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Subunit Composition of *N*-Methyl-D-aspartate Receptors in the Central Nervous System that Contain the NR2D Subunit

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ABSTRACT

The *N*-methyl-D-aspartate (NMDA) receptor is assembled using proteins from two gene families, NR1 and NR2. Although a few studies have examined the composition of NMDA receptors containing NR1, NR2A, and NR2B, the composition of native NMDA receptors that incorporate the NR2D subunit is not known. The goal of the current study was to examine the subunit composition of native NMDA receptors that contain the NR2D subunit in the rat central nervous system by immunoprecipitation of assembled NMDA receptors from rat brain tissues using specific antibodies against NR1, NR2A, NR2B, and NR2D subunits. NMDA receptors were solubilized using either nondenaturing (native) conditions, in which the subunits remain assembled in complexes, or denaturing conditions, in which the NMDA subunits are dissociated from one another. Each of the antibodies selectively and quantitatively immunoprecipitated only the corresponding subunit when the subunits were solubilized using denaturing conditions. In contrast, when NMDA receptors were solubilized under nondenaturing conditions, immunoprecipitation followed by quantitative immunoblot analysis of the resulting pellets show that the majority of the NR2D protein is associated with the NR1 subunit. In addition, the NR2D subunit forms a heteromeric assembly with NR1, as well as with NR2A and/or NR2B subunits, reflecting ternary complex formation. Finally, a binary complex composed of only NR1/NR2D subunits was found in the thalamus but not in the midbrain, where the complexes always contained either NR2A or NR2B, demonstrating that in the central nervous system, different subtypes of NR2D-containing NMDA receptors are present that vary in spatial expression, perhaps indicating distinct physiological and behavioral roles.

The NMDA subtype of glutamate receptor plays pivotal roles in the mammalian central nervous system. This receptor is essential for physiological mechanisms involved in synaptic plasticity, learning, and memory (Bliss and Collingridge, 1993; Komuro and Rakic, 1993; Sheetz and Constantine-Paton, 1994). In addition, the NMDA receptors are thought to be involved in the pathogenesis of neurological and neurodegenerative disorders such as epilepsy, ischemic neuronal cell death, and Parkinson's and Alzheimer's diseases (Choi, 1988; Dingledine *et al*., 1990; Meldrum and Garthwaite, 1990; Ulas *et al*., 1992; Meldrum, 1994).

NMDA receptors are composed of proteins encoded by two subunit families of genes: NR1, consisting of eight alternatively spliced variants (Sugihara *et al*., 1992; Hollmann *et al*., 1993), and NR2, which contains four homologous subunits (NR2A, NR2B, NR2C, and NR2D), with the NR2D subunit consisting of two potential splice isoforms (NR2D-1 and NR2D-2) (Monyer *et al*., 1992; Nakanishi, 1992; Ishii *et al*., 1993). Although little is known about the subunit composition of native NMDA receptors, previous pharmacological studies of NMDA receptors using radioligand binding and electrophysiological techniques have shown evidence for heterogeneity in native NMDA receptors in the vertebrate central nervous system (Beaton *et al*., 1992; Stern *et al*., 1992; Sakurai *et al*., 1993; Laurie and Seeburg, 1994; Buller *et al*., 1997). The differences in functional properties of NMDA receptors observed after analysis of pharmacological and electrophysiological studies with recombinant NMDA receptors *in vitro* are dependent on the subunits used to reconstitute the receptors. Expression studies using *Xenopus laevis* oocytes indicate that homomeric NR1, but not homomeric NR2, receptors form functional NMDA receptor channels responsive to glutamate and NMDA. Coexpression of NR1 and NR2 subunits in these systems elicits even stronger responses to glutamate and NMDA, which is characteristic of native NMDA receptors (Moriyoshi *et al*., 1991; Kutsuwada *et al*., 1992; Ishii *et al*., 1993; Monyer *et al*., 1994; Sheng *et al*., 1994). Native NMDA receptors are therefore thought to consist of heteromeric assemblies of NR1, which is mandatory for channel activity, and NR2 subunit, which modulates the properties of the channel, in unknown stoichiometric ratios of NR1 and NR2 subunits (Ishii *et al*., 1993; Akazawa *et al*., 1994; Monyer *et al*., 1994).

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

In comparison with other NR2 subunits, NR2D is a particularly interesting NMDA receptor subunit because both its mRNA (Monyer *et al*., 1994) and protein (Dunah *et al*., 1996; Wenzel *et al*., 1996) are highly expressed in the prenatal and early postnatal brains suggesting an important role for NMDA receptors containing NR2D subunit in brain development. Moreover, coexpression of NMDA receptors composed of NR1 and NR2D subunits have been shown to possess unique electrophysiological properties, including remarkably long decay times and weak Mg^{2+} block relative to the other NMDA subunits (Monyer *et al*., 1994; Vicini *et al*., in press). These properties may be related to alterations in NMDA receptor function that have been observed in the brain during development (Carmignoto and Vicini, 1992). A few studies have examined the subunit composition of native NMDA receptors with emphasis on the compositions of NMDA receptors containing NR1, NR2A, and NR2B subunits using immunoprecipitations with subunit-specific antibodies. Results from these studies have shown that there are direct interactions *in vivo* among the different subunits of NMDA receptors (Sheng *et al*., 1994; Didier *et al*., 1995; Blahos and Wenthold, 1996; Luo *et al*., 1997). However, no studies have addressed the possibility of interactions *in vivo* involving the NR2D protein with the other NMDA subunits; hence, the compositions of native NMDA receptors that incorporate the NR2D subunit are not known.

In the current study, we used an immunoprecipitation technique with subunit-specific antibodies against NR1, NR2A, NR2B, and NR2D to investigate the composition of native NMDA receptors containing the NR2D subunit in both young and adult rat central nervous system. The results suggest that NR2D-containing NMDA receptors of differing subunit composition are present in the brain that may possess distinct physiological functions, especially in the developing central nervous system.

Experimental Procedures

Materials

The full-length NR2D-2 clone as modified at the first 80 amino acids by selective replacement of the guanine and cytosine residues with adenine and thymine residues so the amino acid sequence was not altered (Monyer *et al*., 1994) was a generous gift of Dr. P. Seeburg (University of Heidelburg, Heidelburg, Germany). TSA-201 (derived from human embryonic kidney 293 cells) cells were obtained from Dr. V. Ramakrishnan (COR Therapeutics, South San Francisco, CA). Protein A-Sepharose, dimethylpimelimidate, and Triton X-100 were from Sigma Chemical (St. Louis, MO). Sodium deoxycholate (S285–100, lot 951636) was from Fisher Scientific (Fair Lawn, NJ). Horseradish peroxidase-linked donkey anti-rabbit and horseradish peroxidase-linked sheep anti-mouse antibodies were purchased from Amersham International (Buckinghamshire, UK). The chemiluminescence detection system (Super Signal) used for the immunoblots was from Pierce Chemical (Rockford, IL). Subunit-specific antibodies for NR1 (Luo *et al*., 1997), NR2A (Wang *et al*., 1995), NR2B (Wang *et al*., 1995), NR2C (Y.-H. Wang and B. B. Wolfe, unpublished observations), and NR2D (Dunah *et al*., 1996) were developed in our laboratory.

Methods

Preparation of P2 tissue. Adult and 7-day-old (P7) rat central nervous system tissues (cortex, striatum, thalamus, midbrain, and cerebellum) from male Sprague-Dawley rats were homogenized twice using a Tekmar Tissuemizer (Cincinnati, OH) in ice-cold 10 mM Tris·HCl buffer, pH 7.4, containing 320 mM sucrose at speed 60 for 10 sec with a 20-sec interval between bursts. The tissue homogenate was centrifuged at 700 × *g* for 10 min at 4°, and the pellet was discarded. The supernatant was centrifuged at 37,000 × *g* at 4° for 40 min. This high-speed pellet (P2) was resuspended in 10 mM Tris·HCl buffer, pH 7.4, using the Tekmar Tissuemizer. Protein concentrations were determined using the BCA protein assay (Pierce) with bovine serum albumin as a standard.

