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The Majority of *N*-Methyl-D-Aspartate Receptor Complexes in Adult Rat Cerebral Cortex Contain at Least Three Different Subunits (NR1/NR2A/NR2B)

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SUMMARY

A monoclonal antibody (R1JHL) against the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor has been developed that recognizes an epitope in the region of the amino-terminal amino acids 341–561 (a region common to all splice variants of NR1). This monoclonal antibody identifies a broad band at 115 kDa in immunoblots using membranes from NR1-transfected cells and from rat brain tissue. No cross-reactivity with any NR2 subunit is seen. With the goal to determine quantitatively the subunit composition of cortical NMDA receptors, we used the monoclonal antibody to NR1 and polyclonal antibodies against the NR2A and NR2B subunits to perform immunoprecipitations of receptor subunits from solubilized adult rat cortical membranes. Solubilization of the receptor subunits was accomplished under both nondenaturing (native) conditions, under which the subunits seem to remain associated with one another, and denaturing conditions, under which the subunits are dissociated from each other. Although each of these antibodies selectively immunoprecipitates only its corresponding (cognate) subunit when the subunits have been solubilized under denaturing conditions, each of the antibodies immunoprecipitates a sizable fraction of the other two NMDA receptor subunits when membranes are solubilized under nondenaturing conditions, indicating an interaction *in situ*. Using quantitative immunoblot analysis of the three subunits in both the pellets and supernatants from the immunoprecipitations, we found 1) the dominant NMDA receptor complex in adult rat cortex contains at least three subunits, NR1/NR2A/NR2B; 2) a smaller fraction of NMDA receptors are composed of only two subunits, NR1/NR2B or NR1/NR2A; 3) there are no complexes that contain NR2A/NR2B that do not contain NR1; 4) only a small fraction of each subunit is not associated with any other NMDA receptor subunit; 5) no coimmunoprecipitation of noncognate subunits occurs unless the subunits are assembled with each other *in situ*; and 6) there is no physical interaction between these NMDA receptor subunits and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor GluR2 or GluR3 subunits. These results suggest that functional studies with recombinant receptors composed of at least three subunits may be the most physiologically meaningful.

In the mammalian central nervous system, NMDA receptors are vital to many physiological and behavioral processes mediated via the excitatory neurotransmitter glutamate (1–3). These receptors are thought to consist of an association between multiple distinct subunits to form a glutamate-gated ion channel that passes sodium, potassium, and, importantly, calcium. Diversity in the molecular composition of NMDA receptors, and thus their function, arises not only from the presence of multiple mRNA splice variants of the single gene coding for the NMDA NR1 subunit (4–6) but also from the expression of four distinct genes coding for the members of the NMDA NR2 gene family (7–10). Many differences in the functional properties of the NMDA receptor are observed in electrophysiological and pharmacological studies with recombinant NMDA receptors depending on which subunits are used to generate the receptors. Although it is clear that in *Xenopus laevis* oocytes expression of only a single splice variant of the NR1 subunit will generate a functional ion channel regulated by glutamate but expression of any single NR2 subunit will not, the NMDA receptors generated by coexpression of both an NR1 and an NR2 subunit are functionally the most similar to native NMDA receptors (7–12). These observations have led to the conclusion that native NMDA receptors are heteromeric complexes assembled using NR1 and NR2 subunits in currently unknown ratios and stoichiometries. Thus, it is clear that a number of distinct NMDA receptors could theoretically be constructed depending on the subunit composition.

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR, glutamate receptor; HEK, human embryonic kidney; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Heterogeneity of native NMDA receptors in brain has been suggested by previous pharmacological studies based on radioligand binding assays (13–16), and some comparisons with data obtained from recombinant heteromeric NMDA receptors have been made (11, 16, 17). Therefore, at least some of the potential molecular compositions of NMDA receptors that display distinct functional properties seem to be physiologically relevant. In addition, a few researchers have attempted to examine the subunit composition of native NMDA receptors using immunoprecipitation with subunit-specific antibodies and have shown direct evidence of *in situ* physical interactions between different subunits of NMDA receptors (18, 19). However, there is still little known about the subunit composition of native NMDA receptors.

In the current study, we examined the subunit composition of native NMDA receptors in adult rat cortex by immunoprecipitating the subunits using three specific antibodies against the NR1, NR2A, and NR2B subunits. We estimated the relative amounts of NMDA receptors with various subunit compositions using quantitative densitometry. These studies indicate that although some NMDA receptors seem to be composed of only two types of subunit (NR1/NR2A or NR1/NR2B), the majority of the NMDA receptor complexes in this tissue contain all three subunits (NR1, NR2A, and NR2B) in a single complex.

Experimental Procedures

Materials

The full-length cDNA clone for NR1A was a kind gift from Dr. S. Nakanishi (Kyoto University, Kyoto, Japan) (4). Sources for the cDNAs for NR2A and NR2B have been given previously (20). Protein A/Sepharose, dimethylpimelimidate, and Triton X-100 were obtained from Sigma Chemical (St. Louis, MO). Sodium deoxycholate was from Fisher Scientific (Pittsburgh, PA). Selective antisera for NR2A and NR2B were developed and characterized in our laboratory (20). The selective antiserum for GluR2/3 was a kind gift from Dr. R. J. Wenthold (21). A clone of monoclonal antibody m1, which was used as a control in this study, was generated in our laboratory using a fusion protein from the amino-terminal region of the m1 muscarinic receptor. Horseradish peroxidase-linked donkey anti-rabbit and horseradish peroxidase-linked sheep anti-mouse antibodies used for immunoblotting were purchased from Amersham International (Buckinghamshire, UK). The chemiluminescence detection system for immunoblot was from Pierce Chemical (Super Signal; Rockford, IL). The plasmid pET 14b was from Novagen (Madison, WI).

