional studies reported to date are ambiguous with regard to both NMDA receptor subunit stoichiometry and quaternary structure. Functional studies indicate that the binding of at least two molecules of both glutamate and glycine is required for NMDA receptor activation (2–4). The glutamate- and glycine-binding sites have been localized to the NR2 (5) and NR1 (6, 7) subunits, respectively, by in vitro mutagenesis, thus suggesting that at least four subunits must co-assemble to allow binding and activation of the receptor by both coagonists.
gated to thyroglobulin by the glutaraldehyde method. The resultant conjugate was used to generate polyclonal antibodies in rabbits. Antibody production and affinity purification were carried out as described previously (18). Immunoblots probed with affinity-purified anti-NR2A-(1381–1394) antibodies detected a single polypeptide species with a molecular mass of 180 kDa in HEK 293 cells transfected with pCISNR2A (see Fig. 1A). The 180-kDa species is coincident with that previously identified as the NR2A subunit (19). This band was abolished by the prior incubation of the antibody with peptide-(1381–1394). Anti-NR2A-(1381–1394) antibodies displayed no immunocross-reactivity with other NMDA receptor subunits when used against HEK 293 cells transfected with the DNAs encoding the NR1-1a, NR2B, NR2C, or NR2D subunit (see Fig. 1A).

Anti-NR1 C2-(911–920), anti-NR2A/B-(1435–1445), and anti-NR2B-(46–60) antibodies were as described previously (11, 18, 19). The anti-NR2A-(1381–1394) antibodies to protein A-Sepharose using an immunopure IgG orientation kit (Pierce). The anti-FLAG M2 monoclonal antibody immunoaffinity column was purchased from Sigma-Aldrich (Dorset, United Kingdom).

Transfection of HEK 293 Cells
HEK 293 cells were cultured and transfected using the calcium phosphate method as described previously (19). Post-transfection, the cells were grown in the presence of 1 mM ketamine (Research Biochemicals International, Natick, MA) and harvested 24 h later. The NMDA receptor subunit clones pCISNR1-1a, pCISNR2A, and pCISNR2B were transfected at a ratio of 1:3:3, where the DNA encoding the NR2B subunit was represented by both the FLAG-tagged and c-Myc-tagged subunit. Twenty hours post-transfection, cell cytotoxicity was determined using the Promega cell cytotoxicity kit.

Cell Cytotoxicity
HEK 293 cells were transfected with pCISNR1-1a in combination with pCISNR2B, pCISNR2BFLAG, or pCISNR2Bc-Myc. Twenty hours post-transfection, cell cytotoxicity was determined using the Promega Cytotox 96™ cytotoxicity assay according to the manufacturer’s instructions and as described previously (9).

Radioligand Binding
[3H]Ro 25-6981 binding (20) was performed with both well washed cell homogenates prepared from HEK 293 cells transfected with either NR1-1a/NR2B or NR1-1a/NR2BFLAG and immunopurified NMDA receptors. The assay was performed in 50 mM Tris citrate, pH 7.1, containing 5 mM EGTA and 5 mM EDTA. Aliquots (100 μl) of the cell homogenates or immunoaffinity-purified NMDA receptor preparations were incubated in the presence of 0.1–50 nM [3H]Ro 25-6981 at 4 °C for 2 h. Nonspecific binding was defined by 1 mM spermidine. The reaction was terminated by rapid filtration through either GF/B filters (cell homogenates) or GF/B filters pretreated with 1% polyethyleneimine for 2 h immediately prior to assay (purified samples), followed by five washes with phosphate buffer, pH 7.4, at 4 °C using a Brandel cell harvester.