Denaturing conditions for protein solubilization. The P2 membrane preparation was denatured and solubilized by being boiled in 2% SDS containing 5% β-mercaptoethanol for 5 min. The denatured membrane protein was diluted ≥20-fold using binding buffer (50 mM Tris·HCl, pH 7.4, 0.1% Triton X-100) and centrifuged at 30,000 × *g* for 10 min at 4°. The supernatant was used for the immunoprecipitation.

Nondenaturing conditions for protein solubilization. The P2 membrane preparation was solubilized by the addition of one tenth volume of 10% sodium deoxycholate in 50 mM Tris·HCl, pH 9.0, followed by incubation at 36° for 30 min. A one-tenth volume of a buffer containing 1% Triton X-100, and 50 mM Tris·HCl, pH 9.0, was added and the preparation was dialysed against binding buffer overnight at 4°. The sample was centrifuged at 37,000 × *g* at 4° for 30 min. The supernatant was used for immunoprecipitation.

Transfection of TSA-201 cells for immunoprecipitation. TSA-201 cells were transiently transfected by the calcium phosphate precipitation technique (Chen and Okayama, 1987). Twenty-four hours before transfection, cells were plated into 100-mm-diameter culture dishes at 40–60% confluency. Ten micrograms of either NR2A, NR2B, or NR2D cDNA was cotransfected with an equal amount of NR1a cDNA, and the cells were harvested 48 hr later. The medium was changed, and kynurenic acid (2 mM) was added to prevent NMDA receptor-mediated cell death 14 hours after transfection. Transfected TSA-201 cells were grown in Dulbecco's modified essential medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin (100 units/ml; Sigma), and streptomycin (100 units/ml; Sigma) in 8% CO_2. Cells from the three triplicate dishes were scraped from the dish, mixed, and collected by centrifugation (1000 × *g* for 10 min at 4°), followed by homogenization in ice-cold buffer containing 10 mM Tris-acetate, 5 mM EDTA, pH 7.4, and 320 mM sucrose using a Tekmar Tissuemizer (Tekmar, Cincinnatti, OH). The tissue homogenate was centrifuged at 700 × *g* for 10 min at 4°. The resulting supernatant was centrifuged at 37,000 × *g* at 4° for 40 min. This high speed pellet (P2) was resuspended in 10 mM Tris·HCl buffer, pH 7.4, using the Tekmar Tissuemizer.

Precoupling antibodies to Protein A-Sepharose. The affinity-purified antibodies were incubated with Protein A-Sepharose at 20 μg antibody/20 μl of hydrated Sepharose beads for 2 hr at room temperature in 100 mM borate buffer, pH 8.0, with gentle rocking and then washed with 200 mM sodium borate, pH 9.0. The Protein A-Sepharose/anti-NR1-coupled beads were incubated with 20 mM dimethylpimelimidate in the sodium borate buffer for 30 min at room temperature. The nonspecific sites on the beads then were blocked using 200 mM ethanolamine, pH 8.0, for 2 hr at room temperature (Harlow and Lane, 1988). The beads were washed with binding buffer several times and used for immunoprecipitation. This covalent coupling procedure was not necessary for the rabbit polyclonal antibodies (anti-NR2A, anti-NR2B, and anti-NR2D).

General conditions for immunoprecipitation. Solubilized membrane proteins were incubated with antibody coupled to Protein A-Sepharose beads at a ratio of ≈100 μg of solubilized membrane protein/20 μg of antibody coupled to Protein A-Sepharose beads in a total volume of 400 μl of binding buffer for 2 hr at room temperature with gentle rotation. An aliquot of the immunoprecipitation reaction was taken, and the remainder of the immunoprecipitation reaction was centrifuged briefly at 10,000 × *g* in a refrigerated Sorvall microcentrifuge. The supernatant was transferred to another tube, and an aliquot was taken. The immunopellet was washed twice with 20 volumes of binding buffer. The aliquots from the immunoprecipita-

tion reaction and the supernatants were diluted with 4× loading buffer (250 mM Tris·HCl, pH 6.8, 8% SDS, 20% dithiothreitol, 30% glycerol) to generate the samples labeled before immunoprecipitation and supernatant (see Figs. 2–6) that are at same dilution as the immunopellets. The proteins from the immunopellets denoted pellet (see Figs. 2–6) were solubilized in a suitable volume of 1× loading buffer to make the pellet directly comparable with the diluted samples labeled before immunoprecipitation and supernatant. All the samples were boiled for 5 min, and the proteins were resolved on SDS-polyacrylamide gels by loading equal volumes of all samples per lane.

Determining the optimal amount of NR2D antibody for immunoprecipitation. Varying amounts of NR2D antibody were coupled to 20 μl of hydrated Protein A-Sepharose and used for immunoprecipitation of 100 μg of solubilized membrane proteins. The protein samples were resolved using SDS-PAGE and immunoblotted with NR2D antibody. The intensities of the bands from the pellets were analyzed using densitometry, and these values were used to determine the percent immunoprecipitation for each anti-NR2D concentration. The optimal immunoprecipitation amounts for antibodies against NR1, NR2A, and NR2B subunits have similarly been determined previously (J. Luo and Y.-H. Wang, unpublished observations).

PAGE and quantitative analysis. SDS-PAGE and the transfer of proteins to nitrocellulose were performed according to the protocols of Towbin *et al*. (1979) with minor modifications as described previously (Wang *et al*., 1995). All Western blots were done with 7.5% acrylamide gels. The concentration of antibodies used for the immunoblots was 1–2 μg/ml.

To generate standard curves, 2-fold dilutions of P7 thalamic membrane proteins ranging from 0.3125 to 20 μg/lane were subjected to immunoblotting at same time as the unknown samples. The integrated intensities from the resulting bands in the standards and unknown samples from pellets and supernatant of the immunoprecipitation were determined as described previously (Wang *et al*., 1995; Dunah *et al*., 1996; Luo *et al*., 1996, 1997).

Results

A subunit-specific affinity-purified polyclonal antibody for the NMDA receptor protein NR2D was developed using peptides from the carboxyl terminus of the rat NR2D subunit and characterized as reported previously (Dunah *et al*., 1996). This antibody along with previously characterized subunit-specific antibodies against NR1 (Luo *et al*., 1997), NR2A (Wang *et al*., 1995), and NR2B (Wang *et al*., 1995) have been used to examine the subunit composition of NMDA receptors that contain the NR2D subunit in the rat brain.

Solubilization efficiency of NMDA receptors under nondenaturing conditions. The efficiency of solubilization of NMDA receptor subunits by 1% sodium deoxycholate under native conditions in the P7 rat thalamus was determined by quantitatively immunoblotting the sample taken before the addition of detergent (Fig. 1, *BEFORE*, *lanes 1 and 2*), as well as the soluble (Fig. 1, *SUPERNATANT*, *lanes 3 and 4*) and insoluble (Fig. 1, *PELLET*, *lanes 5 and 6*) fractions of the thalamic membranes. Aliquots of all the samples were diluted to the same volume, loaded at 2-fold dilutions per lane, and separated by SDS-PAGE. The resultant blots were probed with anti-NR1 (Fig. 1, *top*), anti-NR2A (not shown), anti-NR2B (not shown), or anti-NR2D (Fig. 1, *bottom*). Quantitative densitometric analysis indicates that this approach solubilizes 60–80% of the NMDA receptor subunits from P7 rat thalamus. Similar results were obtained for the adult rat hippocampal membranes (data not shown).

