Molecular Neurobiology

ISSN0893-7648/98/19(2): 151–179/$17.25

Biochemical Studies of the Structure and Function of the *N*-Methyl-D-Aspartate Subtype of Glutamate Receptors

Anthone W. Dunah,** Robert P. Yasuda, Jianhong Luo, Yuehua Wang, Kate L. Prybylowski, and Barry B. Wolfe****

Department of Pharmacology, Georgetown University School of Medicine, Washington, DC

Abstract

The *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors plays a key role in synaptic transmission, synaptic plasticity, synaptogenesis, and excitotocity in the mammalian central nervous system. The NMDA receptor channel is formed from two gene products from two glutamate receptor subunit families, termed NR1 and NR2. Although the subunit composition of native NMDA receptors is incompletely understood, electrophysiological studies using recombinant receptors suggest that functional NMDA receptors consist of heteromers containing combinations of NR1, which is essential for channel activity, and NR2, which modulates the properties of the channels. The lack of agonists or antagonists selective for a given subunit of NMDA receptors has made it difficult to understand the subunit expression, subunit composition, and posttranslational modification mechanisms of native NMDA receptors. Therefore, most studies on NMDA receptors that examine regional expression and ontogeny have been focused at the level of the mRNAs encoding the different subunits using northern blotting, ribonuclease protection, and *in situ* hybridization techniques. However, the data from these studies do not provide clear information about the resultant subunit protein. To directly examine the protein product of the NMDA receptor subunit genes, the development of subunit-specific antibodies using peptides and fusion proteins has provided a good approach for localizing, quantifying, and characterizing the receptor subunits in tissues and transfected cell lines, and to study the subunit composition and the functional effects of posttranslational processing of the NMDA subunits, particularly the phosphorylation profiles of NMDA glutamate receptors.

Index Entries: Antibodies; NMDA; western blot; immunoprecipitation; tyrosine phosphorylation; regional expression; developmental expression; subunit composition.

*Author to whom all correspondence and reprint requests should be addressed.
**Current affiliation: Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114.
***Current affiliation: Medical Molecular Biology Laboratory, Zhejiang Medical University, Hangzhou 310031, China.

Introduction

Neurons communicate by releasing chemical substances into the synapses, which then mediate excitation or inhibition of the target cell. At most excitatory synapses in the mammalian central nervous system (CNS), the released chemical substance is the amino acid L-glutamate. The field of excitatory amino acids began with the discovery of the convulsive effects of L-glutamate and L-aspartate *(1)*, and this was soon followed by the demonstration of the depolarizing and excitatory actions of these amino acids on single brain neurons *(2)*. Glutamate is now widely accepted as the principal excitatory neurotransmitter in the mammalian CNS, where it can bind to and activate a variety of receptors resulting in physiological effects and/or pathological conditions. The diverse functions of glutamatergic neurotransmission in the mammalian CNS are mediated by a variety of glutamate receptors that are classified into two major categories, termed ionotropic and metabotropic glutamate receptors, based on pharmacological, biochemical, and electrophysiological studies *(3)*. The ionotropic glutamate receptors can be subdivided into two distinct types of receptors: *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors. The non-NMDA subtype of glutamate receptors can be further subdivided into two types of receptors: α-amino-3-hydroxy-5-methyl-4-isoxazoepropionate (AMPA) and kainate receptors *(3–5)*.

The NMDA glutamate receptor subtype, which constitutes the subject of this review, is a ligand-gated ion channel characterized by high Ca^{2+} permeability, voltage-dependent Mg^{2+} block, requirement for glycine as a coagonist, and slow activation and deactivation kinetics *(6–9)*. This receptor subtype plays a crucial role in many physiological, behavioral, and pathophysiological functions of the CNS. For instance, the NMDA receptor is important for glutamate-mediated neuronal plasticity and induction of long-term potentiation that is thought to underlie learning and memory *(10–13)*. The NMDA receptor also appears to be involved in pathophysiological processes, such as epilepsy, stroke, and ischemic neuronal cell death, and may be involved in chronic neurodegenerative diseases like Alzheimer's, Parkinson's, and Huntington's diseases *(14–19)*.

Presently, two gene families encoding five NMDA receptor subunits have been cloned and characterized for both rat and mouse brain, and several of their human homologs have also been identified. It is believed that in native NMDA receptors these subunits are arranged in various combinations to generate heteromeric complexes. The exact stoichometry is currently unknown. One of the gene families of the NMDA receptors consists of NMDAR1 (NR1), which was the first NMDA receptor subunit cDNA characterized from the rat brain, and the protein it encodes has a predicted molecular mass of 103 kDa *(5)*. The open reading frame of the cDNA sequence isolated by Moriyoshi et al. *(5)* predicts a total of 938 amino acids with a series of five hydrophobic sequences originally thought to represent four transmembrane domains *(13)*. Following the characterization of the NR1 subunit, a second gene family of NMDA receptor subunits, termed NMDAR2 (NR2), was isolated from both rat and mouse brain *(20–24)*. The NR2 family consists of four members, termed NR2A, NR2B, NR2C, and NR2D for the rat subunits *(22,24)* and ∈1, ∈2, ∈3, and ∈4 for the mouse equivalents *(21, 22)*. Analysis of cDNA clones now suggests the identification of as many as eight functional isoforms of NR1 generated by alternative splicing from the primary transcript of the NR1 gene *(25,26)*. There is also a truncated isoform of the NR1 subunit, containing only the first 181 amino-terminal amino acids, that fails to generate a functional NMDA receptor channel when expressed in *Xenopus* oocytes, even though its mRNA is found in the brain *(25)*. The eight splice variants of the NR1 subunit, each of which generates functional NMDA receptors with different pharmacological properties *(26)*, were independently cloned by several groups and as a

result have been assigned different names *(20,25–30)*. Among the NR2 family, no splice variants have been reported for the NR2A, NR2B, and NR2C subunits. The NR2D subunit, however, exists in two splice isoforms, termed NR2D-1 and NR2D-2, with 1323 and 1356 amino acids, respectively *(24)*.

The expression of the NR1 subunit in *Xenopus* oocytes but not mammalian cell lines forms channels responsive to glutamate and NMDA *(5,20)*, although the electrophysiological responses are very small when compared with current responses observed for oocytes injected with poly(A)$^+$ RNA extracted from rat brain. This may be an indication that the NR1 subunit is capable of forming a homomeric structure to produce a functional NMDA receptor or it may indicate that the oocyte is contributing an NR2-like protein that can assemble with NR1 to form somewhat functional receptors. A recent report has shown that *Xenopus* oocytes express an endogenous protein that is thought to be a unitary glutamate receptor, termed *XenU1*, that interacts with the NR1 subunit to form a functional NMDA receptor *(32)*. In contrast, the expression of one or two NR2 subunits using the *Xenopus* oocyte system was not linked to the generation of an electrophysiological response following the application of glutamate or NMDA, demonstrating that the NR2 subunits may not form functional homomeric or heteromeric receptors *(23,24,31)*. Interestingly, the combined expression of each of the NR1 splice variants, but not the truncated isoform of NR1 with any of the NR2 subunits, produced highly active NMDA receptor channels with stronger responses to glutamate and NMDA *(23–25,31)*. Because of this, native NMDA receptors are believed to consist of heteromeric complexes containing both NR1 and NR2 subunits, of which the NR1 is a fundamental subunit necessary for the formation of functional NMDA receptor channel complex. The combination of NR1 with different subunits of NR2 generates NMDA receptors with functional variability in electrophysiological and pharmacological properties *(9,23,24,31,33)*. The affinities for agonists and antagonists, the kinetics of responses, the sensitivities to Mg^{2+} block, the stimulatory effects of glycine, and the modulatory effects of polyamines and histamines are different, depending on the subunit compositions of the NMDA receptors *(5,23,33)*.

A few studies have attempted to examine the heterogenous nature of NMDA receptors using pharmacological agents that recognize either particular subunits or subtypes of NMDA receptors. For instance, ifenprodil, a noncompetitive NMDA receptor antagonist, has been shown to bind homomeric NR1 and heteromeric NR1/NR2B receptors with high affinities, whereas the binding of ifenprodil to receptors composed of NR1/NR2A, NR1/NR2C, and NR1/NR2D was found to be with low affinity *(34,35)*. Similarly, NMDA receptors formed in transiently transfected HEK293 cells and in Xenopus oocytes from cDNAs encoding NR1 and NR2A were seen to have fast deactivation kinetics and low affinity for blockade by haloperidol, a dopamine receptor antagonist and therapeutically useful antischizophrenic drug. On the other hand, NMDA receptors composed of NR1 and NR2B subunits showed slow deactivation kinetics and high affinity for haloperidol *(36–38)*. These findings suggest that ifenprodil and haloperidol bind to all the cloned subunits of the NMDA receptor, but both agents appear to be more selective for the NR2B subunit.

Although ifenprodil and haloperidol can distinguish two populations of NMDA receptors in the brain, namely high- and low-affinity receptors, the lack of ligands, whether agonists or antagonists selective for a given NMDA receptor subunit, has made it difficult to understand the expression and composition of these receptors. Therefore, most studies on NMDA receptors that examine regional and developmental expressions have been focused only at the level of the mRNAs encoding the different subunits using either northern blot or *in situ* hybridization approaches *(9,23,24)*. However, the data from

these studies do not provide adequate and clear information about the resultant subunit protein. To examine directly the protein product of the NMDA receptor subunit genes, the development of subunit-specific antibodies using synthetic peptides and fusion proteins has been useful for localizing, quantifying, and characterizing the receptor subunits in tissues and transfected cell lines, and for studying the subunit compositions and post-translational modification mechanisms of native NMDA receptors *(38–46)*. To date, antibodies recognizing NR1 *(39–41,46–50)*, NR2A *(39,42,43,45,50,51)*, NR2B *(39,42,43,45,51)*, NR2C *(48,51,52)*, and NR2D *(53,54)* have been reported. The regional distributions *(41–43,45,49,51,52)*, ontogenies *(39,43,45,46,53–55)*, subunit compositions *(39,47,48,52,56,57)*, and phosphorylation profiles *(40,44,58–63)* of some of these subunit proteins have been determined, and the data demonstrate that each protein is unique in these regards. This review focuses on how antibodies have been successfully applied as useful tools in the investigations of the structure and functions of NMDA receptors revolving around hypothetical questions, such as the anatomical and ontogenic expressions, subunit compositions, and functional effects of phosphorylation of NMDA glutamate receptors.

Development and Characterization of NMDA Receptor Antibodies

The development, purification, and characterization of antibodies selective for a protein product of interest using either bacterially expressed fusion proteins or synthetic peptides as antigens has now become a common but important practice in biochemical research. This approach has been successfully and extensively used in the generation of subunit-specific monoclonal and polyclonal antibodies against the NMDA receptor proteins and has proved useful in defining the structure and function of NMDA receptors.

Fusion-Protein-Generated NMDA Receptor Antibodies

The production of fusion proteins primarily involves the subcloning of a cDNA encoding a region of interest from the NMDA receptor gene into a bacterial expression vector. The expression of the fusion protein can then be induced depending on the properties of the expression vector. Antibodies recognizing the NMDA receptor are subsequently purified from the immune serum by affinity chromatography following immunization of such animals as rabbits *(43)* or guinea pigs *(53)* with the fusion protein antigen. The same fusion protein used for immunization of animals typically is coupled to a resin, such as Reacti-Gel (6X) (Pierce, Rockford, IL), to form an affinity matrix for antibodies directed against the NMDA receptor subunit *(43,46)*. Antibodies to the carrier portion of the fusion protein (e.g., glutathione-*S*-transferase) are removed by preincubating the serum with high concentrations of the carrier protein. The main advantage of using a fusion protein as an antigen for the development of antibodies against NMDA receptors and other gene products is that the resulting large protein fragment has more potential epitopes, thereby increasing the probability of obtaining useful antibodies. However, the large size of the fusion protein can also lead to the (undesirable) production of antibodies that crossreact with other proteins.

