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The major tyrosine-phosphorylated protein in the postsynaptic density fraction is *N*-methyl-D-aspartate receptor subunit 2B

(synapse/synaptic plasticity/signal transduction/glutamate receptor)

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ABSTRACT **The postsynaptic density (PSD) is a specialization of the submembranous cytoskeleton that is visible in the electron microscope on the cytoplasmic face of the postsynaptic membrane. A subcellular fraction enriched in structures with the morphology of PSDs contains signal-transduction molecules thought to regulate receptor localization and function in the central nervous system. We have purified a prominent tyrosine-phosphorylated glycoprotein of apparent molecular mass 180 kDa, termed PSD-gp180, that is highly enriched in the rat forebrain PSD fraction. The sequences of four tryptic peptides generated from the protein reveal that it is the 2B subunit of the *N*-methyl-D-aspartate (NMDA) type glutamate receptor. We have confirmed the identity of PSD-gp180 by showing that it reacts with antibodies raised against a unique fragment of the 2B subunit of the NMDA receptor. We also show that the 2B subunit is the most prominently tyrosine-phosphorylated protein in the PSD fraction based upon recognition by an anti-phosphotyrosine antibody. Two types of NMDA receptor subunits have been identified by molecular cloning [Nakanishi, S. (1992) *Science* 258, 597–603]. The single type 1 subunit is expressed throughout the brain and is necessary for formation of the receptor channel. The four type 2 subunits (2A, 2B, 2C, and 2D) are expressed in discrete brain regions, contain unusually long unique C termini, and confer distinct kinetic properties on NMDA receptors that contain them. Our findings suggest that, in the forebrain, NMDA receptor subunit 2B may serve to anchor NMDA receptors at the postsynaptic membrane through its interaction with the PSD. The prominent presence of tyrosine phosphate further suggests that the NMDA receptor may be regulated by tyrosine phosphorylation or that it may participate in signaling through tyrosine phosphorylation and through its ion channel.**

Regulation of synaptic transmission by prior neural activity is an important mechanism of information processing in the central nervous system. Such regulation occurs in part through modulation of transmitter receptors in the postsynaptic membrane (1, 2). Excitatory synapses in the central nervous system contain two major classes of postsynaptic glutamate receptors: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type receptors generate the basal postsynaptic potential; whereas *N*-methyl-D-aspartate (NMDA)-type receptors display unique voltage-dependent activation properties and play a critical role in initiation of long-term potentiation, a form of synaptic plasticity believed to be important for formation of memories (3, 4).

The postsynaptic density (PSD) is a specialization of the submembranous cytoskeleton visible in the electron microscope that appears to represent a tight complex of postsynaptic junctional proteins (for a recent review, see ref. 5). A subcellular fraction from rat forebrain called the PSD fraction is enriched in structures with the morphology of PSDs (6, 7). AMPA receptor subunits are associated with the PSD fraction (8–10) and may be enriched there (8). The fraction also contains high-affinity glutamate binding sites with some of the pharmacological properties of NMDA receptor sites (11) and proteins that may participate in receptor regulation and in regulation of other forms of synaptic efficacy. These include the subunits of Ca^{2+}/calmodulin-dependent protein kinase II (12–15), a src-like protein tyrosine kinase (16), a cAMP-dependent protein kinase anchoring protein (17), and PSD-95, a close homologue of the *Drosophila* discs-large tumor suppressor protein which participates in formation of septate junctions and growth control in developing flies (18). Ca^{2+}/calmodulin-dependent protein kinase II (19) and PSD-95 (18) have been shown to be highly enriched in the PSD fraction and are associated with PSDs *in situ* as determined by immunocytochemical staining of brain synaptosomes (ref. 15 and C. Hunt, L. Schenker, and M.B.K., unpublished observation) reinforcing the notion that the major proteins of the PSD fraction represent a functional protein complex.

Here we show that a 180-kDa glycoprotein highly enriched in the PSD fraction is subunit 2B of the NMDA receptor (NR2B). We show also that NR2B is the most prominently tyrosine-phosphorylated protein in the PSD fraction.