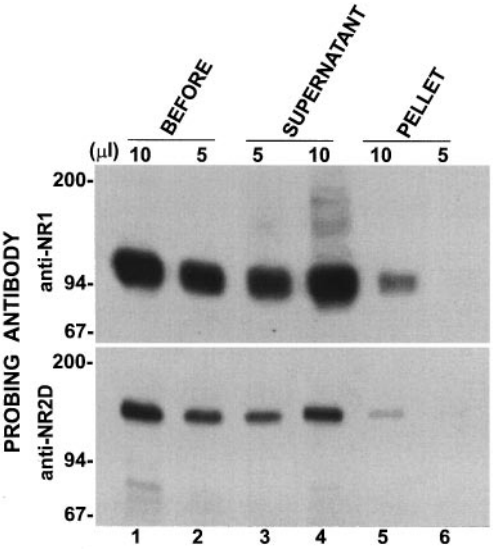

Fig. 1. Efficiency of solubilization of NMDA receptors using nondenaturing conditions. Thalamic P2 membrane fraction from P7 rat brain was solubilized as described in Methods. Aliquots from the resuspended P2 membrane (*BEFORE*; *lanes 1 and 2*), soluble supernatant from the final preparation (*SUPERNATANT*; *lanes 3 and 4*), and the final pellet (*PELLET*; *lanes 5 and 6*) were diluted with 4× loading buffer so all samples are at the same dilution. Then, 2-fold dilutions of all the samples were loaded per lane and subjected to SDS-PAGE. *Left*, immunoblots were probed with either anti-NR1 (*top*) or anti-NR2D (*bottom*).

Determination of the optimal amount of anti-NR2D antibody for immunoprecipitation and generation of standard curves for quantitative immunoblotting. To determine the amount of NR2D antibody required for optimal immunoprecipitation of the receptor from rat central nervous system tissues, equal amounts of thalamic proteins were immunoprecipitated with 2-fold dilutions of NR2D antibody (Fig. 2). Samples from the immunoprecipitates were probed with anti-NR2D (e.g., Fig. 2, *top*). Densitometric analysis was used to calculate the percent of maximal immunoprecipitation. These values were plotted versus the amount of the antibody per 100 μg of protein. A best-fit line was computed for the data as indicated in Fig. 2 (*bottom*). Similar results were obtained when the immunoprecipitation was performed using nondenaturing conditions of receptor solubilization (data not shown). Based on these data, the minimum amount of NR2D antibody that produces optimal immunoprecipitation of the receptor is 20 μg/100 μg of protein. Similar experiments have been done previously to determine the optimal amounts of NR1, NR2A, and NR2B antibodies for maximum immunoprecipitation of the respective receptors; those also were found to be 20 μg/100 μg of protein (J. Luo and Y.-H. Wang, unpublished observations). Consequently, 20 μg of antibody/100 μg of protein was used for subsequent immunoprecipitation experiments.

For the quantification and comparison of the relative amounts of NMDA subunit proteins in the pellets and supernatants of immunoprecipitations, a standard curve was used in each experiment. A series of 2-fold dilutions of solubilized membrane proteins from the P7 rat thalamus were electrophoresed and immunoblotted at the same time as the experimental samples. The integrated intensities of the resulting bands were determined by computer-assisted densitometry, and standard curves were constructed by plotting the inte-

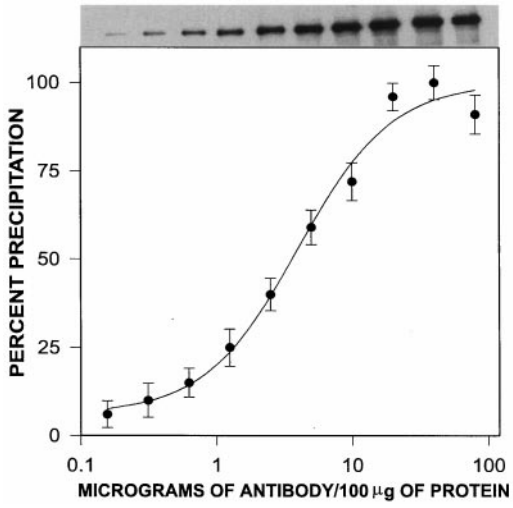

Fig. 2. Determination of the optimal amount of NR2D antibody for immunoprecipitation. Equal amounts (100 μg) of membrane proteins from P7 midbrain solubilized under denaturing conditions were immunoprecipitated using 2-fold dilutions of anti-NR2D antibody ranging from 0.15625 to 80 μg, with each coupled to 20 μl of hydrated Protein A-Sepharose resin. Samples from the pellet were subjected to SDS-PAGE, and the resulting blot was probed with NR2D antibody. *Top*, results of a typical experiment. Densitometric analysis of the resulting immunoblot was used to determine the percentage of NR2D protein immunoprecipitated by the different amounts of antibody. *Bottom*, mean ± standard error percentage precipitation obtained from three separate experiments was plotted versus the amount of antibody (in μg/100 μg of protein).

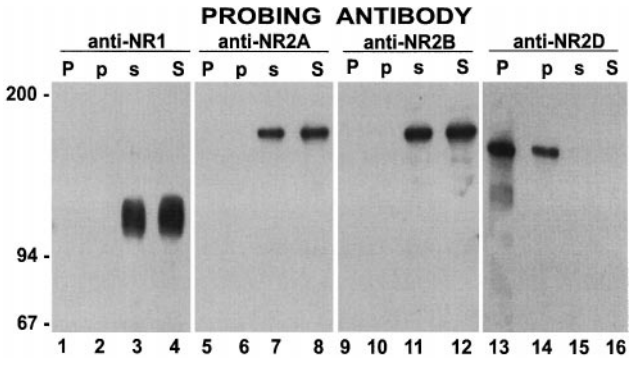

Fig. 3. Anti-NR2D specifically and quantitatively immunoprecipitates NR2D subunit solubilized under denaturing conditions. Membrane proteins from P7 rat midbrain were solubilized under denaturing conditions, and samples were immunoprecipitated using anti-NR2D coupled to Protein A-Sepharose and resolved on SDS-PAGE. *P* and *p* (*lanes 1, 2, 5, 6, 9, 10, 13, and 14*), samples from the immunoprecipitation pellet, with *P* containing twice the load as *p*. *S* and *s* (*lanes 3, 4, 7, 8, 11, 12, 15, and 16*), samples from the supernatant, with *S* having twice the load as *s*. The loads for samples *S* and *P* (20-μg equivalents) were equal, as were the loads for samples *s* and *p* (10-μg equivalents). *Top*, blots were probed with anti-NR1 (*lanes 1–4*), anti-NR2A (*lanes 5–8*), anti-NR2B (*lanes 9–12*), or anti-NR2D (*lanes 13–16*).

grated intensities versus the amount of thalamic protein in micrograms as described previously (Wang *et al*., 1995; Dunah *et al*., 1996; Luo *et al*., 1996, 1997). Similar standard curves were constructed for NR1, NR2A, and NR2B subunits and used to quantify the amounts of these NMDA subunits in the pellets and supernatants of the immunoprecipitations.

NR2D antibody selectively and quantitatively immunoprecipitates only the NR2D subunit protein under denaturing conditions of NMDA receptor solubilization. To determine the selectivity of anti-NR2D, membrane proteins from P7 rat midbrain were solubilized under denaturing conditions to dissociate the NMDA receptor subunits. Aliquots were immunoprecipitated with anti-NR2D, and the immunopellet was resuspended in a volume equal to the supernatant so equal fractional loads could be added per lane. The immunoblots were probed with anti-NR1, anti-NR2A, anti-NR2B, or anti-NR2D as indicated in Fig. 3 (*top*); anti-NR2D did not immunoprecipitate NR1 (*lanes 1 and 2*), NR2A (*lanes 5 and 6*), or NR2B (*lanes 9 and 10*) as indicated by these proteins being found in their respective supernatant (*S* and *s*) fractions. On the other hand, NR2D is found completely in the pellet (*lanes 13 and 14*), demonstrating not only that anti-NR2D is selective for its cognate subunit protein but also that the immunoprecipitation is complete under the conditions of the experiment.