Immunopurification of Recombinant and Native NMDA Receptors

Recombinant Receptors—An anti-NR2A antibody immunopurification column was synthesized by the directional coupling of affinity-purified anti-NR2A-(1381–1394) antibodies to protein A-Sepharose using an immunopure IgG orientation kit (Pierce). The anti-FLAG M2 monoclonal antibody immunopurification column was purchased from Sigma-Aldrich. HEK 293 cells cotransfected with NR1-1a, NR2A, NR2BFLAG, and NR2Bc-Myc, clones were solubilized with 1% Triton X-100 and 0.5 M NaCl in the presence of a mixture of protease inhibitors as described (11). Anti-NR2A antibody immunopurification was performed as described for native forebrain NMDA receptors (12), with antigen elution of the affinity column with 50 mM diethylamine, pH 11.5, containing 0.05% (w/v) sodium deoxycholate. Fractions of 1 ml were collected and immediately quenched to a neutral pH with 2 mM glycine. The eluted NR2A-containing fractions were pooled and assayed for [3H]Ro 25-6981 radioligand binding activity, or alternatively, they were applied to the anti-FLAG antibody affinity column for 2 h, recirculating at 25 ml/h at 4 °C. The column was washed in 50 mM Tris-Cl, pH 7.4, containing 0.5 M NaCl, 5 mM EGTA, 5 mM EDTA, and 1% (v/v) Triton X-100 at 25 ml/h for 2 h at 4 °C. Elution of the anti-FLAG antibody affinity column was as described above. The NR2A/NR2BFLAG-containing fractions were analyzed by immunoblotting using the different NMDA receptor subunit-specific antibodies available. Additionally, the percentage co-association of NR2A and NR2B subunits in the NR1-1a/NR2A/NR2B-transfected cells was determined by quantitative immunoblotting. Thus, equal volumes of the detergent-solubilized cell homogenates and the anti-NR2A antibody affinity column filtrates were...
analyzed by quantitative immunoblotting using anti-NR2A, anti-c-Myc, and anti-FLAG antibodies. The percentage NR2 subunit retention by the anti-NR2A antibody immunoaffinity column was calculated, and the respective immunoreactivities in the anti-NR2A antibody affinity column filtrates were expressed as a percentage of the activities in the original detergent-solubilized extracts.

Native NMDA Receptors—Membranes prepared from adult rat forebrain were solubilized with 1% (w/v) sodium deoxycholate and 0.15 M NaCl, pH 9, for 30 min at 37 °C, and NMDA receptors were purified on anti-NR1 C2 and anti-NR2A antibody affinity columns in series as described (12). The double immunoaffinity-purified material was analyzed by quantitative immunoblotting using affinity-purified anti-NR1 C2 and anti-NR2A-(1381–1394) antibodies. Thus, a fixed volume (100 μl) of the double purified material was precipitated and analyzed by quantitative immunoblotting using a range of primary antibody concentrations (0–50 μg/ml), and the ratio of NR1 to NR2 subunits was determined at the saturating antibody concentrations, i.e., 50 μg/ml for each antibody. Note that ideally, the anti-NR2A/2B-(1435–1445) antibody would have been used for this quantitative analysis; however, when the antibody concentration dependence in immunoblots was studied, saturation was not obtained.

Immunoblotting

SDS-polyacrylamide gel electrophoresis was carried out using 7% polyacrylamide slab gels under reducing conditions. Samples were prepared using the chlorform/methanol method of protein precipitation (19). Anti-NR1 C2, anti-NR2A, and anti-NR2B antibodies were used at a final concentration of 1 μg/ml. Anti-FLAG and anti-c-Myc antibodies were used at final dilutions of 1.500 and 1:1000, respectively. Rabbit and mouse immunoglobulin horseradish-linked whole antibodies (Amersham Pharmacia Biotech) were used at a final dilution of 1:2000. Immunoreactivities were detected using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech). Where applicable, immunoblots were quantified by densitometry using ImageQuant (Molecular Dynamics, Inc., Sunnyvale, California) in the linear range of the film (12).

RESULTS

Characterization of the Epitope-tagged NR2B Subunits

Expression of the FLAG-tagged and c-Myc-tagged forms of the NR2B subunit was verified by immunoblotting. The anti-FLAG and anti-c-Myc antibodies both recognize a species with a molecular mass of 180 kDa in HEK 293 cells transfected with the respectively tagged NR2B subunit (Fig. 1B, lanes 2 and 6). The 180-kDa species is consistent with that previously identified as the NR2B subunit (11). In addition, the anti-c-Myc antibody detected multiple bands with molecular masses lower than that described for the NR2B subunit. These bands were also observed in untransfected HEK 293 cells and thus are cellular proteins that are immunoreactive with the anti-c-Myc antibody. The anti-NR2B-(46–60) antibody recognized a single polypeptide species of 180 kDa in HEK 293 cells transfected with the wild-type NR2B subunit (Fig. 1B, lane 7). However, anti-NR2B-(46–60) antibody immunoreactivity was not detected in HEK 293 cells transfected with either of the epitope-tagged forms of the NR2B subunit (Fig. 1B, lanes 8 and 9). The introduction of the epitope tags between amino acids 53 and 54 of the NR2B subunit was coincident with the peptide sequence used to raise the anti-NR2B-(46–60) antibody; thus, the epitope recognized by the anti-NR2B-(46–60) antibody was abolished. However, quantitative immunoblots of HEK 293 cells transfected with either NR2BFLAG or NR2Bc-Myc probed with the anti-NR2B-(46–60) antibody showed that the two 180-kDa epitope-tagged forms of the NR2B subunit were expressed to similar levels (Fig. 1B, lanes 10–12).