Two distinct types of NMDA receptor subunits were identified recently by molecular cloning (20–22). Like most ionotropic receptors, NMDA receptors are composed of multiple subunits with homologous sequences (23, 24). NMDA receptor subunit 1 (termed NR1) has a molecular mass (106 kDa) close to that of the subunits of the AMPA class of glutamate receptors. In contrast, the NMDA receptor subunits termed 2A, 2B, 2C, and 2D (NR2A, NR2B, NR2C, and NR2D, respectively) are considerably larger, having molecular masses from 135 kDa to 166 kDa. Their larger sizes result from long C termini that are only distantly related to each other in sequence. Phosphorylation sites for protein kinase C have been localized to the extreme C terminus of the NR1 subunit (25), suggesting that the long C-terminal domains of the homologous type 2 subunits also extend into the cytosol where they could mediate additional regulation by phosphorylation (25, 26). NR1 is expressed throughout the brain and is essential for formation of a receptor channel; whereas, the NR2 subunits are not essential for channel formation, have more restricted expression patterns, and appear to modulate receptor channel kinetics in different ways (22–24). The findings reported here suggest that NR2B associates tightly with the PSD structure and thus may mediate specific interactions with PSD proteins and other cytosolic proteins perhaps through tyrosine phosphorylated domains.

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Abbreviations: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; PSD, postsynaptic density; NR1, NR2B, etc., NMDA receptor subunits 1, 2B, etc.; NOG, *n*-octyl glucoside.

MATERIALS AND METHODS

Purification of PSD-gp180. The crude PSD fraction (90 mg; 3–5 mg/ml) was prepared as described (18) by a modification of the method of Carlin *et al.* (7). The PSD fraction was extracted with 1% *n*-octyl glucoside (NOG) at 4°C for 30 min with stirring. Insoluble material was pelleted by centrifugation at 200,000× *g* for 30 min at 4°C. The pellet (NOG-P) (2 mg/ml) was treated with Endoglycosidase F/N-glycosidase F (Boehringer Mannheim) in 0.2% SDS according to the supplier's instructions. To purify individual proteins, the deglycosylated proteins (63 mg) were fractionated on 60 preparative SDS/6% polyacrylamide gels. Each gel was stained with 0.2% Coomassie blue R-250 in 10 mM Tris Cl, pH 8.0/0.01% SDS for 30 min. Bands containing two proteins, PSD-gp180 and a second protein termed PSD-up180, were excised from the gels, pooled, and chopped into 3- to 5-mm pieces. Protein (≈400 μg per sample) was electroeluted from the pieces in an Elutrap device (Schleicher & Schuell) at 250 V in 25 mM *N*-ethylmorpholine, pH 8.5/0.01% SDS overnight at room temperature into ≈6 ml of 0.5% SDS/25 mM *N*-ethylmorpholine. Because the purified proteins still contained a small amount of bound Coomassie dye that interferred with determination of protein by the method of Peterson, protein was estimated after SDS/gel electrophoresis by comparison to a set of Coomassie-stained bovine serum albumin standards.

Preparation of Antibodies Against the C Terminus of NR2B. Bacteriophage clones containing cDNA inserts encoding NR2B were selected from a λgt11 library (Clontech) by screening with an oligonucleotide sequence deduced from peptide sequence 2 (Table 1). A restriction fragment (*Pml* I–*Eco*RI) encoding 334 aa of the C terminus of NR2B (from aa 1149 to the C terminus) was ligated into the pGEX-3X expression vector (Pharmacia). A glutathione *S*-transferase fusion protein containing the NR2B fragment was expressed in *Escherichia coli* and purified from inclusion bodies by electrophoresis through a SDS/6% polyacrylamide gel. The protein was visualized after soaking the gel in 0.25 M KCl. Bands containing the fusion protein were cut from the gel and used to immunize mice from which polyclonal ascites fluid was prepared as described by Ou *et al.* (27). Antibodies from the ascites fluid recognize a single band of ≈180 kDa at a 1:5000 dilution on immunoblots of proteins from a rat brain homogenate (data not shown). Control ascites fluid generated without immunization with NR2B does not react with any proteins in the PSD fraction.

Immunoblots with Anti-NR2B and Anti-Phosphotyrosine. For the experiments described in Figs. 2 and 3, the crude PSD fraction was prepared as described (18), except that 0.1 mM orthovanadate was included to inhibit dephosphorylation of tyrosine residues. Transfer to nitrocellulose and incubation with antibodies was carried out as described by Cudmore and Gurd (28). Antibody 4g10 against phosphotyrosine was purchased from Upstate Biotechnology. Primary antibodies were visualized after incubation with alkaline phosphatase-conjugated rabbit IgG according to the supplier's instructions (Boehringer Mannheim).