A majority of NR2D protein is associated with NR1 subunit in the rat central nervous system. Several lines of evidence from coexpression and electrophysiological studies of recombinant NR1 and NR2 subunits have demonstrated that functional NMDA receptors consist of heteromeric complexes of NR1 and NR2 subunits (Ishii *et al*., 1993; Akazawa *et al*., 1994; Monyer *et al*., 1994). To determine whether the NR2D protein forms a heteromeric complex with NR1 subunit *in vivo*, NMDA receptor subunit proteins solubilized under nondenaturing conditions from P7 thalamus were subjected to immunoprecipitation using a subunit-specific antibody against NR1. Shown in Fig. 4 are the results of immunoblots from three concentrations of samples taken before immunoprecipitation, the immunoprecipitates (pellets), and the supernatants that were separated on SDS-polyacrylamide gel and probed with either anti-NR1 (*top*) or anti-NR2D (*bottom*). The immunopellet was resuspended in a volume equal to the supernatant, and 2-fold dilutions of all the samples were loaded per lane. Loading three concentrations of each sample is intended to help in the visual comparison of the amounts of the proteins in the pellets and supernatants. The samples taken before immunoprecipita-

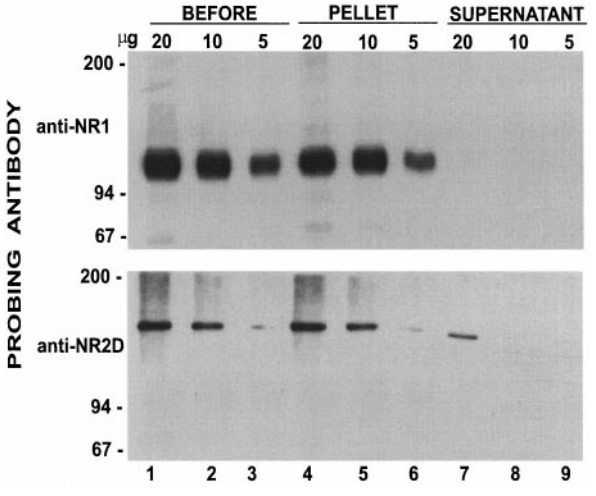

Fig. 4. A majority of NR2D subunit is assembled with NR1 subunit in the rat thalamus. Membrane proteins from P7 rat thalamus solubilized under nondenaturing conditions were immunoprecipitated with anti-NR1 and subjected to SDS-PAGE. The immunopellet was resuspended in a volume equal to that of the supernatant, and amounts loaded in microgram equivalents are shown (*top*). *BEFORE* (*lanes 1–3*), samples taken before the immunoprecipitation. *PELLET* (*lanes 4–6*), samples from resuspended immunoprecipitation pellet. *SUPERNATANT* (*lanes 6–9*), samples from the supernatant. *Left*, immunoblots were probed with either anti-NR1 (*top*) or anti-NR2D (*bottom*).

tion (*lanes 1–3*, *top* and *bottom*) are included to serve as indicators for the presence and amounts of NMDA subunits in the solubilized membrane proteins before immunoprecipitations. The anti-NR1 quantitatively immunoprecipitates the NR1 subunit as indicated by the presence of bands for NR1 protein in the pellet sample at all protein loads (*lanes 4–6*, *top*) and the corresponding absence of same bands in the supernatant (*lanes 7–9*, *top*) even at the highest load of the supernatant (*lane 7*, *top*). In addition, the NR1 antibody immunoprecipitates most of the NR2D protein, as indicated by the appearance of NR2D subunit in the pellet (*lanes 4–6*, *bottom*) after immunoprecipitation with anti-NR1. Because most of the NR2D subunit is immunoprecipitated by anti-NR1, seen by visually comparing the bands for NR2D from the pellet and supernatant, it seems that the majority of NR2D subunit is associated with NR1 in the thalamus of young rats. Similar results were obtained for adult rat thalamus (data not shown).

The NR2C subunit protein is not expressed at measurable levels in rat thalamus. The NR2C mRNA has been shown to be expressed at very high levels in the rat cerebellum and low levels in the olfactory bulb and thalamus (Ishii *et al*., 1993; Monyer *et al*., 1994). The expression of NR2C protein in adult rat cerebellum and transfected TSA-201 cells is shown in Fig. 5. Membrane proteins from untransfected TSA-201 cells, TSA-201 cells coexpressing NR1/NR2C receptor subunits, adult rat cerebellum, P7 rat thalamus, and adult rat thalamus were resolved on SDS-PAGE. The resultant blot was probed with a subunit-specific NR2C antibody (Fig. 5A), and immunoreactive bands were seen only in cells cotransfected with NR1/NR2C cDNAs and cerebellum (Fig. 5, *lanes 2 and 3*, respectively). As a control, the same blot was stripped and probed with anti-NR1 (Fig. 5B). The expression of NR1 protein was seen in cells cotransfected with NR1/NR2C cDNAs, cerebellum, and thalamus at both P7 and P49 (Fig. 5, *lanes 2, 3, 4, and 5*, respectively). Thus, in subsequent experiments, the NR2C subunit was not measured.

The NR2D subunit exists in heteromeric complexes with NR2A and NR2B subunits. To examine the possibility that the NR2D protein may form heteromeric assemblies with NR2A and/or NR2B subunits *in vivo*, immunoprecipitation was performed with anti-NR2D using proteins from P7 rat thalamus as well as adult rat cortex, striatum, and thalamus solubilized under nondenaturing conditions. A typical experiment using adult rat brain tissues is shown in Fig. 6. Equal fractions of the samples before immunoprecipitation, the immunopellet, and the supernatant were loaded per lane for each tissue. The results show that anti-NR2D immunoprecipitates NR2D protein from the cortex and thalamus (*bottom*) under nondenaturing conditions of receptor solubilization, as indicated by the presence of bands for NR2D subunit in pellet for cortex (*lane 2*) and thalamus (*lane 8*); again, this immunoprecipitation by anti-NR2D is quantitative in that no NR2D bands are found in the supernatants for cortex and thalamus (*bottom*, *lanes 3 and 9*, respectively). The striatum provides a control for this experiment because the NR2D subunit has been shown not to be expressed in this area of the rat brain (Dunah *et al*., 1996). Thus, anti-NR2D does not immunoprecipitate NR1, NR2A, or NR2B (*lanes 4–6*) in the striatum. However, anti-NR2D immunoprecipitates NR1 from the cortex and thalamus (*top*, *lanes 2 and 8*, respectively). By visually comparing the NR1 bands in the pellets and supernatants, it seems that anti-NR2D immunoprecipitates a minority (≈10%) of NR1 from the cortex (*top*, compare *lanes 2 and 3*), whereas it precipitates nearly half of the NR1 subunit from the thalamus (*top*, compare *lanes 8*

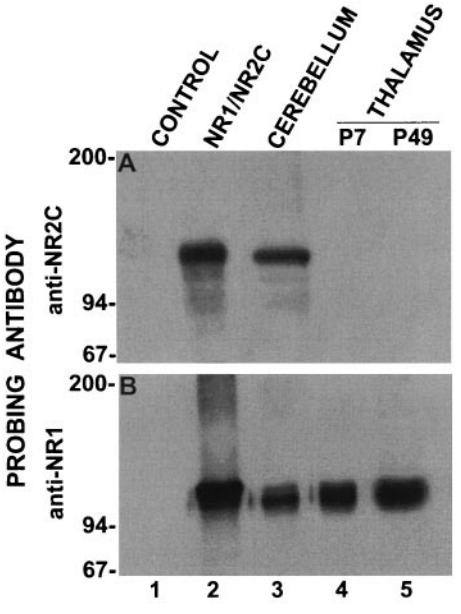

Fig. 5. Anti-NR2C antibody recognizes NR2C protein in transfected cells and cerebellum but not the thalamus of rat brain. Membrane proteins from untransfected TSA-201 cells (*CONTROL*; *lane 1*; 2 μg), TSA-201 cells cotransfected with NR1/NR2C cDNAs (*NR1/NR2C*; *lane 2*; 2 μg), adult rat cerebellum (*CEREBELLUM*; *lane 3*; 20 μg), and P7 and adult rat thalami (*THALAMUS*; *lanes 4 and 5*, respectively; 20 μg) were solubilized using nondenaturing conditions and subjected to SDS-PAGE. The resultant blot was probed with anti-NR2C (A) and stripped and reprobed with anti-NR1 (B).