Generation of Monoclonal Antibodies Against NR1

A fusion protein (fp1-561) was expressed using a vector generated by ligating the cDNA encoding amino acids 1–561 from the NMDA R1A sequence into the *Bam*HI site of the plasmid pET 14b (22). The insert cDNA was a polymerase chain reaction product with *Bam*HI restriction sites on either end, and the template was the NMDA R1A cDNA obtained from Dr. S. Nakanishi. Because this fusion protein was insoluble and could not be purified using conventional procedures over an Ni-NTA column, it was purified by slicing and electroelution of appropriate regions from a preparative (3 mm) SDS-polyacrylamide gel. To visualize the fusion protein in the gel, it was stained with 0.3 M copper chloride, which was removed by washing before elution. Most of the SDS and buffer salts were removed from the sample by dialyzing the eluted sample against 0.2 M ammonium bicarbonate and 0.02% SDS overnight (23). The samples were then aliquoted, lyophilized, and stored at −70° until use. The purified fusion protein (50 μg/injection, resuspended in either Freund's complete adjuvant for the first injection or Freund's incomplete adjuvant for subsequent injections) was used to inject mice. Conventional hybridoma technology was used (23, 24). Clones were screened by immunoblotting using 10 μg of rat cortical membranes/lane. Positive clones were characterized by immunoblotting using rat brain tissues, transfected cells, and a group of fusion proteins; The latter include the fp1-561 antigen itself and three glutathione-*S*-transferase/NR1 fusion proteins expressed using the pGEX system: fp1-254, fp254-340, and fp254-561, corresponding to the indicated regions of amino acids of the NMDA R1A subunit. A clone, R1JHL (a monoclonal antibody that recognizes all splice variants of NR1), used in this study was identified that recognized an epitope in the last but not the first two of these fusion proteins, indicating that it binds to some portion of the region of amino acids 341–561 of NMDA R1A. This region is common to all eight potential splice isoforms of the NMDA R1 subunit. Antibodies from mouse ascites generated using this clone were purified over an affinity column generated by attaching fp1-561 to Reacti-Gel 6X (Pierce, Rockford, IL) by methods described previously (25).

Transfection of HEK 293 Cells

HEK 293 cells or a derived cell line (TSA201) were transiently transfected with the cDNAs encoding the NR1A, NR2A, NR2B, or NR2D subunit alone or in combination using the method of calcium phosphate precipitation (20, 26). Membranes from these cells were used in immunoblots to determine the specificity of the monoclonal antibody and to examine the specificity of immunoprecipitation.

Immunoprecipitation

P2 tissue preparation. Using a Tekmar Tissuemizer (Cincinnati, OH), adult rat cortex from male Sprague-Dawley rats was homogenized twice in 100 volumes (100 × wet weight) of ice-cold 10 mM Tris·HCl buffer, pH 7.4, containing 320 mM sucrose at speed 6 for 10 sec with a 20-sec interval between bursts. The tissue homogenate was centrifuged at 700 × *g* for 10 min at 4°. The pellet was rehomogenized and spun again at 700 × *g*, and the supernatants were combined, transferred to another tube, and centrifuged at 37,000 × *g* at 4° for 40 min. This high-speed pellet (P2) was resuspended in 10 mM Tris·HCl, pH 7.4, using the Tekmar Tissuemizer. Protein concentrations were determined using the BCA protein assay (Pierce) with bovine serum albumin as a standard. Aliquots of this P2 membrane preparation at a protein concentration of 2–3 mg/ml were stored at −70° until use.

Denaturing conditions for membrane solubilization. The P2 membrane proteins were denatured by adding 0.10 volume of 20% SDS containing 50% 2-mercaptoethanol and boiled for 5 min. The denatured membrane preparation was diluted 20-fold into binding buffer (50 mM Tris·HCl, pH 7.4, 0.1% Triton X-100) and centrifuged at 37,000 × *g* at 4° for 10 min. The supernatant was used for immunoprecipitation.

Nondenaturing conditions for membrane solubilization. The P2 membrane preparation was solubilized by the addition of 0.10 volume of 10% sodium deoxycholate in 500 mM Tris·HCl, pH 9.0, and incubation at 36° for 30 min. A 0.10 volume of 1% Triton X-100/50 mM Tris·HCl, pH 9.0, was then added, and the preparation was dialyzed against binding buffer overnight at 4°. After centrifugation at 37,000 × *g* at 4° for 30 min, the protein concentration in the supernatant was determined, and the supernatant was used for immunoprecipitation. This method solubilizes 88 ± 1% (four measurements) of the NMDA receptors from rat cortical membranes, as judged by quantitatively immunoblotting the soluble (supernatant) and the insoluble (pellet, subsequently solubilized in boiling 2% SDS) fractions. All subunits were solubilized equally well.

Precoupling antibodies to protein A/Sepharose. The affinity purified antibodies were incubated with Protein A/Sepharose at 10–20 μg of antibody/10 μl of Sepharose wet beads (i.e., 50 μl of a 20% slurry of protein A/Sepharose) for 2 hr at room temperature in 100 mM borate buffer, pH 8.0, with gentle rocking. After washing

with 200 mM sodium borate, pH 9.0, the beads were incubated with 20 mM dimethylpimelimidate in the sodium carbonate buffer for 30 min at room temperature. The unreacted sites on the beads were then blocked using 200 mM ethanolamine, pH 8.0, for 2 hr at room temperature (23). The beads were washed with binding buffer several times and stored at 4° in a 25% (w/v) slurry. Approximately 75% of the antibody was covalently coupled to the beads with this procedure.