Synthetic Peptide Generated NMDA Receptor Antibodies

Synthetic peptide antigens can induce the production of antibodies reactive with the cognate NMDA receptor subunit, and this approach of antibody development has provided specific antipeptide antibodies that have proved useful in studies of NMDA receptors *(41,42,48,53,54)*. The conventional approach for producing antipeptide antibodies involves the conjugation of a peptide to a known protein or synthetic polymer carrier, such as keyhole

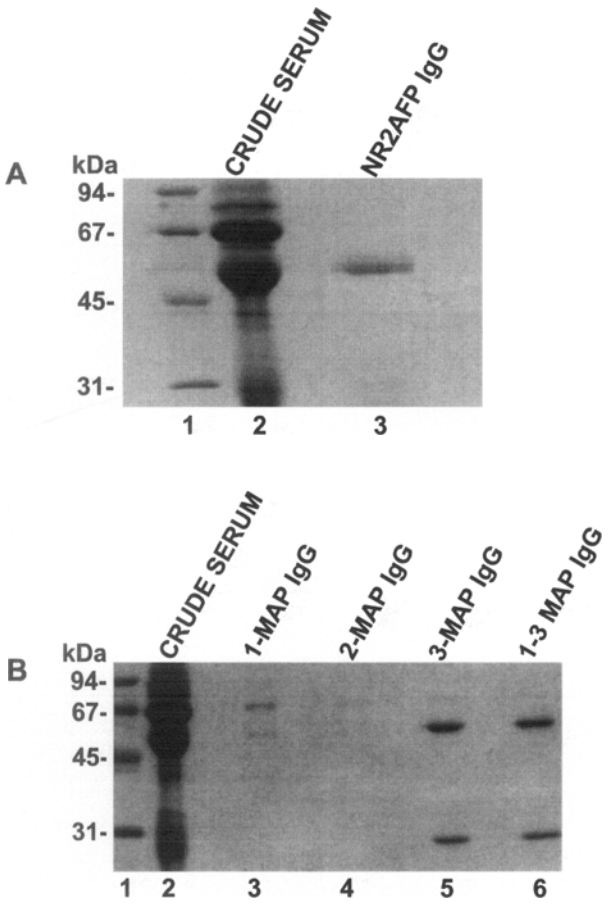

Fig. 1. **(A)** Coomassie blue stain of NR2A antibodies purified using fusion protein affinity columns. The fusion protein used for immunization of rabbits to develop antibodies against the NR2A subunit was coupled to Reacti-Gel (6X) resin (Pierce) to form an affinty column and used in the purification of antibodies directed against the NR2A subunit. Shown is a Coomassie stain of a 2 µL load of the crude antiserum prior to purification (lane 2, crude serum) and antibodies purified using affinity columns generated from the NR2A fusion protein (lane 3, NR2AFP IgG). Molecular-weight standard markers (lane 1) are indicated on the left of the figure. The 55-kDa band in lane 3 represents the heavy chain of IgG. (B) Coomassie blue stain of NR2D antibodies purified using peptide affinity columns. The linear forms of the MAP peptides used for the generation of antibody against NR2D subunit were acetylated at the N-terminus and coupled to Reacti-Gel (6X) resin (Pierce) to form an affinity matrix. Four NR2D affinity columns were made and each was used in the purification of the same volume of crude NR2D rabbit antiserum. The purified antibodies from each of the four affinity columns were resolved on SDS-PAGE, and the gel was stained with Coomassie blue. The Coomassie-stained SDS-PAGE gel shows crude antiserum prior to purification (lane 2, crude serum) and antibodies purified using affinity columns generated from the first peptide (lane 3, 1-IgG), second peptide (lane 4, 2-IgG), third peptide (lane 5, 3-IgG), and a mixture of the three peptides (lane 6, 1–3 IgG). The 55 and 25 kDa bands in lanes 5 and 6 represent the heavy and light chains of IgG, respectively. Lane 1 contains molecular weight markers as indicated on the left of the figure.

limpet hemocyanin, to give a macromolecular structure to the antigen suitable for immunization *(64,65)*. A carrier is needed for most peptide antigens to render them immunogenic, because synthetic peptides are too small to render them visible to the immune system. A novel approach for generating antipeptide antibodies is the use of Multiple Antigen Peptides (MAP), developed by Tam *(64)*. This has been successfully employed in generating antipeptide antibodies against NMDA receptor subunits *(48,53,54)*. In the MAP system, multiple copies, usually eight, of the peptide are synthesized onto a nonimmunogenic lysine backbone *(64,65)*, and the resulting product is directly employed as a peptide antigen for immunizing animals. Antipeptide antibodies are usually purified by affinity chromatography prior to use. The MAP product itself is often not useful in generating an affinity column but the linear form of the antigen used for immunization can be conjugated to a resin, such as Reacti-Gel, to form an affinity column for purification of antibodies. The use of synthetic peptides as antigens has the potential advantage of generating monospecific antibodies with low crossreactivity. A common disadvantage of the synthetic peptide approach of antibody generation is the low probability of correctly choosing a good epitope. Two examples of the successful application of both the fusion protein and synthetic peptide approaches

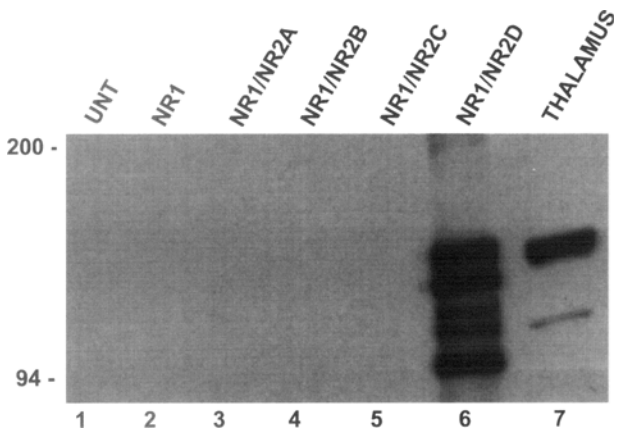

Fig. 2. Characterization of affinity-purified anti-NR2D receptor subunit-specific antibody. Western blot analysis using transfected HEK293 cells and a rat brain tissue was performed. Membrane protein samples from untransfected HEK293 cells (lane 1; UNT), cells transfected with NR1a cDNA (lane 2; NR1), cells cotransfected with NR1a and NR2A cDNAs (lane 3; NR1/NR2A), cells cotransfected with NR1a and NR2B cDNAs (lane 4; NR1/NR2B), cells cotransfected with NR1a and NR2C cDNAs (lane 5; NR1/NR2C), and cells cotransfected with NR1a and NR2D cDNAs (lane 6; NR1/NR2D) and rat thalamus (THALAMUS) were separated on an SDS gel. Each lane was loaded with 1 μg of membrane protein from the cells and 10 μg of thalamic tissue. The positions of molecular weight markers are indicated on the left of the figure. In lane 6 the upper band corresponds to the full-length, glycosylated subunit seen in brain (lane 7), the second band corresponds to unglycosylated, full-length subunit (*see* Fig. 3), and the rest of the bands appear to be truncated or proteolyzed portions of the NR2D protein.

of antibody generation are shown in Fig. 1. Figure 1A shows the immunoglobulins (IgG), obtained from a rabbit and purified from a crude antiserum, that have been immunized with a fusion protein containing a unique portion (carboxy terminus amino acids 934–1442) of the NR2A subunit. Figure 1B shows the IgG purified from a crude antiserum obtained from a rabbit that had been injected with a mixture of three MAPs, all from the carboxy terminus the NR2D subunit. Note that only one (3-MAP; lane 5) of the three antigens produced antibodies.

The production of an NMDA receptor antibody following animal immunization with either the fusion protein or synthetic peptide antigen can be monitored and quantified over time, using the respective antigen, by the enzyme-linked immunosorbent assay (ELISA). However, it is important to determine whether the antibody generated recognizes the native NMDA receptor subunit protein from which the antigen is derived. Expression of the cDNA encoding the receptor subunit in a eukaryotic cell line by either transient or stable transfections yields tissues suitable for screening of the antibody by Western blot. The specificity of the antibody can be determined using membranes from cells transfected with the cDNAs encoding the various NMDA receptor subunits *(43,46,54)*. An example of this approach of antibody characterization is shown in Fig. 2. The molecular size of the resulting immunoreactive band can be compared to the molecular size of the subunit protein as deduced from its amino acid sequence, and questions examining posttranslational modifications, such as N-linked and O-linked glycosylation, can be addressed. The native and recombinant receptor subunits can be compared with regard to molecular size and types of posttranslational modifications.

Biochemical Studies of NMDA Receptors Using Subunit-Specific Antibodies

The advent of antibody development technology has provided an avenue whereby subunit-specific antibodies against the various NMDA receptor proteins are generated and used as biochemical tools for research. In effect, this approach now provides clear information on the structure and function of NMDA glutamate receptor subunit proteins in the mammalian CNS.

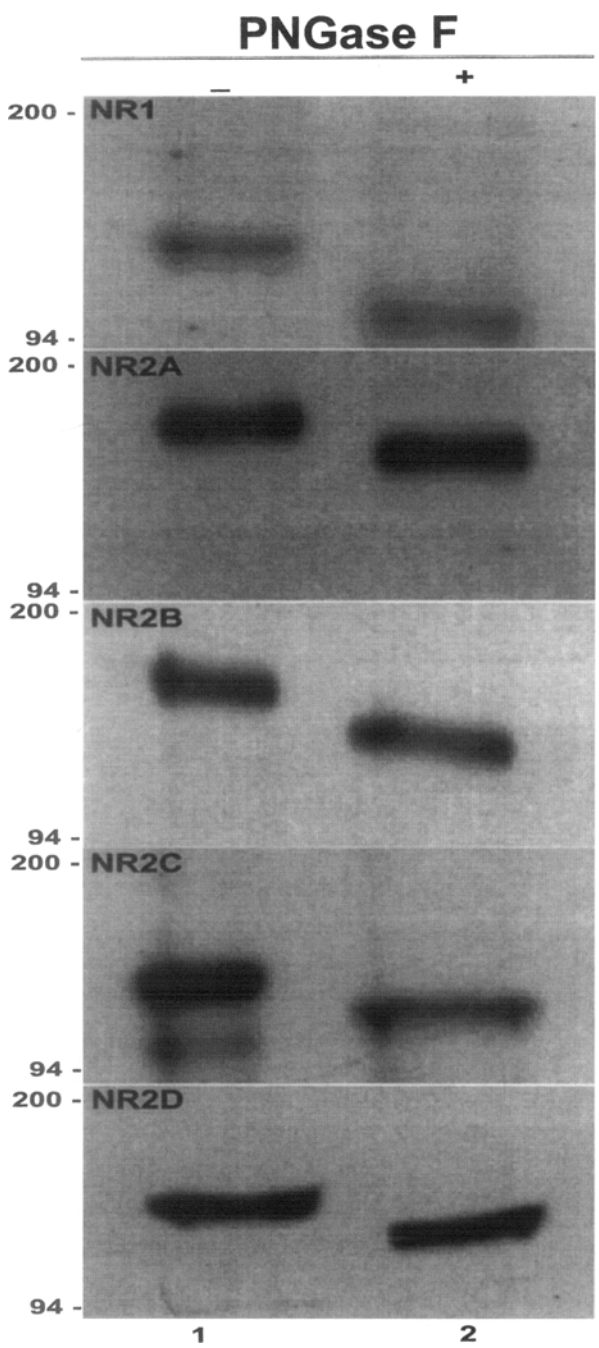

Fig. 3 *(Right).* Enzymatic deglycosylation of NMDA receptor subunits expressed in the rat brain reveals that native NMDA subunits are N-linked glycoproteins. Membrane proteins from adult rat cortex (NR1, NR2A, and NR2B), cerebellum (NR2C), or thalamus (NR2D) were incubated either without (-), or with (+) the glycosidase enzyme (PNGase F). The membrane proteins were resolved on SDS-PAGE and the resultant blots were probed with anti-NR1 (first panel; NR1), anti-NR2A (second panel; NR2A), anti-NR2B (third panel; NR2B), anti-NR2C (fourth panel; NR2C), or anti-NR2D (fifth panel; NR2D). The positions and sizes of molecular weight markers are shown on left of the figure.

Regional Expression of NMDA Receptor Subunit Proteins

NR1 Subunit Protein

The NR1 subunit mRNA has been shown to be ubiquitously expressed in neuronal cells throughout the CNS *(5,9,26,27)*, including the spinal cord *(66)*. On the other hand, the NR1 subunit splice variant mRNAs have been reported to be differentially distributed in various regions of the rat CNS *(33, 67)*.

Biochemical analysis of NMDA receptors in the adult rat brain showed that a subunit-specific antibody against the NR1 subunit detected a single immunoreactive band migrating at an apparent molecular mass of about 115–117 kDa on immunoblots *(46,48)*. The band was consistent with the predicted molecular mass of the NR1 subunit protein as deduced from its amino acid sequence of approx 105 kDa *(5)*, taking into account possible contributions in molecular mass from post-translational modification processes, such as N-linked glycosylation. The deglycosylation of the NR1 protein in both rat brain (Fig. 3; panel NR1) and transfected cells using *N*-glycosidase F enzyme caused a shift in molecular size from about 115 to about 97 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), indicating that the NR1 subunit is an N-linked glycoprotein *(46)*. Studies on the regional distribution of NR1 protein in rat CNS by quantitative immunoblotting technique shows that this subunit is expressed in the cortex, hippocampus, olfactory bulb, midbrain, and cerebellum, as indicated in Fig. 4, with a molecular size of about 115 kDa on immunoblots *(46)*. In a similar investigation that employed conventional

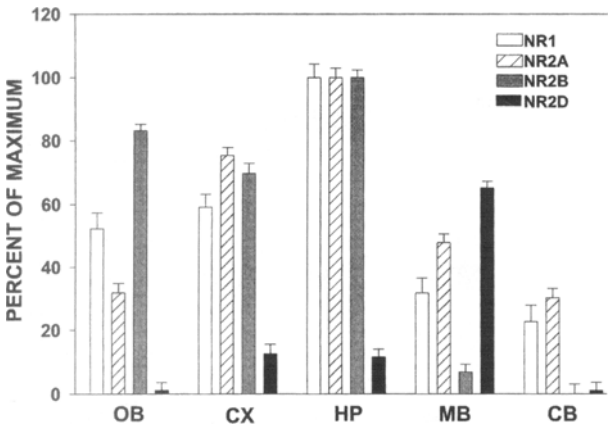

Fig. 4. Regional distribution of NMDA receptor subunits in the adult rat brain. Adult rat brain regions were dissected and homogenized. Membrane proteins from olfactory bulb (OB), cortex (CX), hippocampus (HP), midbrain (MB), and cerebellum (CB) were subjected to SDS-PAGE by loading 5 (NR1, NR2A, and NR2B) and 10 μg (NR2D) of protein per lane, and the immunoblots were probed with antibodies against NR1, NR2A, NR2B, and NR2D subunits. Results of the experiments were analyzed using computer-assisted densitometry. Values from the brain regions that gave the darkest band intensity were used as the maximum (100%) for the subunits. Hippocampus (Hp) gave the maximum values for NR1, NR2A, and NR2B subunits *(43,46)*, whereas thalamus (not shown) gave the darkest band intensity for NR2D *(54)* and was accordingly used as the 100% value for quantitation of the NR2D subunit. Data are mean ± SE (bars) obtained from three rats.