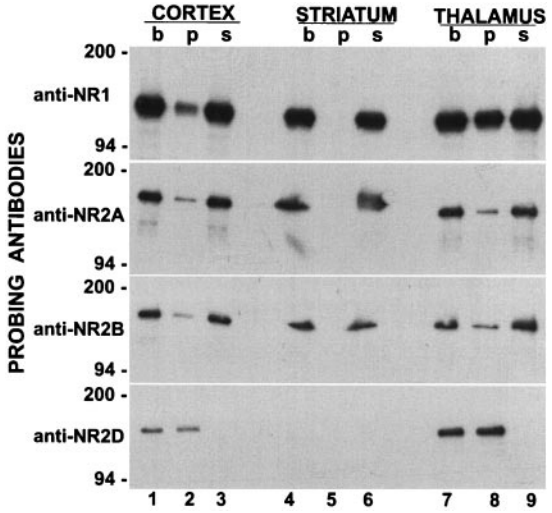

Fig. 6. In addition to the NR1 subunit, the NR2D protein is associated with NR2A and NR2B subunits in the rat brain. Membrane proteins from adult rat cortex (*left*, *lanes 1–3*), striatum (*middle*, *lanes 4–6*), and thalamus (*right*, *lanes 7–9*) were solubilized under nondenaturing conditions. The solubilized proteins were immunoprecipitated with anti-NR2D. Samples from before immunoprecipitation (*b*), the immunoprecipitation pellets (*p*), and supernatants (*s*) were brought to the same volume of loading buffer, and the proteins were separated on SDS-PAGE by loading equal amounts of each sample per lane. Each lane contains 20-μg equivalents of sample. *Left*, blots were probed with anti-NR1 (*top*), anti-NR2A (*second from top*), anti-NR2B (*third from top*), or anti-NR2D (*bottom*).

and 9), which is consistent with the report on the regional expression of NR2D protein in rat brain (Dunah *et al*., 1996; Wenzel *et al*., 1996). In addition, these data agree with the earlier observation (Fig. 4) that NR2D is assembled with NR1, as demonstrated by immunoprecipitation with anti-NR1 antibody. Of particular interest is the finding that anti-NR2D immunoprecipitates NR2A and NR2B subunits from the cortex and thalamus (*second and third from the top*, *lanes 2 and 8*, respectively). The NR2A and NR2B subunits, like NR1, also were not immunoprecipitated from the striatum by anti-NR2D, demonstrating specificity using native conditions of immunoprecipitation of NMDA receptors. Comparison of the amounts of NR2A and NR2B subunits in the pellets and supernatants of the immunoprecipitations shows that anti-NR2D immunoprecipitates only a small fraction of NR2A and NR2B from the cortex and thalamus. It is important to note that the intensities of the bands for NR2A (*second from the top*, *lanes 2 and 8*) and NR2B (*third from the top*, *lanes 2 and 8*) resulting from immunoprecipitation with anti-NR2D are too low for computer-assisted quantitative densitometry. Visual inspection of the bands (*lanes 2, 3, 8, and 9*) and visual comparison with a standard curve indicate that probably <5% of NR2A and NR2B is assembled with NR2D in the adult cortex and thalamus.

However, to quantify more accurately the relative amounts of NR1, NR2A, and NR2B subunits that are immunoprecipitated by anti-NR2D from data such as those shown in Fig. 6, four similar experiments were performed using P7 rat thalamus. This tissue was chosen for the quantitative analysis because it represents the age and region of rat brain for which the NR2D protein expression level is highest (Dunah *et al*., 1996). Standard curves were generated for each of the NMDA subunits as described in Methods and used for the quantification. The relative amounts of NR1, NR2A, NR2B, and NR2D subunits in the pellets and supernatants of immunoprecipitations were calculated, and the percentage of each subunit immunoprecipitated by anti-NR2D is shown in Table 1. It is important to note that NR2C subunit was not quantified in this experiment because the data in Fig. 5 demonstrate that the NR2C antibody (Y.-H. Wang and B. B. Wolfe, unpublished observations) does not recognize an immunoreactive band in this tissue. As shown in Table 1, anti-NR2D immunoprecipitated 93% of NR2D and 48% of NR1 subunit from the thalamus. In addition, anti-NR2D immunoprecipitated 25% of NR2A and 36% of NR2B subunits from the same tissue. These results indicate that NR2D is assembled with NR1, NR2A, and NR2B subunits, which is consistent with the hypothesis that some native NMDA receptors are composed of heteromeric complexes of NR1 and two or more different NR2 subunits (Akazawa *et al*., 1994; Sheng *et al*., 1994).

TABLE 1

Quantitative estimates of NMDA receptor subunits immunoprecipitated by NR2D antibody from P7 rat thalamus solubilized under nondenaturing conditions

Four experiments were performed using anti-NR2D as the precipitating antibody. The antibodies indicated at the top were used to probe the resulting blots. In a situation in which no measurable signal could be detected in a supernatant, the estimated limit of sensitivity of the method (98%) was used in calculation of the mean ± standard error.

	Probing antibody			
	Anti-NR1	Anti-NR2A	Anti-NR2B	Anti-NR2D
Precipitation (%)	48 ± 4	25 ± 2	36 ± 2	93 ± 3

Coimmunoprecipitation of NMDA receptors solubilized using nondenaturing conditions reveals regional variation of NR2D-containing NMDA receptor subtypes in the rat brain. The data from Fig. 6 and Table 1 provide evidence that NR2D is associated with NR1, NR2A, and NR2B subunits in heteromeric complexes, but they do not indicate whether the NMDA subunit combinations specifically are in binary or ternary complexes. With the goal to determine whether NMDA receptor binary complexes consisting of NR1 and NR2D subunits are present only in the mammalian central nervous system, sequential coimmunoprecipitation was performed of NMDA receptor proteins from P7 rat midbrain and thalamus solubilized under nondenaturing conditions using NR2A, NR2B, and NR1 antibodies. The working idea for this experiment is to first immunoprecipitate all the assembled and unassembled NR2A and NR2B subunits from the protein samples with anti-NR2A and anti-NR2B and then immunoprecipitate the remaining NMDA receptors in the supernatant with anti-NR1 and probe the resulting immunopellet with anti-NR2D and anti-NR1 antibodies. After the first immunoprecipitation, all the NR2A and NR2B subunits are cleared from the samples; the resulting supernatant contains NR1 and NR2D subunits not associated with NR2A or NR2B. Any NR2D assembled with NR1 then can be immunoprecipitated with anti-NR1 antibody. The data from such an experiment are shown in Fig. 7. Samples from before immunoprecipitation, the immunopellet, and the supernatant from the first immunoprecipitation were subjected to SDS-PAGE, and the blots were probed with anti-NR1 (first immunoprecipitation midbrain and thalamus, *lanes 1–3*), NR2A (first immunoprecipitation midbrain and thalamus, *lanes 4–6*), NR2B (first IP midbrain and thalamus, *lanes 7–9*), and NR2D (first immunoprecipitation midbrain and thalamus, *lanes 10–12*). The results show that anti-NR2A and anti-NR2B immunoprecipitated all of the NR2A and NR2B proteins from both midbrain and thalamus (first immunoprecipitation midbrain and thalamus, *lanes 5 and 8*, respectively). In addition, both antibodies immunoprecipitated some NR1 and NR2D subunits from both the midbrain and thalamus (first immunoprecipitation midbrain and thalamus, *lanes 2 and 11*, respectively), indicating that NR2D is associated with NR2A and NR2B subunits, which is in excellent correlation with the data from Fig. 6. The bands for NR1 and NR2D proteins present in the supernatants of immunoprecipitation with anti-NR2A/NR2B (first immunoprecipitation midbrain and thalamus, *lanes 3 and 12*, respectively) represent the fractions of NR1 and NR2D that are not assembled with NR2A and NR2B subunits. Bands for NR2A and NR2B proteins are not found in the supernatants of immunoprecipitation with anti-NR2A/NR2B (first immunoprecipitation midbrain and thalamus, *lanes 6 and 9*, respectively), indicating that all the NR2A and NR2B complexes have been precipitated. This result is essential for the experiment to be meaningful. In the second immunoprecipitation (Fig. 7), the supernatants from the first immunoprecipitation for both midbrain and thalamus were immunoprecipitated with anti-NR1, and the immunoblots were probed with anti-NR1 (second immunoprecipitation midbrain and thalamus, *lanes 1–3*) and anti-NR2D (second immunoprecipitation midbrain and thalamus, *lanes 10–12*). NR1 was quantitatively