General conditions for immunoprecipitation. The solubilized membrane preparations were incubated with antibody precoupled to protein A/Sepharose at a concentration of ~100 μg of solubilized membrane protein/20 μl of the settled beads (coupled with ~20-40 μg of purified antibodies) in 250 μl of binding buffer. After incubation at room temperature for 2–3 hr with slow rotation, the reaction was briefly centrifuged at 14,000 rpm in a Brinkman (Brinkman Instruments, Westbury, NY) microcentrifuge, and the supernatant was transferred to another tube. To improve immunoprecipitation efficiency, the supernatant was immunoprecipitated again with another aliquot of the same antibody. A 200-μl aliquot of the final supernatant was then transferred to a new tube, and one-third volume (67 μl) of 4× loading buffer (250 mM Tris·HCl, pH 6.8, 8% SDS, 20% dithiothreitol, 30% glycerol) was added to generate the 5×-concentration supernatant samples for Fig. 3 (*S*). An aliquot (60 μl) of this denatured supernatant was diluted 5-fold with 1× loading buffer to generate supernatant samples (s) at the same dilution as the immunopellets. The immunopellet from the second immunoprecipitation was combined with the pellet from the first immunoprecipitation and washed twice with 20 volumes of binding buffer. The proteins on the beads were solubilized in 1.67 ml of 1× loading buffer to make the pellet samples directly comparable with the diluted supernatant samples. All samples were boiled for 5 min, and the proteins were separated on SDS-polyacrylamide gels with 16.7 μl loaded/lane.

Immunoblot and Quantitative Analysis

SDS-PAGE and transfer of proteins to nitrocellulose membranes were performed according to the method of Towbin *et al*. (27), with minor modifications as previously described (28). The concentration of all antibodies used for immunoblot in this study was 1–2 μg/ml.

To compare the relative amounts of a given protein found in the supernatant or pellet of an immunoprecipitation, a standard curve consisting of a series of 2-fold dilutions of solubilized cortical membranes ranging in protein concentrations from 0.125 to 2 μg/lane was generated. This standard curve was run with the unknown samples, and the integrated intensities of the bands in the standard curve and the unknowns from the supernatants and pellets were determined as previously described (20, 28). Thus, unlike band darkness or integrated intensity, which have nonlinear relationships with the amount of NMDA receptor subunit present in the sample, the integrated intensities of the bands from the unknowns were converted into "μg equivalents of cortex," which can be linearly compared with each other.

Results

Characterization of monoclonal antibody. A monoclonal antibody called R1JHL was generated and identified to target an amino-terminal region of the NMDA R1 subunit between amino acid 341 and amino acid 561. This is near the first putative transmembrane region and is shared with all NMDA R1 splice variants (5, 29). Therefore, the signal obtained by this antibody should be the summation of the signals for all NR1 splice variants in a given sample.

This antibody shows good sensitivity and specificity in recognizing the NR1 subunit on immunoblots, as shown in Fig. 1. R1JHL identified a single broad band with a molecular mass of ~115 kDa in samples from rat cortex (*lane 1*) and

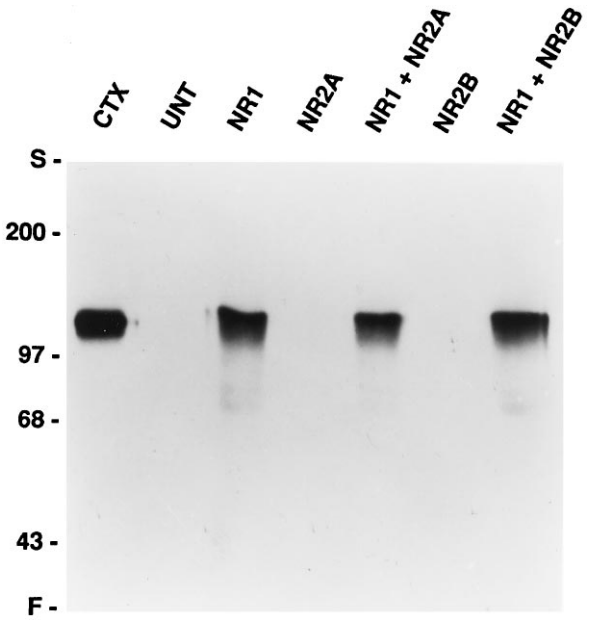

Fig. 1. Monoclonal antibody R1JHL specifically recognizes the NR1 receptor subunit on immunoblots. Membrane protein samples were from adult rat cerebral cortex (*CTX*), untransfected HEK 293 cells (*UNT*), and HEK 293 cells transfected with cDNA encoding NR1, NR2A, NR1 and NR2A, NR2B, or NR1 and NR2B. *Lane CTX* contains 3 μg of membrane protein. Each HEK 293 cell *lane* contains 1.5 μg of membrane protein. The concentration of R1JHL was 1 μg/ml. *S*, Top of the separating gel. *F*, Dye front of the gel. Positions of molecular mass standards are noted.

HEK 293 cells either transfected with NR1A alone (*lane 3*) or cotransfected with NR1A and NR2A (*lane 5*) or NR1A and NR2B (*lane 7*) but not in nontransfected HEK 293 cells (*lane 2*) or in HEK 293 cells transfected with NR2A (*lane 4*) or NR2B (*lane 6*) alone. In addition, a set of immunoblots with this antibody has been performed to detect NR1 protein in different brain regions and peripheral tissues of the rat (data not shown). In the central nervous system, NR1 is found widely distributed, with the highest expression in the hippocampus (28, 30). In 11 peripheral tissues examined (i.e., cardiac ventricle, liver, lung, stomach, kidney, spleen, adrenal gland, salivary gland, ileum, testes, and trachea), no detectable signal was found (data not shown). After deglycosylation with PNGase F, the NR1 subunit from both brain and transfected cells showed a shift in molecular size from ~115 kDa to ~97 kDa on immunoblots probed with R1JHL (data not shown). All of these observations are in agreement with previous reports using other antibodies (28, 31).

Under denaturing conditions of receptor solubilization, each antibody selectively immunoprecipitates only its corresponding subunit. To begin to examine the subunit composition of cortical NMDA receptors, we performed immunoprecipitations with each of the three antibodies and examined both the supernatants and pellets from these immunoprecipitations with each of the three antibodies. In Figs. 2 and 3, labels indicate (*left*) the antibody used to probe the blot or (*top*) the antibody used for immunoprecipitation.