western blot analysis, NR1 subunit expression was found in the cerebral cortex, hippocampal formation, olfactory bulb, thalamus, caudate-putamen, brainstem, and cerebellum *(68)*. These studies demonstrate a ubiquitous expression profile for the NR1 subunit in rat brain. Consistent with the NR1 immunoblot data are immunocytochemical findings showing that the NR1 protein has a distinct anatomical expression in neuronal cells in addition to its widespread distribution in the rat CNS. Thus, using a immunocytochemical technique with subunit-specific antibodies against NR1, Petralia et al. *(41)*, Brose et al. *(49)*, and Aoki et al. *(68)* reported prominent immunostaining for NR1 in all layers of the cerebral cortex, the pyramidal cells of CA1, CA2, and CA3 of hippocampal formation, the mitral cells, olfactory nerve, and granule cells of the olfactory regions, the caudate-putamen, nucleus acumbens, globus pallidus, and substantia nigra of the basal ganglia, neuronal nuclei of the thalamus, hypothalamus, and brainstem, the granule cells of the dentate gyrus, and the granule, Purkinje, and Golgi cells, including the deep cerebellar nuclei of the cerebellum. NR1 immunolabeling was not seen in nonneuronal tissues and glia, suggesting that it may be exclusively localized to neuronal cells and that glial cells do not express NMDA receptors. Most of the immunostainings for NR1 protein were confined to the dendrites, dendritic spines, and postsynaptic membranes of cells *(41,49,68)*, indicating that NR1 is predominantly expressed as a postsynaptic receptor. Noteworthy, however, is the fact that several authors have observed that a sizable fraction of the NR1 protein is found intracellularly *(41,49)*. The functional significance of such a pool of receptors is currently unclear. Collectively, the data on the regional expression of the NR1 subunit protein in the rat CNS determined by either immunoblot or immunocytochemistry match the distribution of the mRNA encoding the NR1 subunit, which has previously been shown to be ubiquitously expressed in the brain by either Northern blot analysis (5) or *in situ* hybridization studies *(5,9,26,69)*.

Also, a good correlation is observed when the immunoblot data on distribution of NR1 is compared to reports on density of NMDA receptors obtained from ligand binding studies in mammalian brain. Cik et al. *(50)* initially showed that when mammalian cells are transfected with either the cDNA encoding NR1 alone or with cDNAs encoding both NR1 and NR2A subunits, binding activities for [^{3}H]MK-801 were obtained from both types of receptors, indicating that the NR1 subunit is involved in [^{3}H]MK-801 binding. In line with

this, ligand binding and autoradiography studies using either either [^{3}H]MK-801 or L-[^{3}H]glutamate in both young and adult rat and mouse brain have revealed several binding sites with differing intensities in the CNS *(43,48,50,70–72)*. These findings reflect the widespread expression of NR1 subunit and suggest that most NMDA receptors in the brain contain at least one NR1 subunit.

Most immunolocalization studies involving immunocytochemistry have revealed only the existence of postsynaptic NMDA receptors on the postsynaptic densities and dendrites, and dendritic spines of neurons in the CNS *(41,49,68)*. An antibody directed against the NR1 subunit showed that the NR1 subunit was exclusively expressed on postsynaptic structures of the CA1 region of the hippocampus on immunocytochemistry *(73)*, which correlates well with the report from Petralia et al. *(41)*. In the spinal cord, however, NR1 immunostaining was found in the presynaptic terminal adjacent to the vesicle release site at the active zone in about 30% of labeled synapses, demonstrating that glutamatergic terminals in the spinal cord dorsal horn neurons express presynaptic NMDA receptors. Further experiments revealed more than 70% of the NR1 immunoreactive terminals to be positive for glutamate, suggesting that the presynaptic NR1 receptor was an autoreceptor that might be important for contributing to neuronal plasticity by increasing the release of neurotransmitters from primary afferent fibers *(73)*. In a separate investigation that involved immunoelectron microscopy, NR1 immunoreactivity was identified along the presynaptic and postsynaptic membranes of adult rat visual cortex *(68)*, demonstrating the existence of NR1 protein as presynaptic and postsynaptic receptor. Such biochemical studies are especially important in providing information about the cellular mechanisms for the involvement of NMDA receptors, particularly in postsynaptic membrane excitability and regulation of neurotransmitter release by neuronal cells of the CNS.

NR2 Subunits

In contrast to the ubiquitous expression of NR1 mRNA, the anatomical expression of the four NR2 subunit mRNAs shows overlapping but restricted patterns in the adult rat brain. For instance, NR2A mRNA is widely distributed in the brain, but high expression levels are found in the cerebral cortex, hippocampal formation, and cerebellar cortex, whereas the NR2B subunit mRNA is more selectively expressed in the forebrain, with high levels of expression in the cerebral cortex, hippocampal formation, septum, caudate-putamen, olfactory bulb, and thalamus *(9,26,27,30)*. The mRNA for NR2C subunit is expressed predominantly in the cerebellum, with weak expression levels in the olfactory bulb and thalamus, whereas the NR2D mRNA expression is mainly confined to the thalamus, brainstem regions, and spinal cord *(9,27)*.

The distinct distributions of NR2A and NR2B subunits have been determined in the rat brain using selective antibodies for NR2A and NR2B by the quantitative immunoblot technique. The NR2A and NR2B subunits were reported to be glycoproteins having full-length apparent molecular masses of approx 175 and 180 kDa, respectively (Fig. 3; panels NR2A and NR2B, respectively). The NR2A protein was found to be expressed at its highest level in the cerebral cortex and hippocampus, with intermediate levels detected in the striatum, olfactory tubercle, midbrain, olfactory bulb, and cerebellum, and lowest levels in the pons-medulla (Fig. 4). On the other hand, the expression of the NR2B protein was seen to be at the highest levels in the olfactory tubercle, hippocampus, olfactory bulb, and cerebral cortex. Intermediate levels of NR2B subunit were expressed in the striatum and midbrain, and low levels were detected in the pons-medulla *(43)*, as shown in Fig. 4. In a similar study, Portera-Cailliau et al. *(45)* reported data on the regional distributions of NR2A and NR2B proteins in the rat CNS which closely matches results from Wang et al. *(43)*. However, the reports on NR2A protein expression in the

cerebellum by the two independent investigators are different. Wang et al. *(43)* observed intermediate levels of NR2A protein in the cerebellum, whereas Portera-Cailliau et al. *(45)* found that low levels of NR2A protein could only be detected after longer exposures of western blot films. Consistent with findings of Wang et al. *(43)* are data from histo-blot studies of the regional distribution of the NR2A and NR2B subunits in rat brain using subunit-specific anti-NR2A and anti-NR2B antibodies, which showed strongest NR2A immunoreactivity in the hippocampus, cerebral cortex, cerebellum, thalamus, and striatum, whereas the NR2B immunoreactivity was restricted to the rat forebrain regions, such as hippocampus, striatum, olfactory bulb, and cerebral cortex *(51)*. In fact, such a discrepancy in biochemical data can be postulated to be because of variations in the sensitivities of the antibodies in recognizing their corresponding receptors, or differences in the processing and denaturing of tissues for immunoblot. It should be noted, though, that data on the regional distributions of mRNA encoding the NR2 subunits in the cerebellum determined by *in situ* hybridization *(26,27,30)* show prominent expression of NR2A mRNA in the cerebellum, which supports the results of Wang et al. *(43)*. The immunoblot data on the regional distribution of the NR2A and NR2B proteins appear to be generally consistent with the distribution of the mRNA encoding their cognate subunits *(26,27,30)* with the exception of the result on NR2A protein expression in the cerebellum reported by Portera-Cailliau et al. *(45)*.

Immunocytochemical studies that examined the anatomical distribution of the NR2A and NR2B subunits in the rat brain using antibodies recognizing both the NR2A and NR2B subunits showed significant immunostaining throughout the CNS. The NR2A and NR2B subunits were prominently immunostained in the olfactory bulb, cerebral cortex, hippocampus, caudate-putamen, brainstem nuclei, and neurons of the spinal cord and sensory ganglia *(42)*, which concurs with the immunoblot data on regional expression of NR2A and NR2B subunits in rat brain *(43,45,51)*. It has been reported that immunolabeling for NR1, NR2A, and NR2B subunits were not observed in non-neuronal tissues. In addition, a clearly defined staining of the glia for NR2A and NR2B subunits was not seen in rat brain *(41,42)*. These results may suggest that the synthesis of NMDA receptors may be restricted only to neuronal cells and that glia cells do not make NMDA receptors. In contrast to the observation that NR1 protein was prominently immunolabeled in the Purkinje cells of the cerebellum *(41)*, the immunostaining for NR2A and NR2B was undetectable in these cells. These findings are in accord with a report from *in situ* hybridization studies showing that significant levels of NR1 mRNA are expressed in Purkinje cells of the cerebellum compared to low or undetectable levels of mRNA for NR2 subunits *(5)*. However, recently Cull-Candy et al. *(74)* have shown that Purkinje cells contain NMDA receptor-mediated responses consistent with NR1/NR2D subunit composition.

Overall, the immunohistochemical distributions of NR2A and NR2B show a similar pattern to that of the NR1 protein, indicating that the NR1 and NR2 subunits are colocalized in most neurons in the CNS. This finding has been further supported by data from *in situ* hybridization studies *(23,24,30)*. The colocalization of NR1 and NR2 subunits in neurons of the brain is pivotal to the formation of functional NMDA receptors of varying subunit compositions, as demonstrated by physiological and pharmacological studies *(9,23,36,38,75)*.

The observation that strongest immunoreactivities for NR2A and NR2B *(43,45,51)* and NR1 *(41,46,49,68)* subunits are seen in the cerebral cortex and hippocampus relative to other brain regions, especially the cerebellum *(43)*, appears to be in good agreement with the distribution data for these subunits obtained from ligand-binding and quantitative autoradiography studies *(70,76–78)*. The results from these studies have shown the presence of very high binding sites, especially for [^{3}H]MK-801, representing the distribution of receptor densities in the cerebral cortex and hippocampus

compared to the cerebellum, suggesting a regulation in the regional expression of NMDA subunits in the CNS.

The expression of NR2C protein has been demonstrated in the mouse cerebellum using antibodies selective for NR2C subunit *(51)*. Similarly, we have identified, using a subunit-specific NR2C antibody, that the NR2C protein, like the other NMDA receptor subunits, is posttranslationally modified by *N*-glycosylation. This is because treatment with *N*-glycosidase F enzyme decreased the apparent molecular size from approx 140 kDa to about 130 kDa in rat brain as shown in Fig. 3 (panel NR2C). A similar result was also obtained in cells transfected with cDNAs encoding NR1 and NR2C subunits and treated with the enzyme *N*-glycosidase F (Wang and Wolfe, unpublished results). In addition, we found that the highest level of NR2C protein expression is in the cerebellum, with undetectable levels in the other rat brain areas examined (Wang and Wolfe, unpublished results).

Moreover, histo-blot analysis of the regional expression of the NR2C protein using a subunit-specific NR2C fusion protein antibody demonstrated that NR2C immunoreactivity was present in only three rat brain regions, with strong expression found in the cerebellum and comparably weak and faint immunostaining seen in the thalamus and olfactory bulb, respectively *(52)*. This result parallels the data on the regional expression of mRNA for NR2C in rat brain, which indicate high expression levels in the cerebellum and low levels in the olfactory bulb and thalamus *(9,27)*. However, our antibody only showed immunoreactivity for NR2C in the cerebellum and did not recognize the receptor in the olfactory bulb and thalamus. This mismatch between the protein and mRNA for NR2C may represent a commonly encountered problem in studies with antibodies, i.e., the sensitivity of the antibody in detecting its cognate protein, especially in brain regions in which the expression of the protein is very low, such as the olfactory bulb and thalamus in this case.

The NR2D is the most recently cloned among the NMDA glutamate receptor subunits. NR2D appears to be a particularly interesting subunit because both its mRNA *(9)* and protein *(53,54)* are highly expressed in neonatal and prenatal brain, suggesting a significant role 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 receptor subunit combinations *(9,27,38)*. These properties may be related to the alterations in function observed in NMDA receptors during development *(78)*. The expression of the NR2D protein has been shown to be mainly restricted to the diencephalic, mesencephalic, and brainstem structures in adult rat brain using both immunoblot and histo-blot methods, with subunit-specific antibodies developed against the NR2D-2 splice isoform of the NR2D subunit *(51,53)*. The NR2D protein signals were found to be strongest in the thalamic regions, including subthalamic nuclei, globus pallidus, and brainstem, whereas only weak to faint bands could be detected in other brain areas, such as olfactory bulb, cerebral cortex, striatum, hippocampal formation, and cerebellum *(51,53)*.

In a similar study, the NR2D subunit was demonstrated to be an N-linked glycoprotein (Fig. 3; panel NR2D), and its relative densities in young and adult rat brains were determined by quantitative immunoblot using a selective antibody for NR2D generated from a peptide antigen common to both splice isoforms of the NR2D subunit *(54)*. In both young and adult rat brains, the NR2D protein was found to be expressed at highest levels in the thalamus, midbrain, medulla, and spinal cord, whereas intermediate levels of this subunit protein were detected in the cortex and hippocampus, and low or undetectable levels of NR2D were seen in the olfactory bulb, striatum, and cerebellum *(54)*. Figure 4 illustrates the regional distribution of the NR2D protein in some regions of the adult rat brain. The immunoblot data on the regional distribution of NR2D pro-

tein shows a good correlation, with the distribution of the mRNA encoding the NR2D subunit from *in situ* hyridization and northern blot studies *(9,27)*.