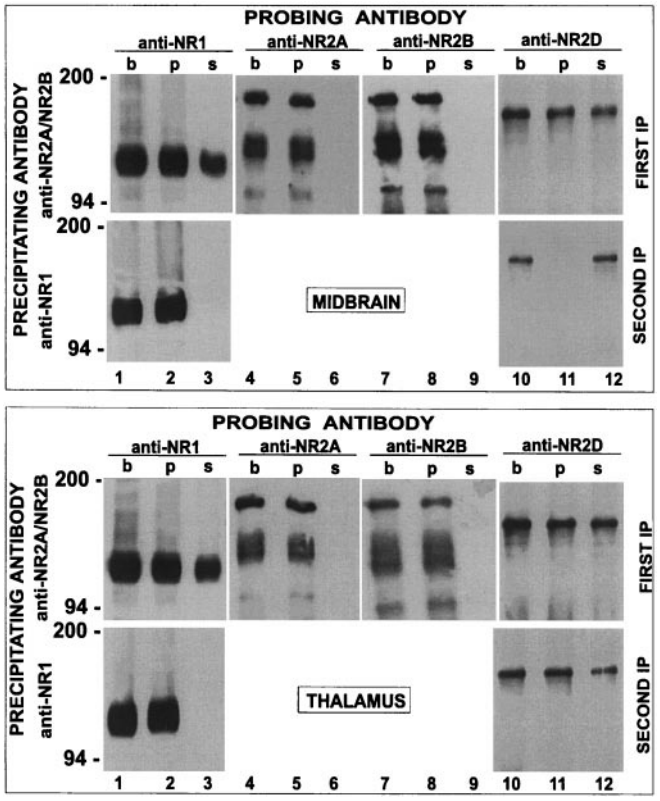

Fig. 7. NMDA receptor binary complex consisting of NR1/NR2D subunits is present in the thalamus but not the midbrain of rat central nervous system. Membrane proteins from P7 rat midbrain (*top*) and thalamus (*bottom*) were solubilized under nondenaturing conditions as described in Methods. The soluble proteins from both rat brain tissues were immunoprecipitated with a combination of anti-NR2A and anti-NR2B (anti-NR2A/NR2B; *FIRST IP*). The supernatants from the first immunoprecipitation were immunoprecipitated using anti-NR1 (anti-NR1; *SECOND IP*). *Top*, probing antibody. *Left*, precipitating antibody. Equal fractions of samples taken before immunoprecipitation (*b*; *lanes 1, 4, 7, and 10*), samples of the pellet (*p*; *lanes 2, 5, 8, and 11*) and supernatant (*s*; *lanes 3, 6, 9, and 12*) were resolved on SDS-PAGE, and the blots were probed with anti-NR1 (*lanes 1–3*), anti-NR2A (*lanes 4–6*), anti-NR2B (*lanes 7–9*), or anti-NR2D (*lanes 10–12*). *Top gels from each panel*, immunoblots resulting from immunoprecipitation with anti-NR2A and anti-NR2B (*FIRST IP*) for midbrain (*top*) and thalamus (*bottom*). *Bottom gels from each panel*, immunoblots of immunoprecipitation with anti-NR1 using supernatants from the first immunoprecipitation for both tissues (*SECOND IP*).

immunoprecipitated from both midbrain and thalamus by anti-NR1 as demonstrated by the appearance of NR1 in the pellets and its absence in the supernatants (second immunoprecipitation midbrain and thalamus, compare *lanes 2 and 3*). A particularly interesting finding is the absence of a band for NR2D subunit in the pellet from the midbrain and its appearance in the supernatant (second immunoprecipitation midbrain, *lanes 11 and 12*, respectively). In the thalamus, however, the NR2D protein was found in both the pellet and supernatant (second immunoprecipitation thalamus, *lanes 11 and 12*, respectively), and a visual comparison of this protein in the pellet and supernatant shows that >50% of NR2D is precipitated by anti-NR1. This experiment, using protein samples precleared of NR2A and NR2B subunits, shows that NR1 is associated with the NR2D subunit, forming a binary complex composed of only NR1 and NR2D subunits in the rat thalamus, and that this binary complex is not present in the midbrain. Although the NR1/NR2D binary complex was not detected in the midbrain, the NR2D subunit has been shown in this study to be associated with NR2A and NR2B subunits in both the thalamus and midbrain, demonstrating the heterogeneity inherent with NMDA receptors in the mammalian central nervous system. The NR2D protein found in the supernatant of the second immunoprecipitation may be the fraction of this subunit that is not (yet) assembled with other NMDA subunits.

Specificity of coimmunoprecipitation of NMDA receptor complexes solubilized under nondenaturing conditions with subunit-specific antibodies against the NMDA subunits. To investigate the possibility that the nondenaturing conditions used for NMDA receptor solubilization may cause the receptor subunits to aggregate, thereby resulting in artifactual coimmunoprecipitation of the NMDA receptor complexes, TSA-201 cells were generated coexpressing NR1/NR2A, NR1/NR2B, or NR1/NR2D receptors. Membranes from the three transfections were harvested, mixed together, and then homogenized and solubilized exactly as described for the brain tissues. As shown in Fig. 8 (*top*), immunoprecipitation of the solubilized NMDA receptors with anti-NR1 (Fig. 8A), anti-NR2A (Fig. 8B), or anti-NR2B (Fig. 8C) antibodies was performed, and the resulting pellets and supernatants were probed with anti-NR2D. In parallel, an-

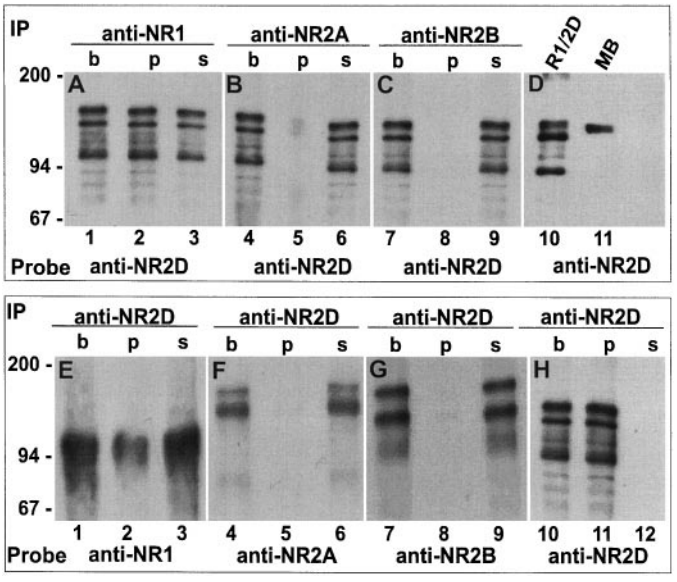

Fig. 8. Specificity of immunoprecipitation of NMDA receptor complexes solubilized using nondenaturing conditions by subunit-specific NMDA receptor antibodies. TSA-201 cells cotransfected with NR1/NR2A, NR1/NR2B, or NR1/NR2D cDNAs were harvested 48 hr after transfection and mixed together. Membranes were prepared and solubilized using nondenaturing conditions as described in Methods. For each immunoprecipitation, equal fractional loads of the sample before immunoprecipitation (*b*), the immunoprecipitation pellets (*p*), and supernatants (*s*) were resolved by SDS-PAGE. *Each lane*, 5-μg equivalents of sample. *Top*, soluble proteins were immunoprecipitated with anti-NR1 (A), anti-NR2A (B), or anti-NR2B (C) as shown (*top of the panels*), and the resulting pellets and supernatants were probed with anti-NR2D as indicated (*bottom of the panels*). D, Membrane proteins from TSA-201 coexpressing NR1/NR2D receptors (*R1/2D*, *lane 10*; 2 μg) and rat midbrain (*MB*, *lane 11*; 10 μg) were subjected to SDS-PAGE, and the blot was probed with anti-NR2D antibody. *Top* (*lanes 10 and 11*), full-length glycosylated NR2D subunit protein (Dunah *et al*., 1996). *Bottom*, another aliquot of the sample was immunoprecipitated with anti-NR2D as indicated (*top of the panels*), and the resultant immunoblots were probed with anti-NR1 (E), anti-NR2A (F), anti-NR2B (G), or anti-NR2D (H) as shown (*bottom of the panels*). Left, positions and sizes of molecular weight markers.