Receptor-mediated cytotoxicity was used to assess whether the epitope-tagged NR2B subunits were capable of co-associating with the NR1-1a subunit to form functional receptors. It was found that HEK 293 cells transfected with NR1-1a/NR2BFLAG or NR1-1a/NR2Bc-Myc gave 94.6 ± 9.9 and 94.8 ± 10.2% of the receptor-mediated cytotoxicity observed with

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\text{NR1-1a/NR2B, respectively. Furthermore, HEK 293 cells transfected with either NR1-1a/NR2B or NR1-1a/NR2BFLAG receptors expressed in HEK 293 cells. HEK 293 cells were transfected with either NR1-1a/NR2B or NR1-1a/NR2BFLAG receptor clones and cultured in the presence of 1 mM ketamine. Cells were harvested 24 h post-transfection; cell homogenates were prepared; and \(^{3}H\)Ro 25-6981 radioligand binding was carried out as described under "Experimental Procedures." A is a saturation binding curve showing the specific binding to NR1-1a/NR2B; B is a saturation binding curve showing the specific binding to NR1-1a/NR2BFLAG. Typical values for total/nonspecific binding were as follows: at 7 nM \(^{3}H\)Ro 25-6981, total = 3075 ± 152 dpm and nonspecific = 1352 ± 526 dpm; and at 22 nM \(^{3}H\)Ro 25-6981, total = 7014 ± 473 dpm and nonspecific = 3945 ± 100 dpm. Values are the means ± S.D. for triplicate determinations. Both saturation binding curves are representative of a typical experiment that was replicated for three independent transfections. Analysis of the saturation data by nonlinear regression (GraphPAD Prism) resolved a single binding site for both NR1-1a/NR2B and NR1-1a/NR2BFLAG with \(K_D = 10.5 ± 1.7\) and 8.2 ± 3.6 nM, respectively.

Solubilization and Immunopurification of Recombinant NMDA Receptors

Anti-NR2A Antibody Immunoaffinity Chromatography—In this study, consistent with results previously reported (11), 1% Triton X-100 and 0.5 M NaCl solubilized ~50% of the NR1 and NR2 subunit immunoreactivities expressed in HEK 293 cells transfected with NR1-1a/NR2A/NR2BFLAG/NR2Bc-Myc clones. The solubilized material was subjected to immunoaffinity chromatography as shown in Fig. 3A, and immunoblotting was used to characterize the purified receptors. Initially, the solubilized preparation was applied to an anti-NR2A antibody immunoaff-
finity column, and the material before and after affinity column application was assayed for anti-NMDA receptor subunit antibody immunoreactivities. The anti-NR2A antibody column was not 100% efficient since only 67 ± 18% (n = 4) of the anti-NR2A antibody immunoreactivity was retained (Fig. 3B, lane 1). Further quantification of the immunoblots revealed that only 20 ± 4% (n = 2) of the solubilized anti-FLAG antibody (Fig. 3C, lane 1 and 2) and 26% (n = 1) of the anti-c-Myc antibody immunoreactivities were retained by the anti-NR2A antibody immunoaffinity column. In the eluted fractions, anti-NR2A antibody immunoreactivity was detected (Fig. 3B, lane 3) in addition to anti-FLAG, anti-c-Myc, and anti-NR1 C2 antibody immunoreactivities were retained by the anti-NR2A antibody immunoaffinity column. The presence of anti-NR1 C2 antibody immunoreactivity in anti-NR2A antibody immunoaffinity-purified material was consistent with the co-association of NR1 and NR2 subunits within the same NMDA receptor complex.