When membrane proteins were solubilized and denatured by boiling in SDS, each of the three subunit-selective antibodies immunoprecipitated only its cognate subunit protein (Fig. 2), demonstrating that each of the antibodies is com-

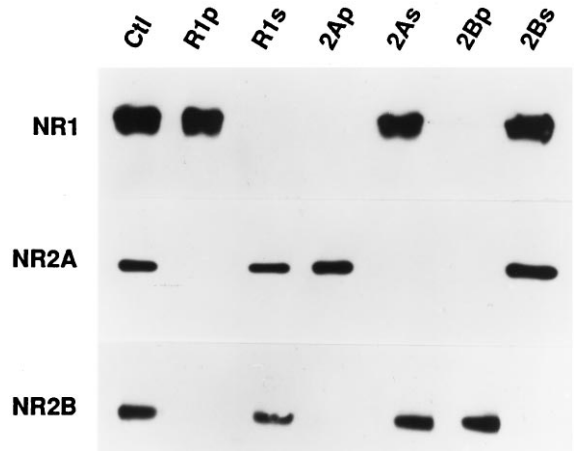

Fig. 2. Specific immunoprecipitation and immunoblotting of each of the NMDA receptor subunits solubilized under denaturing conditions. Cortical membranes were solubilized in SDS and 2-mercaptoethanol as described in Experimental Procedures. After dilution of the SDS into Triton X-100-containing buffer, proteins were immunoprecipitated with R1JHL (*R1p* and *R1s*), anti-NR2A (*2Ap* and *2As*), or anti-NR2B (*2Bp* and *2Bs*). Equal fractions of samples of the pellet (*p*) and supernatant (*s*) from these immunoprecipitations were subjected to SDS-PAGE and immunoblotted. *Lane 1* (*Ctl*), an equivalent amount of denatured cortical membrane proteins (1 μg/lane) without immunoprecipitation. The immunoblots were probed with the three antibodies (*left*), and the photographs were stacked. Thus, *row 1* was probed with R1JHL, *row 2* was probed with anti-NR2A, and *row 3* was probed with anti-NR2B.

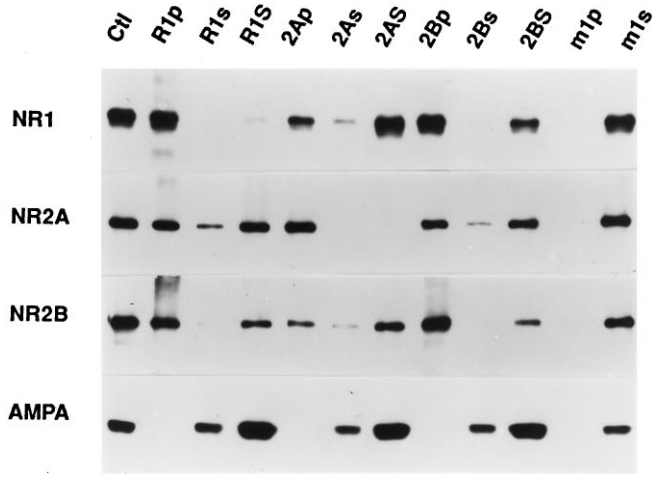

Fig. 3. Coimmunoprecipitation of NMDA receptor subunits solubilized using nondenaturing conditions. Cortical membranes were solubilized under nondenaturing conditions as described in Experimental Procedures. The soluble proteins were either not immunoprecipitated (*lane 1*; *Ctl*) or immunoprecipitated with R1JHL (*lanes 2–4*; *R1p*, *R1s*, and *R1S*), anti-NR2A (*lanes 5–7*; *2Ap*, *2As*, and *2AS*), or anti-NR2B (*lanes 8–10*; *2Bs*, *2Bp*, and *2BS*) or with an antibody directed against a fusion protein corresponding to a portion of the m1 muscarinic receptor (*lanes 11 and 12*; *m1p* and *m1s*). Top, antibody abbreviation has suffix *p* for pellet, *s* for supernatant containing the same fraction of the sample as *p*, and *S* for supernatant with 5-fold the fraction of the sample as *p* or *s* from the same immunoprecipitation. Each *p* or *s lane* in a blot contains 1-μg equivalents of original proteins. *Ctl* contains an equivalent amount (1 μg) of solubilized cortical membrane proteins without immunoprecipitation. Four immunoblots were generated with the same set of immunoprecipitation samples, probed by antibodies (*left*). The four immunoblots were stacked with each immunoblot corresponding to a row. *Row 1* was probed with R1JHL. *Row 2* was probed with anti-NR2A. *Row 3* was probed with anti-NR2B. *Row 4* was probed with anti-GluR2/3.

pletely selective and indicating a completely dissociated status among the three subunits under these denaturing conditions. For example, as shown in Fig. 2 (*top row of bands*; probed with R1JHL), the NR1 protein appears in the pellet of the NR1 immunoprecipitation (*R1p*) but in the supernatants of the immunoprecipitations with NR2A (*2As*) and NR2B (*2Bs*). Conversely, it does not appear in the pellets from the NR2A (*2Ap*) and NR2B (*2Bp*) immunoprecipitations or in the supernatant from the NR1 immunoprecipitation (*R1s*). Thus, the NR1 is quantitatively and selectively immunoprecipitated by R1JHL when the subunits are initially separated by denaturation. Similarly, the NR2A and NR2B proteins are also found precisely where they are expected (the pellets of the corresponding antibody and the supernatants of unrelated antibodies), assuming each antibody selectively immunoprecipitates only its own protein (Fig. 2, *middle and bottom rows of bands*). Thus, under the study conditions, each antibody was not only selective but also quantitative in its ability to immunoprecipitate only its corresponding subunit.