Table 1. Peptides

Peptides	Amino acid sequence	Amino acid positions in NR2B
1	FGTVPXGSTE	683–692
2	SDVSDISTHTVTYGD	1058–1072
3	FYLDQFR	1132–1138
4	NLTNVDWEDR	1200–1208

Peptides were sequenced by the California Institute of Technology Applied Microsequencing Facility on an Applied Biosystems automatic sequencer. Initial yields were 41, 3, 28, and 23 pmol for sequences 1–4, respectively. The sequence numbering for NR2B is based upon the numbering of the protein product of the cDNA (GenBank accession no. M91562). At the position marked X in sequence 1, we obtained no identifiable amino acid derivative; this residue is asparagine (N) in NR2B. In sequence 2, the last residue was identified as aspartate (D), whereas it is asparagine (N) in NR2B. Deamidation of the tryptic peptide may have caused this discrepancy.

Immunoprecipitation. Crude PSD fraction (500 μg) prepared in the presence of 0.1 mM orthovanadate was brought to 0.7% SDS and boiled for 3 min. The solution was adjusted to final concentrations of 10 mM Tris Cl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS (RIPA buffer) in a final volume of 1 ml. Ascites fluid (anti-NR2B or control antibody) was added at a 1:50 dilution, and the mixture was incubated for 4 hr at 4°C with mixing. The solution was cleared by centrifugation for 20 min at 12,000 rpm in an Eppendorf centrifuge at 4°C. Protein A-agarose beads (250 μl, Pierce) washed in RIPA buffer were added to the supernatant and incubation was continued for an additional 4 hr. The beads were collected by centrifugation and washed five times with RIPA buffer. Sample solution for SDS/polyacrylamide gel electrophoresis was added and the mixture was boiled for 3 min. Beads were pelleted by centrifugation and a volume of the supernatant corresponding to 100 μg of the original PSD fraction was applied to each lane of a SDS/6% gel.

Analytical Methods. Except where noted, protein was measured by the method of Lowry *et al.* (29) as modified by Peterson (30). SDS/polyacrylamide gel electrophoresis was performed by the method of Laemmli (31).

RESULTS

Purification of PSD-gp180. A crude PSD fraction was isolated from rat forebrains. We investigated the proteins contained in a broad band at 180 kDa (Fig. 1) because they have previously been shown to contain tyrosine phosphate (32), to be phosphorylated by a calmodulin-dependent protein kinase (33), and to remain associated with the PSD fraction after extraction with the harsh detergent sarkosyl (18, 34). Three proteins associated with this broad band were resolved from one another. The first was selectively removed by extraction of the PSD fraction with 1% NOG (Fig. 1*A*). Enzymatic deglycosylation shifted the electrophoretic mobility of a second protein band (PSD-gp180) to an apparent molecular mass of 160 kDa and did not alter the mobility of a third protein band (PSD-up180). We electroeluted several hundred micrograms of each individual protein from preparative SDS gels for sequencing. Each electroeluted protein appeared as a single band upon SDS/gel electrophoresis (Fig. 1*B*).

Sequencing and Identification of NR2B. Purified PSD-gp180 (≈300 μg) was trypsinized as described (18, 35) and four tryptic peptides were purified and sequenced (Table 1). A search of the GenBank data base performed with the BLAST network service through the National Center for Biotechnology Information (April 1993) revealed that all four sequences are present in NR2B (21, 22, 36, 37). Sequences 2–4 are unique to NR2B; sequence 1 is found in all four type 2 NMDA receptor subunits.

Identification of NR2B by Antibody Binding. We raised antibodies against a portion of the C-terminal domain that is unique to NR2B to verify that PSD-gp180 is NR2B. Immunoblots of the NOG-treated PSD fraction were probed with these antibodies and revealed that NR2B was present at an apparent molecular mass of 180 kDa before deglycosylation. Its mobility shifted completely to an apparent molecular mass of 160 kDa after deglycosylation (Fig. 2). The anti-NR2B antibodies also recognized purified electroeluted PSD-gp180 protein.