**Anti-FLAG Antibody Immunoaffinity Chromatography**—The NR2A-purified material was subjected to further immunopurification using the anti-FLAG antibody affinity column. The results are shown in Fig. 3C. It can be seen that all the anti-FLAG antibody immunoreactivity was retained by the anti-FLAG antibody immunoaffinity column (Fig. 3C, lane 3 and 4). The affinity column eluate was further analyzed by immunoblotting. Anti-NR2A, anti-FLAG, and anti-NR1 C2 antibody immunoreactivities were all detected in the double immunoaffinity-purified preparation (Fig. 4, lanes 3, 9, and 12). More important, the immunoblots also demonstrated in the eluted fractions the presence of anti-c-Myc antibody immunoreactivity (Fig. 4, lane 6). The detection of anti-c-Myc antibody immunoreactivity in material that has been purified on anti-NR2A and anti-FLAG antibody immunoaffinity columns in series demonstrates the presence of a population of recombinant NMDA receptors that contain the NR2A subunit co-associated with NR1-1a and both the FLAG- and c-Myc-tagged forms of the NR2B subunit, i.e., in this receptor population, three NR2 subunits are co-associated within one receptor complex. Interestingly, when the anti-NR2A, anti-FLAG, and anti-c-Myc antibody signals in the double immunopurified material were quantified, they gave a ratio of 1:1:1 (Fig. 4, lanes 3, 6, and 9). It should be pointed out, however, that these measurements were made using different specificity antibodies at single antibody concentrations. Full concentration-dependent immunoblots will be required to verify this ratio.

**Determination of the NR1/NR2 Subunit Ratios in Native and Recombinant NMDA Receptors**

**Recombinant NMDA Receptors**—The above-described results demonstrate that three NR2 subunits can co-assemble within

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**Fig. 3.** Double immunoaffinity purification of recombinant NMDA receptors by sequential anti-NR2A and anti-FLAG antibody immunoaffinity chromatography. A is a schematic diagram of the double immunopurification procedure used in this study. The recombinant receptors were solubilized and subjected to anti-NR2A and anti-FLAG antibody immunoaffinity chromatography in series as described under “Experimental Procedures.” B and C are the results of immunoblotting of samples taken from the different stages of the double immunoaffinity purification procedure. The layout of the lanes is the same for both immunoblots. Lane 1, solubilized material; lane 2, anti-NR2A antibody post-column filtrate; lane 3, NR2A immunoaffinity-purified material; lane 4, anti-FLAG antibody post-column filtrate; lanes 5–9, fractions 2–6 of the anti-NR2A/anti-FLAG antibody immunoaffinity-purified material, respectively. B shows immunoblotting with the anti-NR2A antibody, and C shows immunoblotting with the anti-FLAG antibody. The positions of the molecular mass markers (in kilodaltons) are shown on the right. These blots are representative of at least three immunoaffinity purifications from three independent transfections.
immunoaffinity-purified preparations of cloned NR1-1α/NR2A/NR2BFLAG/NR2Bc-Myc-containing NMDA receptors were purified by anti-NR2A and anti-FLAG antibody immunoaffinity chromatography in series. The double immunoaffinity-purified material was analyzed by immunoblotting as described under “Experimental Procedures.” Lanes 1, 4, 7, and 10, solubilized material; lanes 2, 5, 8, and 11, anti-NR2A antibody immunoaffinity-purified material; lanes 3, 6, 9, and 12, anti-NR1 α/anti-FLAG antibody immunoaffinity-purified material. Lanes 1–3 were immunoblotted with the anti-NR2A antibody; lanes 4–6 with the anti-c-Myc antibody; lanes 7–9 with the anti-FLAG antibody; and lanes 10–12 with the anti-NR1 C2 antibody. The positions of the molecular mass markers (in kilodaltons) are shown on the right. This blot is representative of at least three immunoaffinity purifications from three independent transfections.