Altogether the biochemical results demonstrate that NR2 receptor subunits are differentially expressed in various regions of the rat CNS, which strongly supports the notion that the anatomical and functional variations of the NR2 subunits may provide the molecular basis for the generation of the heterogeneity observed in the physiological and pharmacological properties of the NMDA receptors that is thought to occur in different neuronal cells and brain regions.

Developmental Expression of NMDA Receptor Subunit Proteins

NR1 Subunit

In situ hybridization studies of the mRNA encoding the NR1 subunit of NMDA receptors in the developing rat CNS have shown that the NR1 mRNA is expressed in virtually all neurons at all stages of development *(9)*. This may be related to the role the NR1 plays as the essential subunit for the formation of functional NMDA receptor channels.

The ontogenic profile of the NR1 subunit in the olfactory bulb, cortex, hippocampus, midbrain, and cerebellum of rat brain at postnatal d 2–42 (P2–P42) was determined by quantitative immunoblot using a subunit-specific NR1 antibody that recognized all splice variants of the NR1 subunit. The results indicated that the NR1 protein is expressed widely in but varies quantitatively from region to region and with age. For instance, in all the rat brain regions examined, the levels of NR1 protein are low at birth and rise with a similar pattern two- to fourfold, reaching adult levels by approx 20–30 d after birth *(46)*. Similar observations in the temporal expression profile of NR1 protein were seen in the rat cortex *(39)* and mouse cerebellum *(52)*. Significant differences in the absolute amounts of NR1 protein were seen, with the hippocampus expressing the highest amount of NR1, followed by cerebral cortex, then olfactory bulb and midbrain, and lowest levels of NR1 subunit in the cerebellum. In addition, the absolute levels (pmol/mg) of NR1 obtained from the quantitative immunoblot were observed to be close to those found using [^{3}H]MK-801 binding *(46)*. This may suggest that many of the NR1 subunits expressed in the brain exist in active forms. The temporal expression profile of the NR1 subunit from immunoblot studies in the cerebellum is shown in Fig. 5.

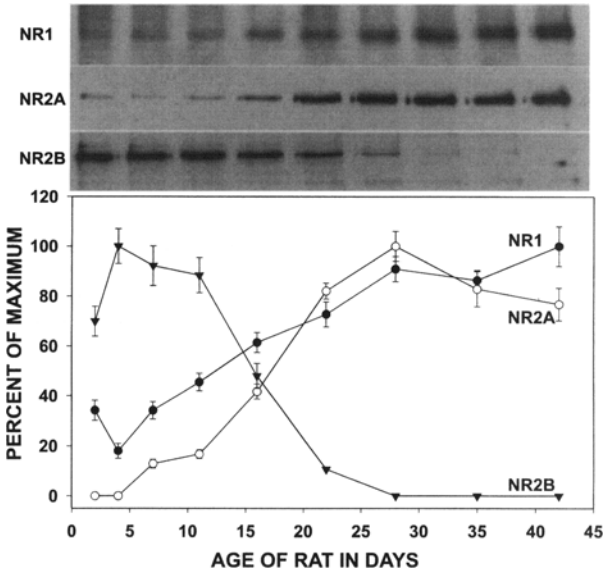

Fig. 5. Ontogenic expression of NMDA receptor subunits NR1, NR2A, and NR2B in the rat cerebellum. Membrane proteins from postnatal (P2–P42) rat cerebellum were subjected to SDS-PAGE by loading 5 μg of protein per lane. The resultant immunoblots were probed with anti-NR1 (upper panel; NR1), anti-NR2A (second panel; NR2A), or anti-NR2B (third panel; NR2B) antibodies. The results were analyzed by computer-assisted densitometry as shown in the lower panel. Values on the abscissa represent the age of rat in days, and values on the ordinate indicate the amount of NMDA subunits given as a percentage of the maximum value obtained for each subunit. Data are mean ± SE (bars) from five rats at each age.

Using the immunocytochemical approach Aoki et al. *(68)* investigated qualitatively the

developmental expression of NR1 protein in the rat visual cortex and observed very dynamic changes in the localization of NR1 protein during the few weeks after birth. At early stages of development, NR1 appeared diffusely localized to the dendrites. During postnatal development, NR1 immunoreactivity appeared in axons and also became localized to spinous postsynaptic densities. At later stages of development NR1 immunoreactivity was also seen in astrocytes *(68)*. A comparison of the biochemical results on temporal expression of NR1 protein with the data from mRNA studies determined by *in situ* hybridization and ligand-binding studies using [^{3}H]MK-801 shows a good correlation. Thus, semiquantitative analysis for developmental studies on expression of the mRNA for NR1 subunit demonstrated that increases in levels of mRNA in cortex, hippocampus, midbrain, and cerebellum occured mainly during the first few weeks of postnatal development *(9,30,71,80,81)*. In particular, the NR1 subunit mRNA was shown to develop 1 wk earlier in the hippocampus relative to the cerebellum and the periods for half maximal development were approx 6 d in the hippocampus and 14 d in the cerebellum *(81)* which matches the NR1 protein data from studies of Luo et al. *(46)*. The quantitative changes may possibly be caused by alterations in the cell types expressing the NR1 protein. Although the time courses of the developmental expression of the NR1 protein appear to be in good agreement with that of the expression of its mRNA, the relative amounts of NR1 protein reported by Luo et al. *(46)* do not correlate well with data on the relative amounts of NR1 mRNA obtained from quantitative solution hybridization studies *(82)*. The relative levels of NR1 mRNA in, for example, the hippocampus, cortex, and cerebellum, were found to be the same *(82)*, whereas protein levels showed marked differences among these rat brain regions, especially between the hippocampus and the cerebellum *(46)*. Similarly, a study that examined the expression of both NR1 mRNA and protein in PC12 cell line demonstrated that these cells expressed NR1 mRNA levels similar to the hippocampus, but expressed much lower NR1 protein levels than the hippocampus *(83)*.

NR2 Subunits

Unlike NR1 mRNA, the expression of the individual NR2 subunit mRNAs are differentially regulated during the development of the CNS *(9,69,82,84–86)*. In the rat brain, the NR2B and NR2D subunit mRNAs are expressed prenatally and at early stages of development, whereas the mRNA encoding NR2A and NR2C subunits are first detected near birth and become pronounced postnatally *(9)*. In addition, dynamic changes in the temporal expression patterns of mRNAs for the NR2 subunits occur during ontogenesis of the CNS, particularly during the first 2 wk after birth *(85)*. For instance, in some regions of the rat CNS, mRNA levels for the NR2A, NR2B, and NR2C subunits have been shown to plateau around P20, whereas the NR2D mRNA level peaks around P7 and then decreases to adult levels *(9)*.

The ontogenic profiles of NR2A and NR2B proteins in the adult rat cerebellum have been determined by quantitative immunoblot using subunit-specific NR2A and NR2B antibodies. As shown in Fig. 5, NR2A was undetectable at P2, whereas NR2B was expressed at amounts easily quantifiable at that age. At about P12, the levels of NR2A were found to rise rapidly and reached adult levels by P22, and at the same postnatal stage (P12), levels of NR2B protein began to decline, reaching undetectable levels by 22 d after birth, as shown in Fig. 5 *(43)*. Similar findings were reported when the developmental expression of NR2A and NR2B subunits in the developing mouse cerebellum were investigated by immunoblot analysis. The NR2A level increased during the first three postnatal weeks and remained almost constant toward adult stages, whereas NR2B protein increased transiently from birth, reaching maximum levels around P9, and then decreased to barely detectable levels toward the end of the third week after birth *(52)*. In the rat cortex,

studies on the temporal expression of NR2A and NR2B subunits using antibodies selective for NR2A and NR2B subunits demonstrated that NR2A was undetectable at birth but the protein level increased progresively over the next 3 wk to reach adult levels, whereas the NR2B was highly expressed at P1 and the protein levels remained fairly constant through adult stages of development *(39)*. Taken together, these results suggest similar patterns in the developmental expressions of NR2A and NR2B proteins in the cortex and cerebellum of rat and mouse, which is consistent with *in situ* hybridization data *(9)* on the ontogeny of the mRNAs encoding NR2A and NR2B subunits.

However, another study that examined the developmental expressions of NR2A and NR2B subunits in four areas of the rat brain, cortex, striatum, cerebellum, and spinal cord using immunoblot analysis with antibodies specific for NR2A and NR2B reported that NR2A protein was not detectable in the cortex and striatum at birth, but its expression increased during the second and third postnatal weeks, and adult levels were attained at about d 26 after birth. In contrast, NR2B was expressed at birth and the protein levels remained comparably constant toward adult stages of development in the cortex and striatum *(45)*. The findings for NR2A and NR2B in the cortex agree with the report of Sheng et al. *(39)* on developmental expression of these NMDA subunit proteins in rat cortex. In the spinal cord, the NR2A subunit was reported to occur at uniform but low expression levels throughout development, whereas NR2B protein expression was seen to be moderate but transient around P7, and then decreased rapidly by the third postnatal week of development. In the cerebellum, the NR2B was reported to be present at low levels during the first postnatal week, followed by a transient increase around the second week of development, and the protein levels subsequently decreased to undetectable levels by P26. This latter result agrees with the report of Wang et al. *(43)* on the developmental expression of NR2A and NR2B subunits in the cerebellum. The authors reported that an immunoreactive band with an approximate molecular mass of 150 kDa was seen around the first week of postnatal development of the cerebellum instead of the full-length 172 kDa band for NR2A subunit. This band was speculated to represent either an immature form or a splice isoform of NR2A, or an unrelated protein containing an epitope that crossreacted with NR2A antibody. This finding is in contrast to that of Wang et al. *(43)*, who demonstrated the presence of full-length 175 kDa NR2A protein during the first week of rat cerebellar development. Possible explanations for such inconsistent observations include differences in detection sensitivities of the antibodies and in experimental protocols with respect to processing and solubilization of membrane proteins for immunoblot.

Histo-blot studies with antibodies that specifically recognize NMDA receptor subunits NR2A and NR2B have further demonstrated changes in the developmental expressions of NMDA receptor subunits NR2A and NR2B in rat brain. The data showed that at birth, NR2A immunoreactivity was low and restricted to the cerebral cortex, hippocampus, and striatum. NR2B protein was also detected at this age, but its expression was virtually ubiquitous. Within the first three postnatal weeks, NR2A protein became abundantly expressed throughout the rat brain, whereas the NR2B subunit became restricted to forebrain regions and both NR2A and NR2B immunoreactivities were found to decrease to adult levels after the third postnatal week *(55)*.

The temporal expression of the NR2C protein in the developing mouse cerebellum has been determined using the immunoblot approach with antibodies specific for NR2C subunit. NR2C protein was not detectable until P9 and its expression increased thereafter, reaching maximum levels around postnatal day 15, and remained constant at adult stages of development *(52)*. However, histo-blot studies with subunit-specific antibody against the NR2C subunit has revealed a more complete

ontogeny of NR2C protein in rat brain. NR2C immunoreactivity was not detected in any region of the brain at birth, but it appeared in the olfactory bulb, thalamus, and vestibular nuclei at P5. The NR2C immunostaining became very intense in the cerebellar granule cells between P10 and P21, and it remained constant toward adult stages of life *(55)*. This result shows excellent correlation with the data on the developmental expression of mRNA for NR2C. In addition, it demonstrates the expression of NR2C protein not only in the cerebellum, but also in the olfactory bulb and thalamus, which also contain the mRNA for NR2C subunit *(9)*.

The developmental expression profile of the NR2D subunit has been analyzed at the protein level in whole rat brain using both immunoblot and histo-blot techniques with a subunit-specific NR2D antibody. NR2D protein level was high at birth and gradually increased, reaching a maximum level at P10, which was followed by a moderate decline in the level of protein during late postnatal stages. However, the NR2D subunit remained detectable at adult stages of development, consistent with mRNA data on temporal expression of NR2D in rat brain determined by ribonuclease protection assay *(53)*. More specifically, immunstaining data shows that at birth, the strongest NR2D immunoreactivity was detected in the thalamus and subthalamic nucleus as well as the midbrain. Weak staining was observed in the cerebral cortex, olfactory bulb, hippocampal formation, deep cerebellar nucleus, and brainstem. NR2D signals were not detected in the striatum and cerebellum except in the deep cerebellar nuclei. The NR2D protein was detected transiently only in certain brain areas, such as the ventrobasal complex of the thalamus, hippocampus, inferior colliculus, and reticular formation of the brainstem, during postnatal stages of development, and significant levels of NR2D were found to be present at adult stages, particularly in the thalamus, subthalamic nucleus, superior colliculus, and globus pallidus. An additional noteworthy observation from this study was the ribonuclease protection assay experiments, indicating that between the two cloned splice variants of the NR2D subunit, only the mRNA for the NR2D-2 isoform was found in the rat brain *(53)*. This may suggest that protein for the NR2D-2 variant represents the predominantly expressed form of NR2D in the brain.