Under nondenaturing conditions of receptor solubilization, each antibody immunoprecipitates multiple subunits. Fig. 3 shows a typical result of immunoprecipitations and immunoblots with multiple selective antibodies using proteins that were initially solubilized under mild, nondenaturing conditions. Again, the precipitating antibody (*top*) and the antibody used to probe the blot (*left*) are indicated. With an initial focusing on the first row (probed with R1JHL) in Fig. 3, it can be seen that the NR1 protein is found not only in the pellet from immunoprecipitation by NR1 antibody (*R1p*) but also to some degree in the pellets from immunoprecipitations using anti-NR2A (*2Ap*) and anti-NR2B (*2Bp*) antibodies, which is in direct contrast to the result shown in Fig. 2. In Fig. 3 (*s*), columns have a fraction of the supernatant loaded that is equivalent to the fraction loaded in the columns from the immunopellet (*p*). On the other hand, in Fig. 3 (*S*), columns have 5-fold the fractional load of the other columns. This assists in visual estimation of the amounts in the pellet and supernatant fractions. Thus, for example, in Fig. 3 (*row 1*, all probed with R1JHL), the band for NR1 in the immunopellet from precipitation with anti-NR2A (*2Ap*) is darker than the band with an equivalent load from the supernatant (*2As*), indicating that >50% of R1 was precipitated by anti-NR2A because if the bands were identical, it would suggest that 50% of the protein was in each fraction. On the other hand, comparison of the same band from the pellet (*2Ap*) with the darker band from the 5× load of supernatant from the anti-NR2A immunoprecipitation (*2AS*) shows that <83% (5/6) of R1 is in the pellet. Thus, if these two bands had been equal it would have indicated that the ratio of subunit would be ~5:1 in the pellet and supernatant, respectively. A similar visual examination of the bands from the precipitation with anti-NR2B (*2Bp*, *2Bs*, and *2BS*) demonstrates that most (>83%) of NR1 is precipitated by anti-NR2B. These results are consistent with the idea that the NMDA receptor subunits have been solubilized with the *in situ* interactions between subunits remaining intact. It shows that most of the NR1 subunits in the cortex are associated with NR2A and NR2B subunits, which is consistent with current theory of NMDA receptor structure.

A more surprising finding comes on inspection of the second (probed with anti-NR2A) and third (probed with anti-NR2B) rows of Fig. 3. The three columns from the anti-NR1

precipitation (*R1p*, *R1s*, and *R1S*) demonstrate that most of the NR2A and NR2B proteins are associated with NR1 because most of NR2A and NR2B are in the immunopellets from NR1 immunoprecipitation. The next three columns from the anti-NR2A precipitation (*2Ap*, *2As*, and *2AS*) demonstrate (*row 2*) that anti-NR2A immunoprecipitates NR2A quantitatively. In the third row of these NR2A precipitations, there is the surprising finding that more than half of the NR2B subunit in adult rat cortex is associated with the NR2A subunit. Thus, comparison of the immunopellet from anti-NR2A (*2Ap*) with an equal load of supernatant (*2As*) indicates that >50% of NR2B is precipitated by anti-NR2A. Examination of the next three columns precipitated by anti-NR2B (*2Bp*, *2Bs*, and *2BS*) demonstrates that similar results are seen. Thus, it is seen (*row 2*) that the majority of NR2A is precipitated by anti-NR2B (i.e., *2Bp* is much darker than *2Bs*). The immunoprecipitation of NR2B by anti-NR2B is nearly quantitative (i.e., *2Bp* is much darker than even *2BS*) (*row 3*). These data, therefore, are consistent with the hypothesis that a large fraction of the NR2A and NR2B proteins are associated with each other as well as with NR1 in adult rat cerebral cortex.

To examine the possibility that such coimmunoprecipitations are artifacts, we performed several control experiments (e.g., Fig. 3, *row 4*). The same samples shown in the top three rows were probed with anti-GluR2/3, an antibody that recognizes both AMPA receptor subunits GluR2 and GluR3 (21). If coprecipitated artifacts were occurring because, for example, all of the membrane proteins developed nonspecific interactions (stickiness) with each other during the deoxycholate solubilization and/or the dialysis into Triton X-100, one would expect any of the antibodies to coprecipitate at least some of several proteins, including AMPA receptor subunits, regardless of whether there is an *in situ* interaction. This is not the result shown (Fig. 3, *row 4*). AMPA receptor subunits are found only in the supernatants, indicating that these proteins not only do not have an interaction *in situ* with NMDA receptor subunits but also do not form any nonspecific interactions due to the solubilization or other handling conditions of the tissue. Another control experiment is shown in Fig. 3 (*two right columns*). The possibility that any antibody coupled to protein A/Sepharose could cause nonspecific immunoprecipitation of NMDA receptor subunits is tested. As can be seen, utilization of a monoclonal antibody that recognizes a fusion protein coding for a portion of the m1 muscarinic receptor did not precipitate any GluR subunit examined. Thus, all of the proteins are found in the supernatant (*m1s*) fraction and none are found in the pellet (*m1p*) fraction. A similar experiment performed with no antibody linked to the protein A/Sepharose had identical results (data not shown).