Fig. 4. Demonstration of the coexistence of three NR2 subunits in the same NMDA receptor complex. Solubilized recombinant NR1-1α/NR2A/NR2BFLAG/NR2Bc-Myc-containing NMDA receptors were purified by anti-NR1 C2 and anti-NR2A antibody affinity columns in series as described under “Experimental Procedures.” Shown are the results of a densitometric scan of an immunoblot where a fixed amount of NR1/NR2A subunit-containing receptors (100 µl) was used as antigen with increasing concentrations of anti-NR1 C2 (●) or anti-NR2A (○) antibody. The inset shows the actual immunoblot, where lanes 1–4 have been probed with both anti-NR1 C2 and anti-NR2A antibodies at 0.1, 1, 10, and 50 µg/ml, respectively. The figure is representative of two independent receptor double immunoaffinity purifications.

an NMDA receptor molecule. If the NMDA receptor has a quaternary pentameric structure, this would imply two NR1 subunits/receptor. To test this prediction, the ratio of NR1 to NR2 subunits in recombinant NMDA receptors was determined by quantitative immunoblotting as described previously for the determination of γ-aminobutyric acid type A receptor subunit stoichiometry (21). Thus, cloned NR1-1α/NR2BFLAG receptors were purified by anti-NR1 C2 followed by anti-FLAG antibody immunoaffinity chromatography. Receptor-containing fractions from the double immunoaffinity-purified material were pooled and analyzed by quantitative immunoblotting using a fixed amount of antigen, but varying the appropriate antibody concentrations, i.e. anti-NR1 C2 and anti-FLAG antibodies. A typical antibody concentration dependence curve and corresponding immunoblot are shown in Fig. 5. For all the quantitative immunoblots, the immunoreactive signal at 50 µg/ml antibody was a linear function of antigen concentration. The NR1/NR2 subunit ratio for cloned receptors determined at 50 µg/ml antibody was 1:1.5 ± 0.5 for three independent double immunoaffinity-purified preparations of cloned NR1-1α/NR2BFLAG receptors (data not shown).

Native NMDA Receptors—It is necessary to determine the NR1/NR2 subunit ratios in native receptors to show that the value obtained for the cloned receptors was not an artifact of the expression system. Thus, NMDA receptors were purified from adult rat forebrain by anti-NR1 C2 followed by anti-NR2A antibody immunoaffinity chromatography. As described above for cloned receptors, the double immunoaffinity-purified material was analyzed by quantitative immunoblotting using anti-NR1 C2 and anti-NR2A antibodies (Fig. 5). The NR1/NR2 subunit ratio determined at 50 µg/ml antibody was 1:1.4 ± 0.2 for two independent double immunoaffinity-purified preparations (three immunoblots/preparation). From our previous work, this receptor population will be a mixture of NR1/NR2A and NR1/NR2A/NR2B receptors, although the latter contributes only ~16% to the total anti-NR1/NR2A antibody double purified population (12). Thus, this 1:1.4 ratio is an underestimate since the NR2 subunit concentration had to be determined using an anti-NR2A antibody (see “Immunopurification of Recombinant and Native NMDA Receptors” under “Experimental Procedures”), thus not taking into account the NR2B subunits present in NR1/NR2A/NR2B receptors.

A pentameric receptor composed of two NR1 subunits and three NR2 subunits would have an NR1/NR2 subunit ratio, 2:3, i.e. 1:1.5. This value is approached for native receptors. More important, in all double immunoaffinity-purified receptor preparations, NR2 immunoreactivity was always greater than that found for NR1.

Fig. 5. Determination of the NR1/NR2 subunit ratio in double immunopurified NR1/NR2A-containing NMDA receptors from adult rat forebrain. NMDA receptors were purified from adult rat forebrain by anti-NR1 C2 and anti-NR2A antibody affinity columns in series as described under “Experimental Procedures.” Shown are the results of a densitometric scan of an immunoblot where a fixed amount of NR1/NR2A subunit-containing receptors (100 µl) was used as antigen with increasing concentrations of anti-NR1 C2 (●) or anti-NR2A (○) antibody. The inset shows the actual immunoblot, where lanes 1–4 have been probed with both anti-NR1 C2 and anti-NR2A antibodies at 0.1, 1, 10, and 50 µg/ml, respectively. The figure is representative of two independent receptor double immunoaffinity purifications.