In a similar study, more complete ontogenic expression profiles of NR2D protein in the rat telencephalon (cortex, hippocampus, and striatum), diencephalon (septum, thalamus, and hypothalamus), and spinal cord were determined using a quantitative immunoblot approach. The NR2D protein was found to be expressed at high levels at embryonic stages of development (E14 and E18), which rose to a peak by d 7 after birth and then decreased during the following 2–3 postnatal weeks but remained at easily quantifiable levels during adult stages of life *(54)*. These results are in good agreement with a report by Wenzel et al. *(53)* showing that in whole rat brain, NR2D receptor protein levels peak between 5 and 10 d after birth. An interesting observation was that the developmental expression patterns of the NR2D subunit in the three rat brain areas examined were similar, but vary in the relative levels of NR2D protein among these three areas. Early in development, the diencephalon and spinal cord have twice as much protein as telencephalon. The levels of NR2D protein in all three rat brain regions begin to decrease around P14, and in the spinal cord the decrease is largest. Thus, at the latest postnatal ages examined the protein levels remain highest in the diencephalon, whereas the telencephalon and spinal cord both have NR2D protein levels that are only half that found in the diencephalon *(54)*. In comparison, the data on the time course of the developmental expression of NR2D qualitatively corresponds with the report on the mRNA-encoding NR2D subunit in that NR2D mRNA levels are highest prenatally and in early postnatal stages relative to that of an adult rat *(9)*. However, the NR2D mRNA was found to be barely detectable at adult stages, which is in contrast to a report by Wenzel et al. *(53)*. Thus, using a ribonuclease

protection assay to measure mRNA from whole rat brain, Wenzel et al. *(53)* showed that levels of NR2D mRNA declined only by about twofold from d 10 to adult, which matches the quantitative data on the temporal expression profile of NR2D protein. It should be noted that the quantitative dissociation between mRNA and protein observed in this case may only suggest that mRNA levels can not be inferred from autoradiographs of *in situ* hybridization studies.

Nevertheless, the reported data on the developmental expression of the NMDA receptor subunit protein clearly suggest that NMDA receptors are composed of different subunits in different regions of the brain and that even in the same neuron, the receptors are likely to exhibit different properties at various times during the development of the brain, possibly because of alterations in the subunit composition of the receptor.

Subunit Composition of Native NMDA Glutamate Receptors

Diversity in the molecular composition of NMDA glutamate receptors and their functions is not only attributed to the presence of multiple mRNA splice variants of the single gene encoding for the NR1 subunit *(5,20,28)*, but also from the expression of four distinct genes that code for the members of the NR2 gene family *(22,25–27)*. Many differences in the functional properties of the NMDA receptors are observed in electrophysiological and pharmacological studies, with recombinant NMDA receptors depending on which subunits are used to generate the receptors. Expression studies using *Xenopus* oocyte translation systems have suggested that homomeric NR1, but not homomeric NR2 subunits, form functional NMDA receptor channels that are responsive to glutamate and NMDA, and coexpression of NR1 and NR2 subunits in these systems elicited even stronger responses to glutamate and NMDA, which are characteristic of native NMDA receptors *(5,9,25,26)*. Native NMDA receptors are therefore thought to consist of heteromeric assemblies of NR1, which is mandatory for channel activity and NR2 subunits, which modulate the properties of the channel in yet unknown stoichiometric ratios of NR1 and NR2 subunits *(9,27,84)*. It is therefore clear that a number of distinct NMDA receptors could thereotically be generated depending on the subunit composition. Moreover, heterogeneity of native NMDA receptors in the CNS has been inferred by previous pharmacological studies using radioligand binding *(76,87–89)*, and some comparisons have been made from data obtained from recombinant heteromeric NMDA receptors *(7,30,88)*. It therefore seems that at least some of the potential molecular compositions of NMDA receptors that display distinct functional properties appear to have physiological relevance. In addition, a few studies have examined the subunit composition of native NMDA receptors using an immunoprecipitation approach with NMDA receptor subunit-specific antibodies.

Coimmunoprecipitation studies with antibodies selective for NR1, NR2A, and NR2B subunits at different postnatal ages has provided some understanding of the changes in the molecular composition of native NMDA receptors containing NR1, NR2A, and NR2B subunits during the development of rat cortex *(39)*. The results from this study indicated that the NR1 antibody immunoprecipitated NR2B from the rat cortical membrane at all the postnatal stages examined, whereas the amount of NR2A precipitated at P1 was not detectable, but increased progressively to adult levels by the third postnatal week. In addition, both NR2A and NR2B were substantially coimmunoprecipitated by antibodies to either of the two subunits from P7 to adult rat cortex *(39)*. These results suggest that in the rat cortex functional NMDA receptors containing NR1, NR2A, and NR2B subunits are present in three different heteromeric NMDA receptor subtypes: two binary complexes consisting of NR1/NR2A and NR1/NR2B subunits, and a ternary complex composed of NR1/NR2A/NR2B subunits. The

key observation is that different subtypes of the NMDA receptors appear at different stages of postnatal development. For example, during the first postnatal week the predominant receptor is the binary complex consisting of NR1/NR2B, and toward the end of this period, i.e., around P7, the expression of NR2A protein began, resulting in the formation of additional receptors, such as NR1/NR2A and NR1/NR2A/NR2B, which continued throughout rat cortical development. This observation is consistent with a report describing a switch in the sensitivity of NMDA receptors for ifenprodil during postnatal development of rat brain *(34)*. Thus, at early postnatal stages the NR1/NR2B receptors were formed that had high sensitivity for ifenprodil, whereas at late postnatal life receptors presumably composed of NR1/NR2A, and NR1/NR2A/NR2B were formed that showed low sensitivity to ifenprodil. It should be noted that the time courses of the expression of NR1, NR2A, and NR2B proteins in this study very closely followed the *in situ* hybridization mRNA data for the developmental expression profiles of these subunits *(9)*.

Recently the quantitative immunoprecipitation technique was developed and used to study the subunit composition of native NMDA receptors in the adult *(47)*. Each of the antibodies selectively immunoprecipitated only its cognate subunit when the immunoprecipitation was performed under denaturing conditions of receptor solubilization in which the receptor subunits were dissociated from one another. When immunoprecipitation was performed under nondenaturing conditions of receptor solubilization in which the receptor subunits appeared to remain associated with one another, each of the antibodies immunoprecipitated a sizeable fraction of the other two NMDA receptor subunits, indicating an *in situ* interaction among these subunits in the rat cortex (Fig. 6). Results from quantitative immunoblot analysis of NR1, NR2A, and NR2B subunits in both the pellets and supernatants from the immunoprecipitations showed that the dominant NMDA receptor complex in adult rat cortex contained at least

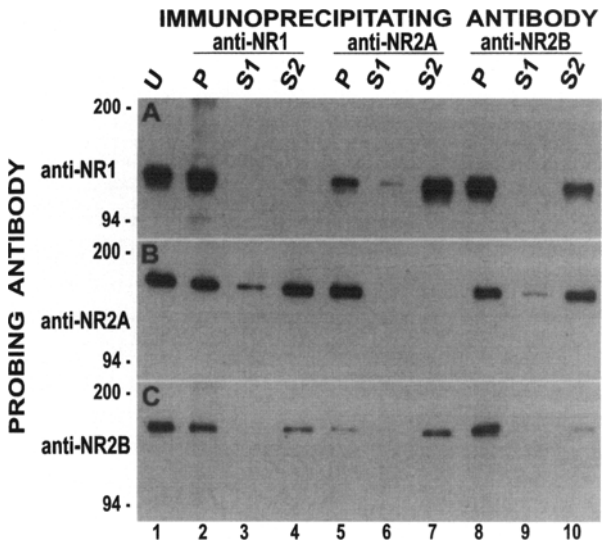

Fig. 6. Coimmunoprecipitation of NMDA receptor subunits solubilized under native conditions using subunit-specific NMDA receptor antibodies. Immunoprecipitation of soluble proteins from cortical membranes solubilized under nondenaturing conditions was performed with anti-NR1 (lanes 2–4), anti-NR2A (lanes 5–7), or anti-NR2B (lanes 8–10) as indicated across the top of the gel. The pellet (P) and supernatant (S1) contain equal fractions of protein, whereas the supernatant (S2) contains five times the fraction of protein sample as P or S1 from the same immmunoprecipitation. Each lane denoted by either P or S1 contains 1 mg equivalents of original proteins. Lane 1 (U) contains 1 μg equivalent of soluble protein without precipitation. All the pellets and supernatants resulting from the immunoprecipitations were resolved on SDS-PAGE and the blots probed with subunit-specific fusion protein antibodies against NR1 **(A)**, NR2A **(B)**, or NR2B **(C)** as shown on the left of the figure. Positions of molecular weight markers are indicated on the left of the figure.

three subunits, NR1/NR2A/NR2B (50–60%), and a smaller fraction of NMDA receptors was composed of only two subunits, NR1/NR2A (10–20%) or NR1/NR2B (25–35%). These findings are in excellent correlation with the results of Sheng et al. *(39)* showing that the most abundant NMDA receptor subtype in the rat cortex is the ternary complex containing NR1, NR2A, and NR2B subunits. These results are

also consistent with the report on immunohistochemical distributions of NR1, NR2A, and NRB in rat brain demonstrating that these NMDA subunits are colocalized in most neurons to form functional receptors *(41,42)*. Collectively, these findings may suggest that functional studies involving ternary NMDA receptors may be the most physiologically relevant. These investigators also found that there were no receptor complexes consisting of NR2A/NR2B without NR1 subunit and only a small fraction of each of the subunit was not associated with any other NMDA receptor subunit, suggesting that most of the NMDA receptors in rat cortex are functionally active. Coimmunoprecipitation of noncognate subunits did not occur unless the subunits were assembled with one another *in situ* and there was no physical interaction between these NMDA receptor subunits and the AMPA receptor GluR2 or GluR3 *(47)*, indicating that NMDA receptors do not form heteromeric complexes with members of non-NMDA receptor families.

Some studies have shown that the pharmacological and physiological properties of NMDA receptor channels are determined by the interaction of the various NR1 splice variants with members of the NR2 family *(25,26,89)*. In this regard, coimmunoprecipitations in the rat forebrain with antibodies selective for the four NR1 cassettes that vary in the NR1 splice isoforms in combination with subunit-specific antibodies for NR2A and NR2B have provided some understanding of the biochemical interactions between the different splice variants of NR1 and the NR2 subunits. Interestingly, preferential coassembly between particular NR1 splice isoforms and NR2A or NR2B subunits was not seen, and at least two NR1 splice variants were found in the same NMDA receptor complex, suggesting that different NR1 splice variants can be part of the same complex *(57)*. In contrast to the previous reports demonstrating that the dominant NMDA receptor complex in adult rat cortex is composed of at least three subunits, NR1/NR2A/NR2B *(39,47)*, these authors found that coimmunoprecipitation with either NR2A or NR2B antibodies showed little precipitation of both subunits, suggesting that majority of the NMDA receptors in rat forebrain consists of binary complexes of NR1/NR2A and NR1/NR2B, and only a minor fraction contains the ternary complex NR1/NR2A/NR2B. This is not an uncommon problem encountered in studies of the subunit composition of native NMDA receptors involving coimmunoprecipitations with NMDA receptor antibodies. However, such disparity can, among other things, be attributed in particular to the differences in the efficiencies of solubilization of NMDA receptors reported by these independent investigators. For instance, Luo et al. *(47)* reported that over 80% of NMDA receptors were solubilized from rat cortical membranes using 1% sodium desoxycholate at pH 9.0, whereas Blahos and Wenthold *(57)* showed only 35% solubilization under the same conditions. It is therefore possible that inadequate solubilization of NMDA subunits can cause significant variations, especially on quantitative studies of subunit compositions of NMDA receptors.

The subunit compositions and the sizes of native NMDA receptor complexes from solubilized synaptic membranes can also be studied by the chemical crosslinking approaches involving immunostaining. Using this approach along with antibodies against NR1, NR2A, and NR2B, a crosslinked product containing three molecular complexes of 603, 700, 750 kDa immunolabeled by these NMDA antibodies were identified in the rat forebrain *(57)*. The cross-linked products appeared to be identical in pattern and size, demonstrating that the NR1, NR2A, and NR2B subunits were present in the same native receptor complex. A similar experiment using only antibodies selective for NR1 subunit showed that this antibody immunostained a protein complex having a molecular mass of 730 kDa *(49)*, indicating that the NR1 subunit is part of the receptor protein complex in the rat brain. These results support the data from coimmunoprecipitation studies *(39,47)* demonstrating that in the rat forebrain the NR1, NR2A, and NR2B subunits

are assembled with one another to form functional receptors.