To further address the possibility that under nondenaturing conditions of solubilization an artifactual stickiness may occur that is specific for NMDA receptor subunits (i.e., NMDA receptor subunits specifically stick to other NMDA receptor subunits even though they may not have been assembled together *in situ*), we generated transfected cells expressing NMDA receptors composed of NR1A/NR2A, NR1A/NR2B, or NR1A/NR2D. Membranes from these three cells were harvested and mixed together. The mixed membranes were homogenized, processed, and solubilized exactly as described for brain. The solubilized receptors were then immunoprecipitated with anti-NR1, anti-NR2A, anti-NR2B, or anti-NR2D (32) antibodies, and the resulting supernatants and pellets were probed with anti-NR2A and anti-NR2B (Fig. 4). The cell line used for transfection was TSA201 (derived from HEK 293) because this cell line expresses very high levels of NMDA receptors; thus, strong signals are observed. Because of the high level of expression, multiple bands are seen. The highest molecular mass band (<) runs at the same molecular size as the cognate protein in cerebral cortex (~180 kDa). The next band corresponds to the size seen on deglycosylation of NR2A or NR2B (20). The bottom molecular mass bands seen in the NR1/NR2A-transfected cells are presumably proteolytic products of NR2A because no bands are seen in TSA201 cells with either antibody in untransfected cells (data not shown). Note that long exposure times were used to more clearly demonstrate the lack of a signal in inappropriate lanes. In Fig. 4, *B* lanes (*lanes 1*, *4*, *7*, and *10*) contain a sample from before the immunoprecipitation; *P* lanes (*lanes 2*, *5*, *8*, and *11*) contain a sample from the resolubilized immunopellet; and *S* lanes (*lanes 3*, *6*, *9*, and *12*) contain a sample from the supernatant. All samples represent equal fractions of the reaction. Examination of Fig. 4 (*lanes 1–3*) demonstrates that immunoprecipitation with R1JHL (NR1) precipitates both NR2A and NR2B nearly quantitatively as would be expected because the NR2 subunits were coexpressed (and presumably assembled *in situ*) with NR1 in the same cell. Immunoprecipitation with anti-NR2A (*lanes 4–6*) quantitatively precipitates NR2A (*top*) but fails to bring any NR2B into the pellet (*lane 5*, *bottom*). Similarly, immunoprecipitation with anti-NR2B (*lanes 7–9*) fails to bring any NR2A into the pellet (*lane 8*, *top*) but precipitates nearly all the NR2B (*lane 8*, *bottom*). As a further control, a recently characterized antibody to NR2D (which quantitatively and selectively immunoprecipi-

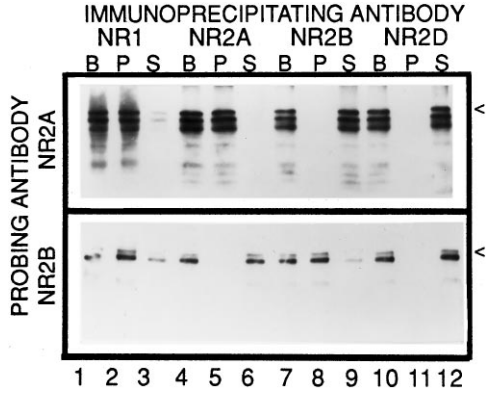

Fig. 4. Noncognate NMDA receptor subunits precipitate only if they are assembled with cognate subunits *in situ*. Three culture dishes of TSA201 (HEK-derived) cells were transfected with (*1*) NR1A/NR2A, (*2*) NR1A/NR2B, or (*3*) NR1A/NR2D cDNAs. Forty-eight hours later the cells were scraped up, the contents of the three dishes were mixed together and homogenized, and a high-speed pellet was collected. The pellet was solubilized under nondenaturing conditions as outlined in Experimental Procedures, and the high-speed supernatant (detergent-solubilized proteins) was collected. Aliquots of this soluble supernatant were immunoprecipitated with anti-NR1 (R1JHL), NR2A, NR2B, or NR2D antibodies (top). The pellet was resuspended in the same volume as the supernatant, and equal volumes were loaded in lanes marked for pellet (*P*) or supernatant (*S*). A sample was taken before immunoprecipitation (*B*), and a load equal to *P* and *S* was applied. Proteins were separated on SDS-PAGE and blotted to nitrocellulose, and the blots were probed with either (*top*) anti-NR2A or (*bottom*) anti-NR2B (*left*). Large loads and long exposure times were used to more convincingly demonstrate the absence of a protein in a lane.

tates NR2D; Ref. 32) failed to immunoprecipitate either NR2A (*lane 11*, *top*) or NR2B (*lane 11*, *bottom*).

To provide a more quantitative analysis of data such as shown in Fig. 3, four similar experiments were performed, and standard curves (see Refs. 20 and 28) were generated at the same time using dilutions of cortical membranes (Fig. 5). The relative amounts of each protein in each supernatant and pellet fraction were calculated, and the percentage of the protein found in the immunopellet of each experiment is listed in Table 1. As can be seen, anti-NR1 precipitated most of the NR1 (96%), NR2A (76%), and NR2B (84%) subunits, which is consistent with the idea that NMDA receptors contain NR1 subunits. Also, anti-NR2A and anti-NR2B immunoprecipitated their cognate subunits with >90% efficiency (Table 1). These antibodies, on average, also precipitated under these nondenaturing conditions approximately half of the other NR2 subunit, indicating that a substantial population of NMDA receptors contain at least one copy of each of the three (NR1, NR2A, and NR2B) subunits examined in this study.

The current working model of NMDA receptor subunit composition postulates that all receptors have at least one copy of the NR1 subunit. The fact that R1JHL precipitated only 76% of NR2A and 84% of NR2B could be consistent with several possibilities, two of which can be readily distinguished: 1) some complexes of NR2A and NR2B do not contain NR1, or 2) the NR2A and NR2B subunits not precipitated by anti-NR1 are "free," unassembled proteins. To address these possibilities, aliquots of the supernatant from a sample immunoprecipitated with R1JHL were immunoprecipitated with either anti-NR2A or anti-NR2B. The pellets from these immunoprecipitations were analyzed using the three antibodies. Under these conditions, anti-NR2A precipitated all of the NR2A and none of the NR2B or NR1, whereas anti-NR2B precipitated all of the NR2B but none of the NR2A or NR1 (data not shown). Thus, it seems that complexes between NR2A and NR2B do not form in the absence of NR1 and the NR2 subunits that are not immunoprecipitated by R1JHL are "free," unassembled subunits.