Fig. 6. Demonstration that the co-association of three NR2 subunits in the double immunoaffinity-purified preparation is not an artifact of receptor solubilization. HEK 293 cells transfected with pCISNR1-1α, pCISNR2A, pCISNR2B-FLAG, or pCISNR2B-c-Myc were pooled, solubilized, and purified by anti-NR2A antibody immunoaffinity chromatography, and the resultant fractions were analyzed by immunoblotting as described under “Experimental Procedures.” Lanes 1, 4, 7, and 10, solubilized material; lanes 2, 5, 8, and 11, anti-NR2A antibody post-column filtrate; lanes 3, 6, 9, and 12, anti-NR2A antibody immunoaffinity-purified material. Lanes 1–3 were immunoblotted with the anti-NR2A antibody; lanes 4–6 with the anti-c-Myc antibody; lanes 7–9 with the anti-FLAG antibody; and lanes 10–12 with the anti-NR1 C2 antibody. The positions of the molecular mass markers (in kilodaltons) are shown on the right. This blot is representative of the results observed in at least three immunoaffinity purifications from three independent transfections.
Demonstration That the Co-association of Three NR2 Subunits Is Not an Artifact of the Solubilization Procedure

To discount the possibility that the co-immunopurification of three NR2 subunits is an artifact of the solubilization procedure, control experiments were carried out in which the NMDA receptor subunits NR1-1a, NR2A, NR2B, and NR2C clones were expressed individually in HEK 293 cells. Homogenates prepared from cells transfected with the different individual subunits were pooled, solubilized, and subjected to anti-NR2A antibody immunoaffinity purification as described under “Experimental Procedures.” The results are the means ± S.D. from at least three independent transfections performed in triplicate. Note that for each transfection, NMDA receptor subunit expression was verified using the appropriate subunit-specific antibody. B–D, HEK 293 cells were cotransfected with NR1-1a, NR2A, and NR2B clones, and cell homogenates were solubilized and purified by anti-NR2A antibody affinity chromatography as described under “Experimental Procedures.” The results of these experiments are representative of functional, native NMDA receptors. To address this problem, the binding of the NR2B-specific radioligand [3H]Ro 25-6981 to immunopurified receptors was utilized. [3H]Ro 25-6981 is an NR1/NR2B-selective, activity-dependent, noncompetitive antagonist. It has been shown in functional studies to antagonize NR1/NR2B receptors with a >5000-fold selectivity compared with other binary subunit combinations (22). Here, [3H]Ro 25-6981 binding was carried out in HEK 293 cells transfected with either single or double combinations of NMDA receptor clones. We observed high affinity [3H]Ro 25-6981 binding to NR1-1a/NR2B receptors, with no detectable binding to NR1-1a, NR2A, or NR2B subunits expressed alone.
or to NR1-1a/NR2B or NR1-1a/NR2C hetero-oligomers (Fig. 7A), confirming the selectivity found by Fischer et al. (22) and Hawkins et al. (23). In this study, we have determined the NR2 subunit copy number of NR1-1a/NR2A/NR2B-containing NMDA receptors. If these receptors, a subunit combination that is known to exist in vivo albeit as a minor subset (12), retain the ability to bind $[^3H]Ro$ 25-6981, it would demonstrate that NR1-1a/NR2A/NR2B receptors must be appropriately assembled. Thus, HEK 293 cells were cotransfected with NR1-1a, NR2A, and NR2B clones. Cell homogenates were prepared and solubilized with 1% Triton X-100 and 0.5 M NaCl, and receptors were purified by anti-NR2A antibody affinity chromatography. The eluted fractions, i.e. NR2A subunit-containing NMDA receptors that will comprise NR1-1a/NR2A, NR1-1a/NR2A/NR2B, and even monomeric NR2A, were analyzed by both immunoblotting and $[^3H]Ro$ 25-6981 radioligand binding. Representative results are shown in Fig. 7 (B–D). In Fig. 7B, it can be seen that specific $[^3H]Ro$ 25-6981 binding was detected in the fractions eluted from the affinity column. Furthermore, the elution profile of $[^3H]Ro$ 25-6981-specific binding paralleled that for the NR1, NR2A, and NR2B immunoreactivities, which were all detected in the purified receptor preparation (Fig. 7C). The copurification of the NR2B subunit on the anti-NR2A antibody affinity column is not an artifact of receptor aggregation, solubilization, or immunocross-reactivity since control experiments as described above showed that no anti-NR2B antibody immunoreactivity was retained and eluted from an anti-NR2A antibody affinity column when HEK 293 cells transfected with single subunit NMDA receptor clones were pooled, solubilized, and purified (see “Demonstration That the Co-association of Three NR2 Subunits Is Not an Artifact of the Solubilization Procedure”).