The coassembly of NR1 and NR2 subunits in the mouse cerebellum during postnatal development has been determined by the immunoprecipitation approach using antibodies selective for NR1, NR2A, NR2B, and NR2C subunits. The results showed that following immunoprecipitation with NR2B antibody, a ternary complex consisting of NR2B subunit assembled with NR2A and NR1 (NR1/NR2A/NR2B) was present at 6 and 12 d after birth *(52)*. This finding concurs with the report from Sheng et al. *(39)* in the developing rat cortex. The ratio of NR2A assembled with NR2B subunit in the ternary complex was seen to be reduced after P12, and by 3 wk after birth the NMDA receptors became mostly binary complexes of NR1/NR2A and NR1/NR2B. Also, the NR2B antibody did not precipitate NR2C subunit at any of the postnatal stages examined. On the other hand, the NR2C antibody immunoprecipitated more than 50% of NR1 but not NR2A or NR2B from P1 through P21, indicating that NMDA receptors composed of NR1/NR2C are present, whereas heteromeric complexes containing NR1/NR2A/NR2C or NR1/NR2B/NR2C were not detected at any stages of the mouse development *(52)*. These data suggest that at early postnatal stages of the mouse cerebellar development the dominant NMDA receptor is a ternary complex containing NR1/NR2A/NR2B subunits. At late postnatal stages, however, the binary complex composed of NR1/NR2C becomes the predominant NMDA receptor. The switch in the composition of NMDA receptors may be related to the very high levels of expression of NR2C mRNA *(27,51)* and protein *(51;* Wolfe and Wang, unpublished observation) seen in the adult cerebellum. The binary complexes composed of NR1/NR2A and NR1/NR2B are also present in the cerebellum but seem to represent only a minor fraction of NMDA receptors relative to NR1/NR2C receptors. In contrast to the observation that ternary complexes containing NR1/NR2A/NR2C subunits were not detected in the mouse cerebellum are reports from studies in which three NMDA subunits were transfected in a mammalian cell line to generate heterotrimeric NMDA receptors whose pharmacological profile was found to be similar to that found in adult mouse cerebellum. Thus, Chazot et al. *(48)* demonstrated that the affinity of [^{3}H]MK-801 for binding to the ternary receptor complex NR1/NR2A/NR2C transiently expressed in HEK293 cells was similar to those found in the mouse cerebellum and was dissimilar to binary receptors composed of NR1/NR2A and NR1/NR2C subunits. This result suggests the presence of a heteromeric NMDA receptor composed of NR1/NR2A/NR2C subunits in the mouse cerebellum. Additionally, these investigators have shown by coimmunoprecipitation in transfected HEK 293 cells that the ternary receptor containing NR1/NR2A/NR2C subunits can form if all mRNAs are expressed in the same cell *(90)*.

Experimental evidence demonstrating the subunit composition of native NMDA receptors that contain the NR2D protein in the rat CNS using quantitative immunoprecipitation with selective antibodies against NR1, NR2A, NR2B, and NR2D has been reported *(56)*. The data from immunoprecipitations of NMDA receptor-subunit proteins from rat cortex, striatum, thalamus, and spinal cord solubilized under nondenaturing conditions followed by quantitative immunoblot analysis of NR1, NR2A, NR2B, and NR2D subunits:

1. Showed that the NR2D protein is predominantly associated with NR1 subunit and only a small fraction of NR2D did not form heteromeric complexes with NR1 subunit (data from thalamus are shown in Fig. 7), consistent with the existence of functional NMDA receptors in the brain;
2. Showed that the NR2D subunit forms a heteromeric assembly with NR1, NR2A, and NR2B subunits, which appears to reflect at least ternary complexes composed of NR1/NR2A/NR2D and NR1/NR2B/NR2D subunits in the spinal cord, cortex, and thalamus of adult rat brain (data from spinal cord are shown in Fig. 8), consistent with the hypothesis that native NMDA receptors in the brain are composed of heteromeric complexes of NR1 and two or more different NR2 subunits *(24)*;

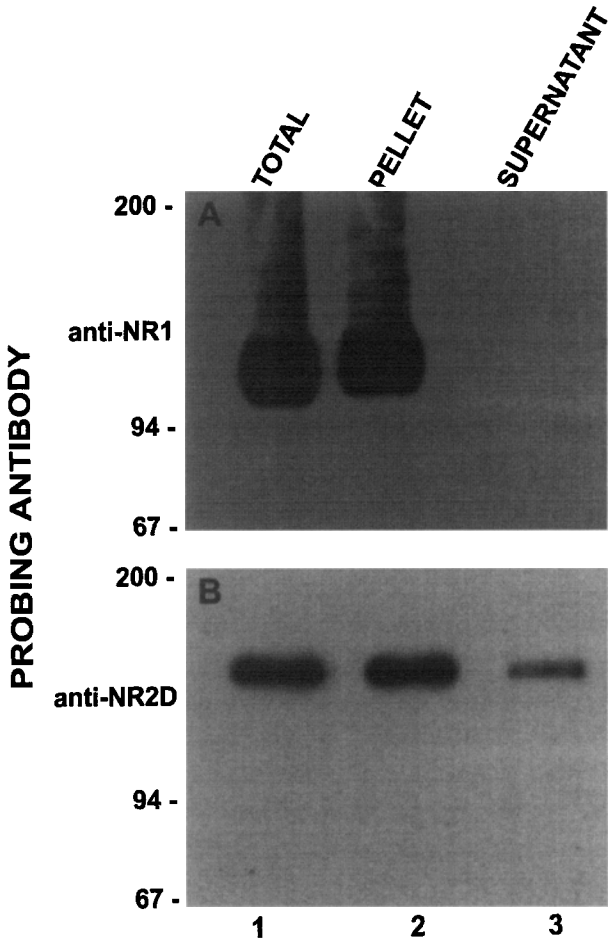

Fig. 7. The NR2D protein is mostly assembled with the NR1 subunit in the thalamus of rat. Membrane proteins from the thalamus of adult rat were solubilized using nondenaturing conditions and the solubilized proteins were immunoprecipitated with anti-NR1 antibody coupled to protein A sepharose beads. The immunoprecipitates were electrophoresed on SDS-polyacrylamide gel, and the resultant immunoblots were probed with either anti-NR1 **(A)** or anti-NR2D **(B)** as indicated on the left of the figure. Each lane contains 10 µg equivalent protein load of all the samples. The lane marked TOTAL (lane 1) contains soluble proteins without immunoprecipitation, whereas lane marked PELLET (lane 2) contains samples from the resuspended immunoprecipitates, and the lane marked SUPERNATANT (lane 3) contains samples from the supernatant.

3. Supported the results of Sheng et al. *(39)* and Luo et al. *(47)* showing that the most abundant and physiologically important NMDA receptor subtype in the brain are ternary complexes containing NR1 and NR2 subunits; and
4. Showed that NMDA receptor binary complex comprised of NR1/NR2D subunits was only found in the thalamus, not the midbrain, where the complexes also contained either NR2A or NR2B, consistent with a report indicating that certain thalamic nuclei express NMDA receptors whose pharmacological properties are similar to that of NR1/NR2D receptor subtype *(87,88)*.

Taken together, the reports on subunit compositions of native NMDA receptors suggest that different subtypes of NMDA receptors are present in the CNS, which vary in both spatial and temporal expression. These differences may reflect distinct physiologic and behavioral roles these receptor subtypes play in the CNS.

Phosphorylation of NMDA Glutamate Receptors

Protein phosphorylation is a posttranslational modification process that alters the functional properties of many proteins and is widely recognized as a major regulatory mechanism involved in the control of neuronal and synaptic functions in the CNS *(91–94)*. Protein phosphorylation involves the transfer of the phosphate group of adenosine triphosphate (ATP) to a serine, threonine, or tyrosine amino acid residue of a substrate protein, often resulting in alterations of its functional properties *(95, 96)*. The process of protein phosphorylation is catalyzed by protein kinases, which usually have selectivity for serine, threonine, or tyrosine residues. Tremendous interest has been focused on tyrosine phosphorylation of proteins because of its potentially important role in the regulation of cellular processes, such as growth, differentiation, and maturation *(97, 98)*. Moreover, several studies have shown that tyrosine kinases and phosphatases are widely expressed in the CNS and found particularly at high levels in neuronal synapses, suggesting

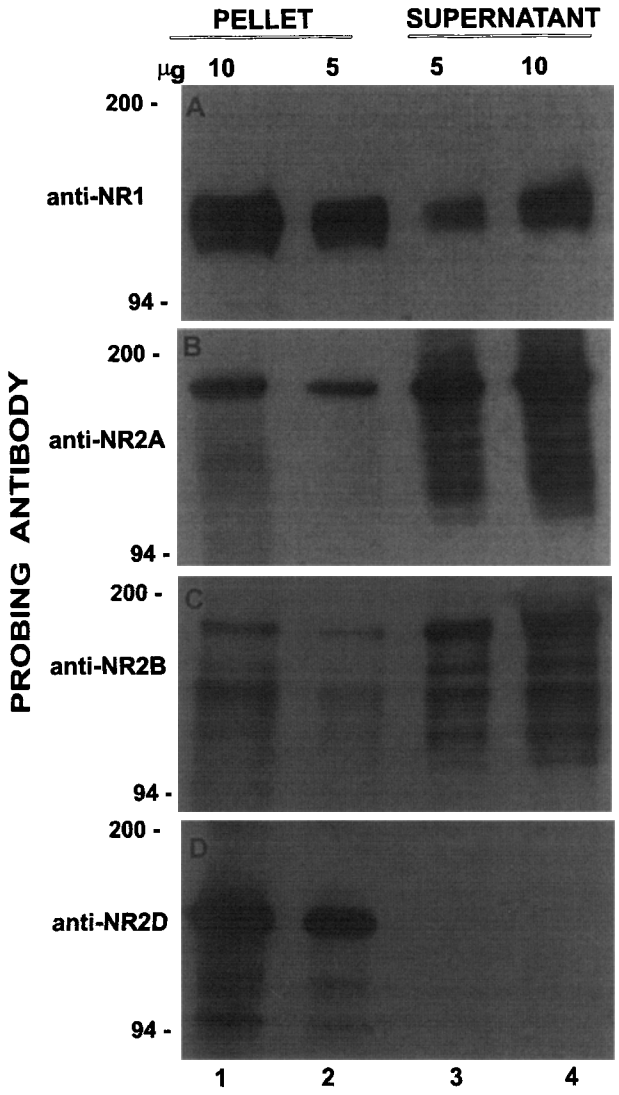

Fig. 8. Some of the NR2D protein is complexed with NR2A and NR2B subunits in addition to the NR1 subunit in the spinal cord of rat. Spinal cord membrane proteins of adult rat were solubilized under native conditions. The solubilized proteins were immunoprecipitated with anti-NR2D antibody coupled to protein A sepharose beads. The resulting pellet and supernatant samples were separated on SDS-PAGE by loading 10 and 5 μg equivalents of each sample per lane as indicated across the top of the figure. Lanes marked PELLET (lanes 1 and 2) contain samples from the immunoprecipitation pellet, and lanes marked SUPERNATANT (lanes 3 and 4) contain samples from the supernatant. The blots were probed with anti-NR1 **(A)**, anti-NR2A **(B)**, anti-NR2B **(C)**, or anti-NR2D **(D)** as indicated on the left of the figure.

that tyrosine phosphorylation may be involved in the regulation of neuronal processes, including synaptic transmission and plasticity *(98–101)*.

Some studies have provided evidence that tyrosine phosphorylation of ion channels modulates their functions *(61,62,102–109)*. The regulation of glutamate receptors, especially the NMDA subtype, by protein tyrosine phosphorylation has only been recently investigated. For instance, long-term potentiation (LTP) in the hippocampus, a brain region implicated in learning and memory, was shown to be blocked by inhibitors of protein tyrosine kinases *(107)*, suggesting that tyrosine kinase activity might be a requirement for induction of long-term synaptic plasticity in the hippocampus. Electrophysiological studies in the dorsal horn of the spinal cord and hippocampal neurons demonstrated that NMDA receptor-mediated responses were potentiated by protein phosphatase inhibitors or by protein tyrosine kinase phosphorylation, and NMDA receptor activity was found to be attenuated by protein tyrosine kinase inhibitors *(108)*. Also, in vitro electrophysiological studies using recombinant NMDA receptors have shown that protein tyrosine kinases of the *src* family increased glutamate-activated currents without affecting desensitization and deactivation kinetics in NR1/NR2A receptors and had no effect on NR1/NR2B, NR1/NR2C, and NR1/NR2D receptor channels *(102)*. The endogenous tyrosine kinase *src* was reported to regulate the activity of NMDA channels in membrane patches from rat spinal dorsal horn *(109)*. These authors speculated that the observed responses of NMDA receptors to protein tyrosine kinases, protein phosphatase inhibitors, and tyrosine kinase inhibitors might be caused by regulation of NMDA receptors by protein tyrosine phosphorylation resulting from the direct phosphorylation of the receptors on tyrosine amino acid residues. In this regard, recent studies employing antiphosphotyrosine antibodies along with selective NMDA receptor subunit antibodies have provided evidence for the direct tyrosine phos-

phorylation of some NMDA receptor subunit proteins.

NR1 Subunit

Although the NR1 subunit has been shown to be phosphorylated predominantly on serine residues and to some extent on threonine residues *(40,58)*, data from immunoprecipitation of rat cerebral cortical synaptic plasma membranes with antiphosphotyrosine and NR1 antibodies have shown that the NR1 subunit was not phosphorylated on tyrosine residues *(44)*. In a similar study that used a [^{32}P] metabolic labeling approach along with antiphosphotyrosine, NR1, and NR2 antibodies to examine the surface expression and phosphorylation of NMDA receptor subunits in rat hippocampal primary culture cells, basal phosphorylation of NR1 subunit was not detectable, whereas high basal phoshorylation levels were obtained for the NR2 subunits *(58)*. These data suggest that the NR1 protein is not tyrosine phoshorylated. In this regard, an attempt was made to determine whether NR1 subunit is phosphorylated at serine and threonine residues in cells transiently expressing recombinant NR1 protein and primary cultures of cortical neurons using NR1 antibody. The results showed detectable levels of basal phosphorylation of NR1 protein, which was found to increase upon activation of protein kinase C with phorbol esters. However, phosphopeptide tryptic digest assay of the phosphorylated NR1 protein confirmed that protein kinase C phosphorylation occured mostly on serine amino acid residues with only a trace amount of phosphorylation seen on threonine residues *(40)*, suggesting that the NR1 subunit is predominantly serine phosphorylated.