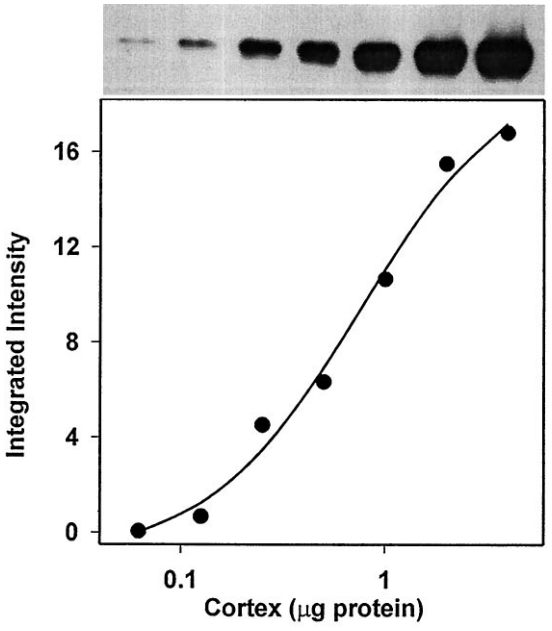

Fig. 5. Standard curve of cerebral cortex using R1JHL. Membrane proteins from cerebral cortex were solubilized, separated on SDS-PAGE, and transferred to nitrocellulose. The blot was probed with R1JHL, and the resulting film was densitometrically scanned and analyzed as described in Experimental Procedures and Refs. 20 and 28. *Lanes* were, loaded with 2-fold dilutions of the proteins ranging from 0.0625 to 4 μg. *Top*, scan of the film. *Bottom*, plot of integrated intensity versus protein concentration. Similar standard curves were included with all experiments in which quantification was performed.

TABLE 1

Quantitative estimates of coimmunoprecipitation of NR1, NR2A, and NR2B subunits with specific antibodies

Four experiments were performed. Antibodies indicated at the top were used for immunoprecipitation. Antibodies indicated at the left were used to probe the resulting blots. When no measurable signal could be detected in a supernatant (e.g., *S* in Fig. 3) lane, the estimated limit of sensitivity of the method (98%) was used in the calculation of the mean ± standard error.

Immunoprecipitated with	NR1	NR2A	NR2B
		% Precipitation	
NR1 probed	96 ± 2	56 ± 7	75 ± 5
NR2A probed	76 ± 4	97 ± 1	59 ± 8
NR2B probed	84 ± 4	48 ± 9	94 ± 2

To address the similar question of how much NR1 subunit is not complexed with either NR2A or NR2B, samples that had been solubilized under nondenaturing conditions were immunoprecipitated with a mixture of anti-NR2A and anti-NR2B. The pellets from these immunoprecipitations contained 83 ± 5% (four measurements) of the NR1, indicating that ≤17% of NR1 is unassembled or assembled with either NR2C and/or NR2D in the absence of NR2A and NR2B (Table 2). In this experiment, it should be noted that both NR2A and NR2B were quantitatively immunoprecipitated, with neither having detectable levels in the supernatant.

These experiments allowed us to begin to calculate the percentages of each of the measured subunits that exist in various combinations in the adult rat cortex. Thus, because R1JHL precipitated 76% of NR2A and this subunit does not form complexes in the absence of NR1, ~24% of NR2A is "free" (Table 2). Similarly, because R1JHL precipitated 84% of NR2B, ~16% of this subunit is "free" (Table 2). The fact that anti-NR2A precipitated 48% of NR2B demonstrates that ≥48% of NR2B is in the form NR1/NR2A/NR2B/*X* (Table 2), where *X* can be nothing or one of the NR2 subunits not measured in this study (i.e., NR2C or NR2D). Because anti-NR2B precipitated 59% of NR2A (Table 1), that percentage of the latter also seems to be in the form NR1/NR2A/NR2B/*X* (Table 2). Similarly, one can deduce the amounts of each NR2 subunit complexed with NR1 but not complexed with the other measured NR2 subunit. Thus, the 83% of NR1 that is precipitated by the addition of both anti-NR2A and anti-NR2B must be in NR1/NR2A/NR2B/*X*, NR1/NR2A/*X*, or NR1/

TABLE 2

Distribution of NR1, NR2A, and NR2B subunits in NMDA receptor complexes with varying compositions in rat cerebral cortex

Values are calculated from numbers found in Table 1 as described in the text and are presented as the percentage of a given subunit found in a given subunit composition. The value under R1/*X* indicates the percentage of NR1 not associated with NR2A or NR2B, whereas values under 2A and 2B indicate the percentage of these subunits not associated with NR1. *X* indicates that NR2C and NR2D have not been measured and may be bound to these species as well.

	R1/2A/2B/*X*	R1/2A/*X*	R1/2B/*X*	R1/*X*	2A	2B
NR1	48	8	27	17		
NR2A	59	17			24	
NR2B	48		36			16

NR2B/*X*, whereas the 56% of NR1 precipitated by anti-NR2A (Table 1) must be in either NR1/NR2A/NR2B/*X* or NR1/NR2A/*X*. Subtraction of the two values gives 27% of NR1 in the form NR1/NR2B/*X* (Table 2). Similarly, the 75% of NR1 precipitated by anti-NR2B (Table 1) is in the form NR1/NR2A/NR2B/*X* or NR1/NR2B/*X*. Subtraction of this 75% from the 83% of NR1 precipitated by antibodies to both NR2 subunits gives 8% of NR1 represented by complexes of NR1/NR2A/*X*. Finally, because all of the percentages for a given subunit must add up to 100%, the fraction of NR1 associated with NR1/NR2A/NR2B/*X* is 48% (100% − 17% − 27% − 8% = 48%; Table 2). Using similar logic, because the 76% of NR2A that is precipitated by R1JHL (Table 1) represents NR1/NR2A/NR2B/*X* and NR1/NR2A/*X* and the 59% of NR2A that is precipitated by anti-NR2B (Table 1) represents NR1/NR2A/NR2B/*X*, the difference is 17% of NR2A that is in the form NR1/NR2A/*X* (Table 2). Finally, because the 84% of NR2B precipitated by R1JHL (Table 1) represents NR1/NR2A/NR2B/*X* and NR1/NR2B/*X* and the 48% of NR2B that is precipitated by anti-NR2A represents the percentage of NR2B in NR1/NR2A/NR2B/*X*, the difference indicates that 36% of NR2B is in the form NR1/NR2B/*X* (Table 2).