Saturation analysis revealed that $[^3H]Ro$ 25-6981 bound with a single high affinity site, $K_D = 10.9 \text{ nM} \ (n = 2)$, to the anti-NR2A antibody-purified receptors (Fig. 7D). Thus, the detection of high affinity binding of the use-dependent NMDA receptor antagonist to receptors purified by anti-NR2A antibody affinity chromatography implies binding to NR1/NR2A/NR2B receptors since the other subunit combinations copurified on this affinity column do not bind $[^3H]Ro$ 25-6981 (Fig. 7A). These receptors must be functional since, to bind $[^3H]Ro$ 25-6981, the receptor must be appropriately assembled.

**DISCUSSION**

This study reports the isolation of recombinant NMDA receptors in which three NR2 subunits are co-assembled within the same NMDA receptor complex. Furthermore, the NR1/NR2 subunit ratio was determined for both native and recombinant receptors; this ratio suggested a greater number of NR2 compared with NR1 subunits per receptor. Thus, in context with previous studies that support the conclusion that at least two copies of the NR1 subunit co-assemble within the NMDA receptor complex (15–17), these results suggest that the NMDA receptor is at least pentameric in structure. This is, however, at variance with the NR2 subunit copy number reported from electrophysiological studies. In one study, wild-type and mutant NR2B subunits were coexpressed with wild-type NR1 in Xenopus oocytes. Agonist activation of the resultant receptors identified three discrete receptor populations that corresponded to wild-type, mutant, and composite wild-type/mutant receptors. Analysis of the relative proportions of the three receptor populations formed by varying the ratio of the mutant to the wild-type NR2B subunit clones transfected was indicative of two NR2 subunits/NMDA receptor complex (17). Using an alternative mutant NR2B, NR2B(N589Q), Premkumar and Auerbach (16) also concluded that there are two copies of the NR2 subunit/NMDA receptor complex. However, Behe et al. (15) commented that their results from similar studies could not be explained by the presence of just two NR2 subunits. Similar methods were used to determine the number of NR1 subunits/receptor. The results again were inconclusive, with either two (15, 17) or three (16) NR1 subunits/NMDA receptor complex. Attempts to resolve subunit copy numbers by the analysis of channel properties is limited by the elucidation of clearly defined phenotypes for all subunit combinations formed, which may explain the different results in the literature. In this study, biochemical methods were used to identify the subunit composition of recombinant NMDA receptors; and thus, the constraints of functional analysis are negated. It is important to note, however, that although we clearly identify a population of NMDA receptors in which three NR2 subunits are co-associated, the efficiency of solubilization and immunooaffinity purification dictates that these receptors are only representative of the total receptor population. Furthermore, our results have shown that when three NMDA receptor subunit clones are coexpressed, the result is a heterogeneous population of receptors with regard to their subunit compositions. This heterogeneity is in agreement with a previous study in which Chinese hamster ovary cells were transfected with the NR1, NR2A, and NR2B subunit clones. Of the channels studied, 23% displayed properties distinct from those of either NR1/NR2A or NR1/NR2B receptors, consistent with the coassociation of NR1/NR2A/NR2B subunits within the same receptor complex (24).

It may be possible that expressed NMDA receptors are also heterogeneous with respect to subunit ratios within receptors, and it cannot therefore be excluded that a subpopulation of receptors that contain only two copies of NR2 is present. There is the further possibility that the isolated receptors could be an abnormal assembly of subunits caused by their high expression in transfected cells. Whatever the inconsistencies in the literature in the determination of NR1/NR2 subunit ratios, it is universally agreed that functional, native NMDA receptors contain NR1 and NR2 subunits. NR1 and NR2 subunits are both found in the double immunooaffinity-purified preparations described here despite the fact that only anti-NR2 subunit antibody affinity columns are used for NMDA receptor isolation. Furthermore, the control experiments that were carried out eliminated the possibility of nonspecific aggregation between NR1 and NR2 subunits during solubilization and purification. Significantly, we have also demonstrated that HEK 293 cells transfected with NR1-1a, NR2A, and NR2B clones and purified by anti-NR2A antibody affinity chromatography retain the ability to bind with high affinity the activity-dependent antagonist $[^3H]Ro$ 25-6981. Thus, the detection of high affinity $[^3H]Ro$ 25-6981 binding to receptors purified by anti-NR2A antibody affinity chromatography implies binding to NR1/NR2A/NR2B receptors and that these receptors are indeed functional.

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