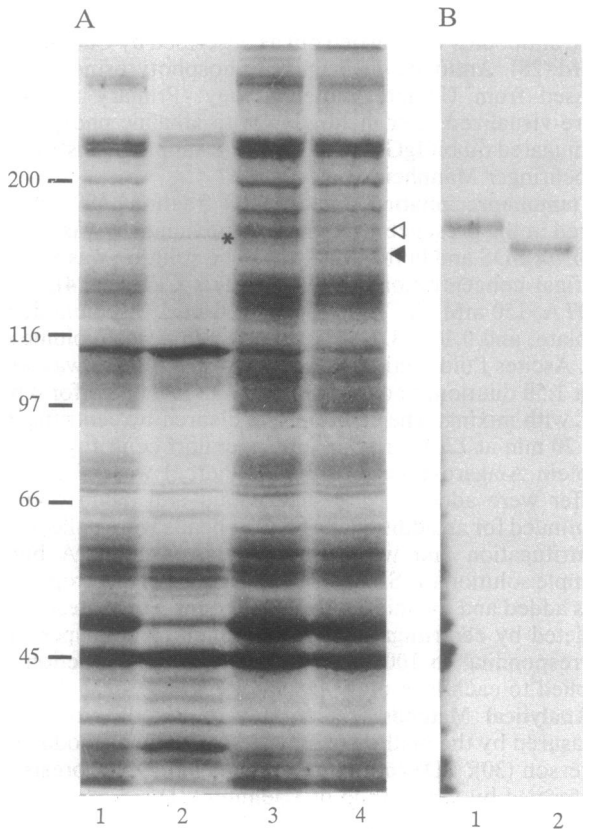

FIG. 1. Purification of PSD-gp180. (*A*) Separation of three protein bands with apparent molecular masses of 180 kDa. A crude PSD fraction (lane 1) was extracted with NOG. One of the 180-kDa proteins (*) was solubilized during the extraction (lane 2); two others (◁, PSD-up180; ◀, PSD-gp180) remained associated with the NOG-insoluble pellet (lane 3). Treatment of the pellet with a mixture of endoglycosidases shifted the mobility of PSD-gp180; the two proteins were then separable by SDS/polyacrylamide gel electrophoresis (lane 4). Proteins in each fraction were separated by electrophoresis on a 6% SDS gel and stained with Coomassie blue. (*B*) Electroeluted PSD-gp180 and PSD-up180. After deglycosylation of the NOG-insoluble pellet, PSD-up180 and PSD-gp180 were purified to near homogeneity by electroelution from preparative SDS gels. A 2-μg aliquot of each protein was electrophoresed in a SDS/6% gel and stained with Coomassie blue. Lanes: 1, PSD-up180; 2, PSD-gp180. Positions of molecular size standards (in kilodaltons) are at left.

Reaction of NR2B with Anti-Phosphotyrosine Antibodies. It has been reported (30) that a 180-kDa glycoprotein from the PSD fraction is a tyrosine-phosphorylated protein. To test whether NR2B contains tyrosine phosphate, blots of the PSD fraction were probed with monoclonal antibody 4g10 against phosphotyrosine (Fig. 2). The major tyrosine-phosphorylated protein band in the PSD fraction comigrated at 180 kDa with NR2B and shifted to an apparent molecular mass of 160 kDa after deglycosylation (lanes 4 and 5), in parallel with NR2B (lanes 1 and 2). In addition, purified electroeluted PSD-gp180 reacted with the anti-phosphotyrosine antibody (lane 6). Preabsorption of the anti-phosphotyrosine antibody with 2 mM phosphotyrosine eliminated staining of the 180- and 160-kDa bands as expected; whereas preabsorption with phosphoserine or phosphothreonine did not reduce the staining (data not shown). To eliminate the possibility that a distinct protein comigrating with PSD-gp180 might contain the tyrosine phosphate, we immunoprecipitated NR2B from the PSD fraction after solubilization and found that the immunoprecipitated protein contains phosphotyrosine (Fig. 3). Indeed, immunoprecipitation from the solubilized PSD fraction of nearly all the protein reacting with anti-NR2B antibodies removed more than two-thirds of the anti-