In a recent study, Tingley et al. *(59)*, examined the phosphorylation of the NR1 subunit in vitro and *in situ* using site-specific antiphosphopeptide NR1 antibodies that recognized the NR1 protein only when specific serine amino acid residues were phosphorylated. The results demonstrated that in addition to protein kinase C, protein kinase A also phosphorylated of the carboxyl terminus of NR1 subunit. Whereas protein kinase C specifically phosphorylated NR1 protein at threonine 879 and serines 890 and 896, the protein kinase A phosphorylated NR1 at serine 897, but only phosphorylation of NR1 at serine 890 by protein kinase C was found to induce the dispersal of clusters of NR1 protein, suggesting that protein kinase C and protein kinase A might differentially regulate NR1 subunits in the CNS.

NR2 Subunit

Experimental data from immunoprecipitation of rat forebrain proteins using either antiphosphotyrosine or subunit-specific NR2B antibodies revealed that the NR2B subunit was not only highly enriched in the postsynaptic density but it also represented the major tyrosine-phosphorylated protein in the rat forebrain postsynaptic densities *(60)*. In another investigation, immunoprecipitation with antiphosphotyrosine antibodies and antibodies selective for NR1, NR2A, and NR2B subunits was used to determine whether NMDA receptors are tyrosine phosphorylated in the CNS. The results showed that both NR2A and NR2B subunits but not the NR1 subunit were phosphorylated on tyrosine amino acid residues in the rat cerebral cortex, and the tyrosine phosphorylation was found to vary quantitatively for both NR2A (2.1%) and NR2B (3.6%) subunits *(44)*. These biochemical findings were supported by data from immunocytochemical staining revealing the presence of abundant levels of phosphotyrosine proteins that colocalized with the NMDA receptor subunits at excitatory synapses in cultured rat hippocampal neurons *(44)*. The observation that NR2A and NR2B but not NR1 is tyrosine phosphorylated supports the notion that the NR2 subunits are required for modulation of NMDA receptor functions, whereas the NR1 subunit is important for its fundamental activities. The finding that tyrosine phosphorylation levels of NR2B are more than that of NR2A supports the reports of Moon et al. *(60)* showing that NR2B is the major tyrosine-phospho-

rylated protein in the postsynaptic densities of rat forebrain. In contrast to the results showing that NR2A and NR2B but not NR1 are tyrosine phosphorylated in rat forebrain *(44,60)*, Hall and Soderling *(58)* demonstrated in a related study that the tyrosine phosphorylation of these NMDA subunits was not detectable in primary cultures of the rat hippocampus. This suggests that neither NR1 nor NR2 subunits are tyrosine phosphorylated when hippocampal neurons are grown in primary cultures, and it is likely that some factors responsible for inducing tyrosine phosphorylation of NR2 subunits might be present in the brain but absent from primary hippocampal cultures.

Phosphorylation of NMDA receptors has been implicated in the process of LTP. The effects of changes in the levels of tyrosine phosphorylation of the NR2B subunit in relation to the induction of LTP in the dentate gyrus of adult rats were investigated using immunoprecipitations with antiphosphotyrosine and NR2B antibodies. Interestingly, the amount of tyrosine-phosphorylated NR2B protein was found to increase without a change in the total NR2B protein levels after induction LTP in the dentate gyrus of rat hippocampal formation *(61, 62)*. This indicates that induction of LTP caused an increase in the levels of tyrosine-phosphorylated NR2B and not an increase in *de novo* synthesis of NR2B subunit. The blockade of the LTP with an NMDA receptor antagonist inhibited the observed increase in tyrosine-phosphorylated NR2B protein *(61)*, suggesting that LTP results from direct phosphorylation of NMDA receptors. It was also observed that an increase in tyrosine phosphorylation of NR2B protein was not detectable at 5 min but was easily measurable by 15 min after induction of LTP, after which the increase persisted for more than 3 h *(61, 62)*. This supports the hypothesis that tyrosine phosphorylation of NMDA receptors is involved in the maintenance of LTP.

The phosphorylation profile of the NMDA receptor subunit NR2C still remains to be investigated. However, immunoprecipitation with subunit-specific antibody against the NR2D along with an antiphosphotyrosine antibody have been used to determine quantitatively the developmental profile of the tyrosine phosphorylation of NR2D subunit in the rat CNS *(63)*. NMDA receptor proteins from the thalamus at P1, P7, P21 and P49 were solubilized using denaturing conditions and used for immunoprecipitations with anti-NR2D and antiphosphotyrosine antibodies. The NR2D protein in the resulting immunopellets was subjected to quantitative immunoblot analysis. The results indicated that the NR2D subunit is tyrosine phosphorylated in rat brain, which is consistent with previous reports demonstrating the tyrosine phosphorylation of NR2A and NR2B subunits in the rat CNS *(44,60)*. The quantified data of the developmental profile of tyrosine phosphorylation of NR2D in the thalamus showed that at P1 1.2 ± 1.2% of the receptors were phosphorylated, which increased to 14.4 ± 1.5% at P49. These results seem to implicate development in the regulatation of tyrosine phosphorylation of NR2D protein in vivo.

Overall, the phosphorylation data are consistent with the presence of serine, threonine, and tyrosine consensus phosphorylation sites for protein kinases in the amino acid sequences of these subunits based on reports from molecular cloning studies of the NMDA glutamate receptor subunits *(9,27)*. The results strongly suggest that tyrosine phosphorylation is important for regulating the functions of NMDA receptor subunit in the mammalian CNS.

Conclusion

Prior to the advent of antibody development technology, the lack of ligands, whether agonists or antagonists, that selectively recognized a given subunit of NMDA receptor posed a tremendous limitation to investigations into the molecular biology of NMDA glutamate receptors. The availability of a few techniques that allow for the development of NMDA receptor subunit-specific monoclonal and polyclonal antibodies using either fusion protein or synthetic peptide approaches has over

the past few years provided a better understanding of the structure and function of the NMDA receptor channels, especially in the areas of regional and developmental expression, subunit assembly, and posttranslational modification mechanisms of NMDA receptors in the mammalian CNS.

Although subunit-specific NMDA receptor antibodies constitute a panel of important and useful probes for investigating the structure and function of the NMDA subtype of ligand-gated ion channels either in the CNS or in recombinant receptor expression systems, the procedures involved in the generation of antibodies are obviously not inexpensive or trivial. The choice and design of the antigen, whether synthetic peptide or fusion protein, and the purification and characterization of the resulting antibodies need to be performed carefully and properly to obtain meaningful experimental data. Despite the relatively high economic and labor-intensive requirements for antibody production, biochemical studies of NMDA receptors using subunit-specific antibodies have advantages over conventional techniques, such as northern blot analysis and *in situ* hybridization, that examine only the mRNA encoding the NMDA subunits. This is because mRNA studies do not provide clear and adequate information about the resulting subunit protein, whereas subunit-specific antibodies allow for direct examination of the protein product of the NMDA receptor subunit genes, thereby providing a useful approach for localizing, quantifying, and characterizing the receptor subunits, and to allow study of the subunit assembly and posttranslational modification mechanisms of native NMDA receptors in both brain tissues and eukaryotic cell lines.

Acknowledgments

A significant part of the research presented in this review, particularly the work from which all the figures are derived, was done in the laboratory of Dr. B. B. Wolfe. The work was supported by grants NS36246, NS28130, and AG09973 from the National Institutes of Health and by the American Health Assistance Foundation.

References

1. Hayashi T. (1954) Effects of sodium glutamate on the nervous system. *Keio J. Med.* **3,** 183–192.
2. Curtis D. R., Phillis J. W., and Watkins J. C. (1959) Chemical excitation of spinal neurons. *Nature* **183,** 611, 612.
3. Monaghan D. T., Bridges R. J., and Cotman C. W. (1989) The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *Ann. Rev. Pharmacol. Toxicol.* **29,** 365–402.
4. Collingridge G. L. and Lester R. A. J. (1989) Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.* **41,** 143–210.
5. Moriyoshi K., Masu M., Ishii T., Shigemoto R., Mizuno N., and Nakanishi S. (1991) Molecular cloning and characterization of the rat NMDA receptors. *Nature* **354,** 31–37.
6. Ascher P. and Nowak L. (1988) The role of divalent cations in the N-methyl-D-aspartate responses of mouse central neurons. *J. Physiol.* **399,** 247–266.
7. 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. London Ser. B.* **250,** 271–277.
8. Burnashev N., Schoepfer R., Monyer H., Ruppersberg J. P., Gunther W., Seeburg P. H., and Sakmann B. (1992) Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptors. *Science* **257,** 1415–1419.
9. Monyer H., Burnashev H., Laurie D. J., Sakmann B., and Seeburg P. H. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12,** 529–540.
10. Bliss T. V. P. and Collingridge G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361,** 31–39.
11. Komuro H. and Rakic P. (1993) Modulation of neuronal migration by NMDA receptors. *Science* **260,** 95–97.

12. Sheetz A. J. and Constantine-Paton M. (1994) Modulation of NMDA receptor function: implications for vertebrate neuronal development. *FASEB J.* **8,** 745–752.
13. McBain C. J. and Mayer M. L. (1994) N-methyl-D-aspartic acid receptor structure and function. *Physiol. Rev.* **74,** 723–760.
14. Choi D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1,** 623,624.
15. Dingledine R., McBain C. J., and McNamara J. O. (1990) Excitatory amino acid receptors in epilepsy. *Trends Pharmacol. Sci.* **11,** 334–338.
16. Meldrum B. and Garthwaite J. (1990) Excitatory amino acid neurotoxicity and degenerative diseases. *Trends Pharmacol. Sci.* **11,** 379–387.
17. Ulas J., Brunner L. C., Geddes J. W., Choe W., and Cotman C. W. (1992) N-methyl-D-aspartate receptor complex in the hippocampus of elderly, normal individuals and those with Alzheimer's disease. *Neuroscience* **49,** 45–61.
18. Lipton S.A., Choi Y. B., Pan Z. H., Lei S. Z., Chen H. S., Sucher N. J., Loscalzo J., Singel D. J., and Stamler J. S. (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* **364,** 626–632.
19. Meldrum B. S. (1994) The role of glutamate in epilepsy and other central nervous system disorders. *Neurology* **44,** 14–23.
20. Yamazaki M., Mori H., Araki K., Mori J., and Mishina M. (1992) Cloning, expression and modulation of a mouse NMDA receptor subunit. *FEBS Lett.* **300,** 39–45.
21. Ikeda K., Nagasawa M., Mori H., Araki K., Sakimura K., Watanabe M., Inoue Y., and Mishina M. (1992) Cloning and expression of the e4 subunit of the NMDA receptor channel. *FEBS Lett.* **313,** 34–38.
22. 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* **358,** 36–41.
23. Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H., Burnashev N., Sakmann B., and Seeburg P. H. (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science.***256,** 1217–1221.
24. 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.
25. 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.
26. 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.
27. Anantharam V., Panchal, R. G., Wilson A., Kolchine V. V., Treistman S. N., and Bayley H. (1992) Combinatorial RNA splicing alters the surface charge on the NMDA receptor. *FEBS Lett.* **305,** 27–30.
28. Durand G. M., Gregor P., Zheng X., Bennett M. V. L., Uhl G. R., and Zukin R. S. (1992) Cloning of an apparent splice variant of the rat N-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. *Proc. Natl. Acad. Sci. USA* **89,** 9359–9363.
29. Durand G. M., Bennett M. V. L., and Zukin R. S. (1993) Splice variants of the N-methyl-D-aspartate receptor NR1 identify domains involved in regulation by polyamines and protein kinase C. *Proc. Natl. Acad. Sci. USA* **90,** 6731–6735.
30. Nakanishi S. (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science* **258,** 597–603.
31. Meguro M., Mori H., Araki K., Kushiya E., Kuzuwada T., Yamazaki M., Kumanishi T., Arakawa T., Sakimura K., and Mishina M. (1992) Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature* **357,** 70–74.
32. Soloview M. M. and Barnard E. A. (1997) Xenopus oocytes express a unitary glutamate receptor endogenously. *J. Mol. Biol.* **273,** 14–18.
33. Laurie D. J. and Seeburg P. H. (1994) Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. *Eur. J. Pharmacol.* **268,** 335–345.
34. 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.
35. Williams K. (1995) Pharmacological characterization of recombinant N-methyl-D-aspartate receptors containing the e4 (NR2D) subunit. *Neurosci. Lett.* **184,** 181–184.