other sample was immunoprecipitated with anti-NR2D antibody, and the blots were probed with anti-NR1 (Fig. 8E), anti-NR2A (Fig. 8F), anti-NR2B (Fig. 8G), or anti-NR2D (Fig. 8H) as indicated in Fig. 8 (*bottom*). Anti-NR1 immunoprecipitated NR2D subunit (Fig. 8A, *lane 2*) as anticipated because NR2D was cotransfected and coexpressed with NR1 in the cells. Similarly, anti-NR2D precipitated some NR1 subunit for the same reason (Fig. 8E, *lane 2*). Immunoprecipitation with anti-NR2D antibody quantitatively precipitates the NR2D subunit (Fig. 8H, *compare lanes 11 and 12*). However, immunoprecipitation with anti-NR2A and anti-NR2B antibodies did not precipitate any detectable NR2D subunit as indicated by the absence of band for NR2D (Fig. 8B, *lane 5*, and Fig. 8C, *lane 8*, respectively). Similarly, immunoprecipitation with anti-NR2D antibody did not show any detectable levels of NR2A (Fig. 8F, *lane 5*) or NR2B (Fig. 8G, *lane 8*) in the immunopellets. These data demonstrate the specificity of immunoprecipitation of NMDA receptor complexes by the subunit-specific antibodies and seem to exclude the possibility of artifactual interactions between NMDA receptor subunits occurring due to the conditions of receptor solubilization in this study.

Discussion

Although little is known about the subunit composition and stoichiometry of native NMDA receptors, experimental studies have demonstrated clearly that native NMDA receptors exist as heteromeric complexes that form distinct functional ligand-gated ion channels whose pharmacological and electrophysiological properties are determined by the subunit composition of the receptors (Ishii *et al*., 1993; Williams, 1993; Laurie and Seeburg, 1994; Monyer *et al*., 1994; Vicini *et al*., in press). A few studies involving the immunoprecipitation of NMDA receptors from rat brain and expression systems have demonstrated that the NR1 subunit can be assembled with NR2A, NR2B, or NR2C subunits, or a combination, to form functional NMDA receptors that fit both the binary and ternary complex models. For instance, Wafford *et al*. (1993) showed the preferential coassembly of NR1/NR2A/NR2C subunits whose pharmacological properties differed from the binary complexes of these subunits in *in vitro* expression systems. The differential expression and coassembly of NMDA receptors composed of NR1/NR2B, NR1/NR2C, and NR1/NR2B/NR2C heteromers was demonstrated in the mouse cerebellum during postnatal development (Didier *et al*., 1995). In addition, Chazot *et al*. (1994) reported the existence of NMDA receptors in the adult mouse cerebellum with a pharmacological profile similar to that displayed by the coexpression of NR1/NR2A/NR2C subunits in a mammalian cell line. Sheng *et al*. (1994) and Luo *et al*. (1997) reported data that are consistent with the coexistence of a ternary complex consisting of NR1/NR2A/NR2B subunits in the adult rat cortex. Furthermore, it has been shown that there is no preferential coassembly between particular splice isoforms of NR1 and the NR2 subunits, and different NR1 splice variants can be part of the same NMDA receptor complex (Blahos and Wenthold, 1996). However, the composition of native NMDA receptors containing the NR2D subunit has not been investigated.

Previous studies using *in vitro* coexpression systems have demonstrated that recombinant NR1/NR2D receptors form ligand-gated ion channels that respond to glutamate and NMDA (Monyer *et al*., 1994; Williams, 1995; Vicini *et al*., in press). These data are consistent with the hypothesis that functional NMDA receptor channels are formed by assembly of the principal subunit NR1 with the different modulatory NR2 subunits. Moreover, it has been reported that certain thalamic nuclei express NMDA receptors with pharmacological properties consistent with the NR1/NR2D subtype receptors (Beaton *et al*., 1992; Buller *et al*., 1997). In this regard, the subunit composition of NR2D-containing receptors in the thalamus and other rat brain regions was investigated. The results indicate that most of the NR2D protein is assembled with NR1 subunit (Fig. 4). This finding is consistent with the hypothesis that functional NMDA receptors are composed of heteromeric complexes of NR1 and NR2 subunits.

In addition, the possibility that the NR2D subunit may form ternary complexes with NR1 and NR2A or NR2B subunits *in vivo* was investigated in three regions of rat brain (Fig. 6). The results demonstrate that NR1, NR2A, and NR2B subunits each were immunoprecipitated by anti-NR2D from the cortex and thalamus but not the striatum. This finding suggests that NR2D is associated with NR2A and NR2B, in addition to the NR1 subunit, forming ternary NMDA receptor assemblies containing at least NR1/NR2A/NR2D and NR1/NR2B/NR2D subunits. This result in part correlates with a previous report describing the presence of NMDA receptors in the medial thalamus of rat brain with pharmacological properties characteristic to those observed for heterotrimeric coexpression of NMDA receptors comprising a ternary complex of NR1/NR2B/NR2D subunits (Buller *et al*., 1997). In addition, these investigators reported the pharmacological properties exhibited by this ternary complex were distinct from those shown by the coexpression of binary complexes containing either NR1/NR2D or NR1/NR2B subunits. Thus, the data are consistent with the existence of heterotrimeric NMDA receptors containing the NR2D subunit in the central nervous system. The observations that a higher fraction of of NR1 protein was immunoprecipitated by anti-NR2D from the thalamus compared with the cortex and that NR1 subunit was not immunoprecipitated from the striatum of adult rat brain are in good agreement with previous reports on the regional distribution of NR2D protein in rat central nervous system (Dunah *et al*., 1996; Wenzel *et al*., 1996), which show high levels of NR2D protein expression in the thalamus, intermediate levels in the cortex, and undetectable levels in the striatum. The amounts of NR1, NR2A, and NR2B subunits immunoprecipitated by anti-NR2D antibody, which should represent the relative amounts of these NMDA subunits assembled with NR2D protein, were quantified in the P7 rat thalamus. This tissue was chosen because previous data on the regional and developmental expression of NR2D in rat brain (Dunah *et al*., 1996; Wenzel *et al*., 1996) indicate that it expresses the most of this protein. Thus, P7 rat thalamus may be a tissue in which maximal amounts of the other NMDA receptor subunits are associated with NR2D *in vivo*. As shown in Table 1, 48% of NR1, 25% of NR2A, and 36% of NR2B are associated with NR2D subunit in this tissue. These results demonstrate that NR2D is associated with NR1, NR2A, and NR2B subunits in some regions of the rat brain, which is consistent with the hypothesis that native NMDA receptors are heteromeric complexes composed of NR1 and one or two different subunits of the NR2 family in stoichiometric ratios that are not yet known.

The results described above and presented in Fig. 6 and Table 1 are consistent with the existence in the rat brain of native NMDA receptors that are ternary complexes consisting of NR1/NR2A/NR2D and NR1/NR2B/NR2D subunits. However, these data do not provide clear information as to whether binary complexes of NMDA receptors composed of NR1 and NR2D subunits only also are present *in vivo*. Interestingly, the results from Fig. 7 demonstrate that a subtype of NMDA receptor containing only NR1/NR2D subunits is present in the thalamus but not the midbrain of rat central nervous system. An alternate explanation of these data, however, could be that the NR2D protein is assembled not only with NR1 but also with NR2C subunit. Although published data on NR2C mRNA in rat brain indicate high expression levels in the cerebellum and low levels in the olfactory bulb and thalamus (Ishii *et al*., 1993; Monyer *et al*., 1994), our antibody to NR2C subunit does not show any immunoreactive band in the thalamus (Fig. 5). This may suggest either a problem with sensitivity of the NR2C antibody or a dissociation between NR2C mRNA and its protein. Nevertheless, our data are consistent with the formation of NR1/NR2D binary complexes in the thalamus but not in the midbrain.