Discussion

We previously reported the development and characterization of polyclonal antibodies that selectively recognize the NR2A and NR2B subunits (20). Here, we report the development and characterization of a monoclonal antibody directed against an epitope found in the amino-terminal region common to all splice variants of NR1 (amino acids 341–561; Ref. 5). This antibody recognizes a single band of an appropriate molecular size on Western blots (Fig. 1), immunoprecipitates only the corresponding subunit (Fig. 2), and recognizes only the NR1 subunit in transiently transfected HEK 293 cells using immunocytochemical techniques (data not shown). Each of these antibodies was required for the current study.

It is clear that NMDA receptors exist as heteromeric assemblies of subunits that form ligand-gated ion channels. The physiological and pharmacological properties of the channels that form depend on the subunit composition of the channel (7–12, 16). Thus, for example, NMDA receptors formed in transiently transfected HEK 293 cells from cDNAs encoding the NR1A and NR2A subunits have fast deactivation kinetics and low affinity for blockade by haloperidol, whereas NMDA receptors formed from NR1A and NR2B subunits have slow deactivation kinetics and high affinity for haloperidol (33). Little is known about the function or pharmacology of NMDA receptors that contain three distinct types of subunits such as NR1/NR2A/NR2B, but a recent abstract (34) reports that such receptors have unique properties in that they have slow deactivation kinetics (dominated by NR2B) but low affinity for haloperidol (dominated by NR2A).

Immunoprecipitation of NMDA receptors solubilized under nondenaturing conditions has been used by previous investigators to demonstrate the existence of receptors assembled from three distinct subunits (19, 35, 36). Although the methodology used in these previous studies was similar to that described for the current study, no attempts to quantify the relative levels of the various species were made.

To be able to quantify assembled species by using the methods presented here, the antibodies must be completely selective and quantitative in immunoprecipitating the corresponding subunit. Data in Fig. 2 demonstrate such selectivity and completeness of immunoprecipitation for the antibodies used in this study. Also, the immunoprecipitation of noncognate subunits must occur only if the subunit has been assembled with the cognate subunit *in situ*. This requirement seems to be demonstrated by the data in Fig. 4 and the controls in Fig. 3.

Thus, the use of a standard curve to quantify the amounts of each subunit precipitated when the receptors were solubilized under nondenaturing conditions allows the calculation of the percentage of each subunit associated with each other subunit (Fig. 3, Tables 1 and 2). From these results, we conclude that the species of NMDA receptor of highest abundance in adult rat cerebral cortex contains all three of these subunits in a single, ternary complex (NR1/NR2A/NR2B) (Table 2). Furthermore, binary species such as NR1/NR2A and NR1/NR2B seem to be present at much lower levels. It should be noted, however, that because there is no way of measuring the NR2C or NR2D subunits, it is possible that these subunits are also complexed with what we are calling ternary or binary complexes as well as being complexed with what we are calling "free" NR1.

In potential contrast to the data presented here are results from studies in which the relative levels of NMDA receptors displaying high and low affinity for ifenprodil were quantitatively examined in rat cerebral cortex as a function of development. Thus, Williams *et al*. (37) demonstrated that (^{3}H)-MK-801 binding to NMDA receptors in membranes from cerebral cortex of 3-day-old rats showed a different sensitivity to ifenprodil than was seen in adult rats. The NMDA receptors from young rats showed a homogeneous population with high affinity for ifenprodil, whereas the receptors from adult rats were heterogeneous with approximately half the receptors having high affinity and half having low affinity for ifenprodil. These data were interpreted to reflect a change in subunit composition of the NMDA receptor that occurred during development. Consistent with this interpretation is the fact that in the neonatal rat cortex, expression of the mRNA encoding the NR2B subunit is high and expression of mRNA for NR2A is very low, but NR2A mRNA levels increase dramatically during the first 3 weeks after birth (12). Sheng *et al* (19) reported and we confirmed[2] that the expression levels of the NR2A and NR2B proteins also follow a very similar pattern in the rat cerebral cortex. Because NR1/NR2B-type receptors have high affinity for ifenprodil and NR1/NR2A-type receptors have low affinity for ifenprodil (38), it is possible that the switch from all receptors having high affinity at day 3 after birth to only half of the receptors having high affinity for ifenprodil in the adult cortex corresponds to a switch from all receptors being in the form of NR1/NR2B at birth to half being in the form NR1/NR2B and half in the form NR1/NR2A in adulthood. Our data, however, do not support such a model in the adult cortex. However, if we assume that ternary receptors of the form NR1/NR2A/NR2B will have low affinity for ifenprodil as they do for haloperidol (34) and we further assume that NR2C and NR2D are such minor players in the adult cerebral cortex that they do not contribute significantly to the assembled

[2] Y. Wang and B. B. Wolfe, unpublished observations.

receptor pool (making the "free" NR1 truly free and making the binary receptors truly binary), the data in Table 2 predict that ~67% of the *assembled* receptors would have low affinity for ifenprodil (NR1/NR2A/NR2B (57%) and NR1/NR2A (10%) and ~33% would have high affinity for ifenprodil [NR1/NR2B (33%)]. These values may not be different from those (51% high and 49% low) reported by Williams *et al*. (37).

In conclusion, we presented direct evidence that NMDA receptors composed of at least three distinct subunit compositions (NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B) exist in the adult rat cerebral cortex. Furthermore, our data are most consistent with the hypothesis that the species of highest abundance is the ternary complex containing NR1, NR2A, and NR2B. Thus, it seems important to generate model systems expressing such receptors so that their properties can be determined and compared with those found for NMDA receptors in the rat cerebral cortex.

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