36. Williams K., Russell S. L., Shen Y. M., and Molinoff P. B. (1993) Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. *Neuron* **10,** 267–278.
37. Ilyin V., Whittermore E. R., Guastella J., Weber E., and Woodward R. M. (1996) Subtype-selective inhibition of N-methyl-D-aspartate receptors by haloperidol. *Mol. Pharmacol.* **50,** 1541–1550.
38. Vicini S., Wang J. F., Li J. H., Zhu W. J., Wang Y. H., Luo J. H., Wolfe B. B., and Grayson D. R. (1998) Functional and pharmacological differences between recombinant NMDA receptors. *J. Neurophysiol.* **79,** 555–566.
39. Sheng M., Cummings J., Roldan L. A., Jan Y. N., and Jan L. Y. (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* **368,** 144–147.
40. Tingley W. G., Roche K. W., Thompson A. K., and Huganir R. L. (1993) Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. *Nature* **364,** 70–73.
41. Petralia R. S., Yokotani N., and Wenthold R. J. (1994) Light and electron microscope distribution of the NMDA receptor subunit NMDA R1 in the rat nervous system using a selective anti-peptide antibody. *J. Neurosci.* **14,** 667–696.
42. Petralia R. S., Wang Y.-H., and Wenthold R. J. (1994) The NMDA receptor subunits NR2A and NR2B show histological and ulstructutural localization patterns similar to those of NR1. *J. Neurosci.* **14,** 6102–6120.
43. Wang Y., Bosy T. Z., Yasuda R. P., Grayson D. R., Vicini S., Pizzorusso T., and Wolfe B. B. (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.
44. Lau L-F. and Huganir R. L. (1995) Differential tyrosine phosphorylation of N-methyl-D-aspartate receptor subunits. *J. Biol. Chem.* **270,** 20,036–20,041.
45. Portera-Cailliau C., Price D. L., and Martin L. J. (1996) N-methyl-D-aspartate receptor proteins NR2A and NR2B are differentially distributed in the developing rat central nervous system as revealed by subunit-specific antibodies. *J. Neurochem.* **66,** 692–700.
46. Luo J., Bosy T. Z., Wang Y., Yasuda R. P., and Wolfe B. B. (1996) Ontogeny of NMDA R1 subunit protein expression in five regions of rat brain. *Dev. Brain Res.* **92,** 10–17.
47. Luo J., Wang Y. H., Yasuda R. P., Dunah A. W., and Wolfe B. B. (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.
48. Chazot P. L., Cik M., and Stephenson F. A. (1992) Immunological detection of the NMDAR1 glutamate receptor subunit expressed in human embryonic kidney 293 cells and in rat brain. *J. Neurochem.* **59,** 1176–1178.
49. Brose N., Gasic G. P., Vetter D. E., Sullivan J. M., and Heinemann S. F. (1993) Protein chemical characterization and immunocytochemical localization of the NMDA receptor subunit NMDA R1. *J. Biol. Chem.* **268,** 22,663–22,671.
50. Cik M., Chazot P. L., and Stephenson F. A. (1993) Optimal expression of cloned NMDAR1/NMDAR2A heteromeric glutamate receptors: biochemical characterization. *Biochem. J.* **296,** 877–883.
51. Wenzel A., Scheurer L., Kunzi R., Fritschy J. M., Mohler H., and Benke D. (1995) Distribution of NMDA receptor subunit proteins NR2A, 2B, 2C and 2D in rat brain. *NeuroReport* **7,** 45–48.
52. Didier M., Xu M., Berman S. A., and Bursztajn S. (1995) Differential expression and co-assembly of NMDAz1 and e subunits in the mouse cerebellum during postnatal development. *NeuroReport.* **6,** 2255–2259.
53. 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.
54. Dunah A. W., Yasuda R. P., Wang Y-H., Luo J., Davila-Garcia M. I., Gbadegesin M., Vicini S., and Wolfe B. B. (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.
55. Wenzel A., Fritschy J. M., Mohler H., and Benke D. (1997) NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. *J. Neurochem.* **68,** 469–478.
56. Dunah A. W., Luo J., Wang Y-H., Yasuda R. P., and Wolfe B. B. (1998) Subunit composition of NMDA receptors in the rat central nervous sys-

tem that contain the NR2D subunit. *Mol. Pharmacol.* **53,** 429–437.
57. Blahos J. II and Wenthold R. J. (1996) Relationship between N-methyl-D-aspartate receptor NR1 splice variants and NR2 subunits. *J. Biol. Chem.* **271,** 15,669–15,674.
58. Hall R. A. and Soderling T. R. (1997) Differential surface expression and phosphorylation of the N-methyl-D-aspartate receptor subunits NR1 and NR2 in cultured hippocampal neurons. *J. Biol. Chem.* **272,** 4135–4140.
59. Tingley W. G., Ehlers M. D., Kameyama K., Doherty C., Ptak J. B., Riley C. T., and Huganir R. L. (1997) Characterization of protein kinase A and protein kinase C phosphorylation of the N-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *J. Biol. Chem.* **272,** 5157–5166.
60. Moon I. S., Apperson M. L., and Kennedy M. B. (1994) The major tyrosine-phosphorylated protein in the postsynaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. *Proc. Natl. Acad. Sci. USA* **91,** 3954–3958.
61. Rostas J. A. P., Brent V. A., Voss K., Errington M. L., Bliss T. V. P., and Gurd J. W. (1996) Enhanced tyrosine phosphorylation of the 2B subunit of the N-methyl-D-aspartate receptor in long term-potentiation. *Proc. Natl. Acad. Sci.* USA **93,** 10,452–10,456.
62. Rosenblum K., Dudai Y., and Richter-Levin G. (1996) Long-term potentiation increases tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit 2B in rat dentate gyrus *in vivo*. *Proc. Natl. Acad. Sci.* USA **93,** 10,457–10,462.
63. Dunah A. W., Yasuda R. P., and Wolfe B. B. (1998) Developmental regulation of the tyrosine phosphorylation of the NR2D NMDA glutamate receptor subunit in the rat central nervous system. *J. Neurochem.* **71,** 1926–1934.
64. Tam P. J. (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl. Acad. Sci. USA* **85,** 5409–5413.
65. Posnett D. N., McGrath H., and Tam P. J. (1988) A novel method for producing anti-peptide antibodies. *J. Biol. Chem.* **263,** 1719–1725.
66. Tolle T. R., Berthele A., Zieglgansberger W., Seeburg P. H., and Wisden W. (1993) The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal grey. *J. Neurosci.* **13,** 5009–5028.
67. Standaert D. G., Testa C. M., Young A. B., and Penny J. B. Jr. (1994) Organization of N-methyl-D-aspartate glutamate receptor gene expression in the basal ganglia of the rat. *J. Comp. Neurol.* **343,** 1–16.
68. Aoki C., Venkatesan C., Go C.-G., Mong J. A., and Dawson T. M. (1994) Cellular and subcellular localization of NMDA-R1 subunit immunoreactivity in the visual cortex of adult and neonatal rats. *J. Neurosci.* **14,** 5202–5222.
69. Watanabe M., Inoue Y., Sakimura K., and Mishina M. (1993) Distinct spatio-temporal distributions of the NMDA receptor channel subunit mRNAs in the brain. *Ann. NY Acad. Sci.* **707,** 463–466.
70. Monaghan D. T. and Cotman C. W. (1985) Distribution of N-methyl-D-aspartate-sensitive L-[H]glutamatebinding sites in rat brain. *J. Neurosci.* **11,** 2909–2919.
71. Boje K. M. and Skolnick P. (1992) Ontogeny of glycine-enhanced [^{3}H]MK-801 binding to N-methyl-D-aspartate receptor-coupled ion channels. *Dev. Brain Res.* **65,** 51–56.
72. Morin A. M., Hattori H., Wasterlain C. G., and Thomson D. (1989) [^{3}H]MK-801 binding sites in neonate rat brain. *Brain Res.* **487,** 376–379.
73. Liu M., Wang H., Sheng M., Jan L. Y., Jan Y. N., and Basbaum A. I. (1994) Evidence for presynaptic N-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn. *Proc. Natl. Acad. Sci.* USA **91,** 8383–8387.
74. Cull-Candy S. G., Brickley S. G., Misra C., Feldmeyer D., Momiyama A., and Farrant M. (1998) NMDA receptor diversity in the cerebellum: identification of subunits contributing to functional receptors. *Neuropharmacology* **37,** 1369–1380.
75. Buller A. L. and Monaghan D. T. (1997) Pharmacological heterogeneity of NMDA receptors: characterization of NR1a/NR2D heteromers expressed in *Xenopus* oocyte. *Eur. J. Pharmacol.* **320,** 87–94.
76. Sakurai S. Y., Penny J. B., and Young A. B. (1993) Regional distinct N-methyl-D-aspartate receptors distinguished by quantitative autoradiography of [3H]MK-801 binding in rat brain. *J. Neurochem.* **60,** 1344–1353.
77. Subramaniam S. and McGonigle P. (1991) Quantitative autoradiographic characterization of the binding of (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine ([^{3}H]MK-801) in rat brain: regional effects of polyamines. *J. Pharmacol. Exp. Ther.* **256,** 811–819.

78. Monaghan D. T. (1991) Differential stimulation of [^{3}H]MK-801 binding to subpopulations of NMDA receptors. *Neurosci. Lett.* **122,** 21–24.
79. Carmignoto G. and Vicini S. (1992) Activity-dependent decrease in NMDA responses during development of the visual cortex. *Science* **258,** 1007–1011.
80. Pujic Z., Matsumoto I., and Wilce P. A. (1993) Expression of the gene coding for the NR1 subunit of NMDA receptor during rat brain development. *Neurosci. Lett.* **162,** 67–70.
81. Riva M. A., Tascedda F., Molteni R., and Racagni G. (1994) Regulation of NMDA receptor subunit mRNA expression in the brain during postnatal development. *Mol. Brain Res.* **25,** 209–216.
82. Zhong J., Carrozza D. P., Williams K., Pritchett D. B., and Molinoff P. B. (1995) Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain. *J. Neurochem.* **64,** 531–539.
83. Sucher N. J., Brose N., Deitcher D. L., Awobuluyi M., Gasic G. P., Bading H., Cepco C. L., Greenberg M. E., Jahn R., Heinemann S. F., and Lipton S. A. (1993) Expression of endogenous NMDAR1 transcripts without receptor protein suggests post-translational control in PC12 cells. *J. Biol. Chem.* **268,** 22,299–22,304.
84. 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.
85. Watanabe M., Inoue Y., Sakimura K., and Mishina M. (1992) Developmental changes in the distribution of NMDA receptor channel subunit mRNAs. *NeuroReport* **3,** 1138–1140.
86. Watanabe M., Mishina M., and Inoue Y. (1994) Distinct spatiotemporal expressions of five NMDA receptor channel subunit mRNAs in the cerebellum. *J. Comp. Neurol.* **343,** 513–519.
87. Buller A. L., Larson H. C., Schneider B. E., Beato J. A., Morrisett R. A., and Monaghan D. T. (1994) The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. *J. Neurosci.* **14,** 5471–5484.
88. Beaton J. A., Stemsrud K., and Monaghan D. T. (1992) Identification of a novel N-methyl-D-aspartate receptor population in the rat medial thalamus. *J. Neurochem.* **59,** 754–757.
89. Hollmann M. and Heinemann S. (1993) Cloned glutamate receptors. *Annu. Rev. Neurosci.* **17,** 31–108.
90. Chazot P. L., Coleman S. K., Cik M., and Stephenson F. A. (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,** 24,403–24,409.
91. Hunter T. (1987) A thousand and one protein kinases. *Cell* **50,** 823–829.
92. Pang D. T., Wang J. K. T., Valtorta F., Benfenati F., and Greengard P. (1988) Protein tyrosine phosphorylation in synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **85,** 762–766.
93. Huganir R. L. and Greengard P. (1990) Regulation of neurotransmitter receptor by desensitization protein phosphorylation. *Neuron* **5,** 555–567.
94. Chan A. C., Desai D. M., and Weiss A. (1994) The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Ann. Rev. Immunol.* **12,** 555–592.
95. Edelman A. M., Blumenthal D. K., and Krebs E. G. (1987) Protein serine/threonine kinases. *Ann. Rev. Biochem.* **56,** 567–613.
96. Swope S. L., Moss S. J., Blackstone C. D., and Huganir R. L. (1992) Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity. *FASEB J.* **6,** 2514–2523.
97. Hunter T. and Cooper J. A. (1988) Protein tyrosine kinases. *Ann. Rev. Biochem.* **54,** 897–930.
98. Nairn A. C., Hemmings H. C., and Greengard P. (1985) Protein kinases in the brain. *Annu. Rev. Biochem.* **54,** 931–976.
99. Huganir R. L., Miles K., and Greengard P. (1984) Phosphorylation of the nicotinic acetycholine receptor by an endogenous tyrosine-specific protein kinase. *Proc. Natl. Acad. Sci. USA* **81,** 6963–6972.
100. Maness P. F., Aubry M., Shores C. G., Frame L., and Pfenninger K. H. (1988) c-src gene product in developing rat brain is enriched in growth cone membranes. *Proc. Natl. Acad. Sci. USA* **85,** 5011–5005.
101. Wagner K. R., Mei L., and Huganir R. L. (1991) Protein tyrosine kinases and phosphotases in the nervous system. *Curr. Opin. Neurobiol.* **1,** 65–73.
102. Kohr G. and Seeburg P. H. (1996) Subtype-specific regulation of recombinant NMDA

receptor-channels by protein tyrosine kinases of the *src* family. *J. Physiol.* **492(2),** 445–452.
103. Levitan I. B. (1994) Modulation of ion channels by protein phosphorylation and dephosphorylation. *Ann. Rev. Physiol.* **56,** 193–202.
104. Siegelbaum S. A. (1994) Ion channel control by tyrosine phosphorylation. *Curr. Biol.* **4,** 242–245.
105. Roche K. W., Tingley W. G., and Huganir R. L. (1994) Glutamate receptor phosphorylation and synaptic plasticity. *Curr. Opin. Neurobiol.* **4,** 383–388.
106. Moss S. J., Gorrie G. H., Amato A., and Smart T. G. (1995) Modulation of GABA receptors by tyrosine phosphorylation. *Nature* **377,** 344–348.
107. O'Dell T. J., Kandel E. R., and Grant S. G. N. (1991) Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature* **353,** 558–560.
108. Wang Y. T. and Salter M. W. (1994) Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature* **369,** 233–235.
109. Yu X-M., Askalan R., Keil G. J. II., and Salter M. W. (1997) NMDA channel regulation by channel-associated protein tyrosine kinase Src. *Science* **275,** 674–678.