These results suggest that different subtypes of NR2D-containing NMDA receptors representing both the binary and ternary complex models of NMDA receptor structure exist in the rat central nervous system. These subtypes seem to vary in spatial location and may be related to their functional roles in the brain. One of the possibilities generated by these data (Figs. 6 and 7, Table 1) is that quaternary (e.g., NR1/NR2A/NR2B/NR2D) NMDA receptors also are assembled in the brain. However, our experimental methods are not appropriate to address this possibility, so for now at least, this subunit combination remains only theoretical.

In conclusion, this study presents data describing for the first time a qualitative and quantitative determination of the subunit composition of native NMDA receptors containing the NR2D subunit in the mammalian central nervous system. The results demonstrate the coexistence of NR2D with other NMDA subunits forming both binary and ternary heteromers of NMDA receptors that differ in subunit composition and vary in regional localization in the central nervous system. These distinct NMDA receptor subtypes are likely to have discrete physiological and behavioral functions for NR2D-containing NMDA receptors in developing and adult brain.

References

Akazawa C, Shigemoto R, Bessho Y, Nakanishi S, and Mizuno N (1994) Differential expression of five *N*-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *J Comp Neurol* **347:**150–160.

Beaton JA, Stemsrud K, and Monaghan DT (1992) Identification of a novel *N*-methyl-D-aspartate receptor population in the rat medial thalamus. *J Neurochem* **59:**754–757.

Blahos II J and Wenthold RJ (1996) Relationship between *N*-methyl-D-aspartate receptor NR1 splice variants and NR2 subunits. *J Biol Chem* **271:**15669–15674.

Bliss TVP and Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature (Lond)* **361:**31–39.

Buller AL and Monaghan DT (1997) Pharmacological heterogeneity of NMDA receptors: characterization of NR1a/NR2D heteromers expressed in *Xenopus* oocyte. *Eur J Pharmacol* **320:**87–94.

Carmignoto G and Vicini S (1992) Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. *Science (Washington DC)* **258:**1007–1011.

Chazot PL, Coleman SK, Cik M, and Stephenson FA (1994) Molecular characterization of *N*-methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule. *J Biol Chem* **269:**24403–24409.

Chen C and Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* **7:**2745–2751.

Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1:**623–624

Didier M, Xu M, Berman SA, and Bursztajn S (1995) Differential expression and co-assembly of NMDAζ1 and ϵ subunits in the mouse cerebellum during postnatal development. *Neuroreport* **6:**2255–2259.

Dingledine R, McBain CJ, and McNamara JO. (1990) Excitatory amino acid receptors in epilepsy. *Trends Pharmacol Sci* **11:**334–338

Dunah AW, Yasuda RP, Wang Y-H, Luo J, Davila-Garcia MI, Gbadegesin M, Vicini S, and Wolfe BB (1996) Regional and ontogenic expression of the NMDA receptor subunit NR2D protein in rat brain using a subunit-specific antibody. *J Neurochem* **67:**2335–2345.

Harlow E and Lane D (1988) *Antibodies: A Laboratory Manual*, Cold Spring Laboratory, Cold Spring Harbor, NY.

Hollmann M, Boulter J, Maron C, Beasley L, Sullivan J, Pecht G, and Heinemann S (1993) Zinc potentiates agonist-induced currents at certain splice variants of NMDA receptor. *Neuron* **10:**943–954

Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M, and Nakanishi S (1993) Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. *J Biol Chem* **268:**2836–2843.

Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, Meguro H, Masaki H, Kumanishi T, Arakawa M, and Mishina M (1992) Molecular diversity of the NMDA receptor channel. *Nature (Lond)* **358:**36–41.

Komuro H and Rakic P (1993) Modulation of neuronal migration by NMDA receptors. *Science (Washington DC)* **260:**95–97.

Laurie DJ and Seeburg PH (1994) Ligand affinities at recombinant *N*-methyl-D-aspartate receptors depend on subunit composition. *Eur J Pharmacol* **268:**335–345.

Luo J, Bosy TZ, Wang Y, Yasuda RP, and Wolfe BB (1996) Ontogeny of NMDA R1 subunit protein expression in five regions of rat brain. *Dev Brain Res* **92:**10–17.

Luo J, Wang Y-H, Yasuda RP, Dunah AW, and Wolfe BB (1997) The majority of *N*-methyl-D-aspartate receptor complexes in adult rat cerebral cortex contain at least three different subunits (NR1/NR2A/NR2B). *Mol Pharmacol* **51:**79–86.

Meldrum BS (1994) The role of glutamate in epilepsy and other central nervous system disorders. *Neurology* **44:**14–23.

Meldrum B and J Garthwaite J (1990) Excitatory amino acid neurotoxicity and degenerative diseases. *Trends Pharmacol Sci* **11:**379–387.

Monyer H, Burnashev H, Laurie DJ, Sakmann B, and Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12:**529–540.

Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, Burnashev N, Sakmann B, and Seeburg PH (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science (Washington DC)* **256:**1217–1221.

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, and Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptors. *Nature (Lond)* **354:**31–37.

Nakanishi S (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science (Washington DC)* **258:**597–603

Sakurai SY, Penny JB, and Young AB (1993) Regional distinct *N*-methyl-D-aspartate receptors distinguished by quantitative autoradiography of [^{3}H]MK801 binding in rat brain. *J Neurochem* **60:**1344–1353.

Sheetz AJ and Constantine-Paton M (1994) Modulation of NMDA receptor function: implications for vertebrate neuronal development. *FASEB J* **8:**745–752.

Sheng M, Cummings J, Roldan LA, Jan YN, and Jan LY (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature (Lond)* **368:**144–147.

Stern P, Behe P, Schoepfer R, and Colquhoun D (1992) Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comparison with native receptors. *Proc R Soc Lond Ser B Biol Sci* **250:**271–277.

Sugihara H, Moriyoshi K, Ishii T, Masu M, and Nakanishi S (1992) Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochem Biophys Res Commun* **185:**826–832.

Towbin H, Staehelin T, and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:**4350–4354.

Ulas J, Brunner LC. Geddes JW, Choe W, and Cotman CW (1992) *N*-Methyl-D-aspartate receptor complex in the hippocampus of elderly, normal individuals and those with Alzheimer's disease. *Neuroscience* **49:**45–61.

Vicini S, Wang JF, Li JH, Zhu WJ, Wang YH, Luo JH, Wolfe BB, and Grayson DR (in press) Functional and pharmacological differences between recombinant NMDA receptors. *J Neurophysiol*.

Wafford KA, Bain CJ, LeBourdelles B, Whiting PJ, and Kemp JA (1993) Preferential co-assembly of recombinant NMDA receptors composed of three different subunits. *Neuroreport* **4:**1347–1349.

Wang Y, Bosy TZ, Yasuda RP, Grayson DR, Vicini S, Pizzorusso T, and Wolfe BB (1995) Characterization of NMDA receptor subunit-specific antibodies: distribution of NR2A and NR2B receptor subunits in rat brain and ontogenic profile in the cerebellum. *J Neurochem* **65:**176–183.

Wenzel A, Villa M, Mohler H, and Benke D (1996) Developmental and regional expression of NMDA receptor subtypes containing the NR2D subunit in rat brain. *J Neurochem* **66:**1240–1248.

Williams K (1993) Ifenprodil discriminates subtypes of the *N*-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. *Mol Pharmacol* **44:**851–859.

Williams K (1995) Pharmacological characterization of recombinant *N*-methyl-D-aspartate receptors containing the ϵ4 (NR2D) subunit. *Neurosci Lett* **184:**181–184.

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