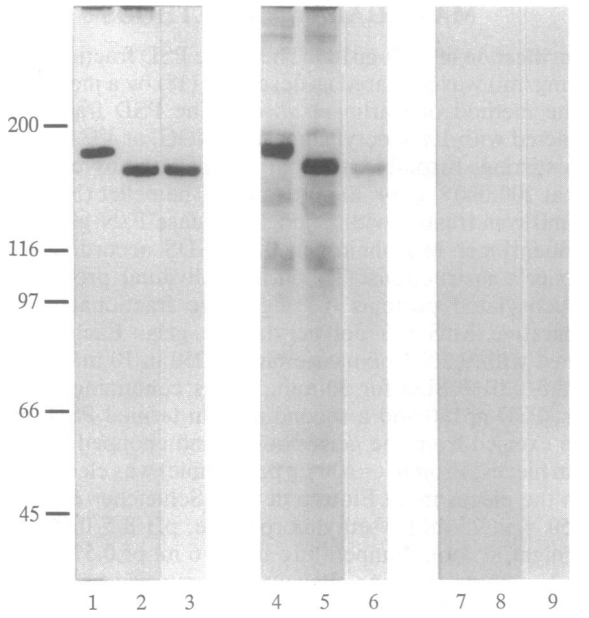

FIG. 2. Reaction of proteins in the PSD fraction with anti-NR2B and anti-phosphotyrosine antibodies. The PSD fraction before (lanes 1, 4, and 7) and after (lanes 2, 5, and 8) deglycosylation (20 μg) and purified electroeluted PSD-gp180 (lanes 3, 6, and 9; 0.2 μg) were probed with antibodies raised against NR2B (lanes 1–3; 1:5000 dilution), with antibodies against phosphotyrosine (lanes 4–6; 4g10, 1:2000 dilution), and with control ascites fluid (lanes 7–9; 1:5000 dilution). Lanes: 1, 2, 4, 5, 7, and 8, NOG-treated PSD fractions; 3, 6, and 9, electroeluted PSD-gp180 (purified in the absence of orthovanadate). Positions of molecular size standards (in kilodaltons) are at left.

phosphotyrosine immunoreactivity at 180 kDa (data not shown). Thus the data in Figs. 2 and 3 demonstrate that NR2B is the most highly tyrosine-phosphorylated protein in the PSD fraction based upon recognition by the anti-phosphotyrosine antibody.

Enrichment of NR2B in the PSD Fraction. If NR2B is specifically associated with the PSD, it should be highly enriched in the PSD fraction, as are two other proteins previously identified as significant components of the PSD, the α subunit of Ca^{2+}/calmodulin-dependent protein kinase II and PSD-95 (15, 18). To assess the level of enrichment of NR2B in the PSD fraction, we prepared immunoblots of rat forebrain homogenates, synaptosomes, and three PSD fractions extracted with successively harsher detergent washes, probing with anti-NR2B antibodies (Fig. 4). NR2B was highly enriched in the PSD fractions, appearing ≈10-fold more concentrated than in synaptosomes (compare lanes 2, 4, and 5) and 30- to 50-fold more concentrated than in the homogenate (compare lanes 1, 4, and 5).

DISCUSSION

We have shown that NR2B is significantly enriched in the PSD fraction and is the most highly tyrosine-phosphorylated protein present there. The latter conclusion is based upon the assumption that antibody 4g10, which was raised against a phosphotyramine conjugate, reacts uniformly with tyrosine-phosphorylated proteins. This assumption is supported by studies demonstrating that 4g10 binds to a broad range of tyrosine-phosphorylated proteins on immunoblots, although differences in the affinities of 4g10 for tyrosine-phosphorylated proteins have not been strictly ruled out (38). NR2B may be identical to the tyrosine-phosphorylated protein termed gp180 described by Gurd (32); however, we have not compared it directly with the protein purified by his protocol.

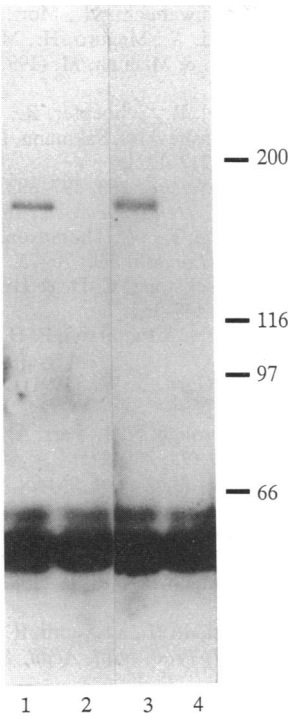

FIG. 3. Reaction of immunoprecipitated PSD-gp180 with anti-phosphotyrosine antibody. PSD-gp180 protein was immunoprecipitated from the crude PSD pellet (prepared in the presence of orthovanadate) with anti-NR2B antibodies. Proteins immunoprecipitated by anti-NR2B (lanes 1 and 3) and proteins immunoprecipitated by the control ascites fluid (lanes 2 and 4) were probed with anti-NR2B antibodies (lanes 1 and 2) or anti-phosphotyrosine antibody (lanes 3 and 4) as described in Fig. 2. Positions of molecular size standards (in kilodaltons) are at right.

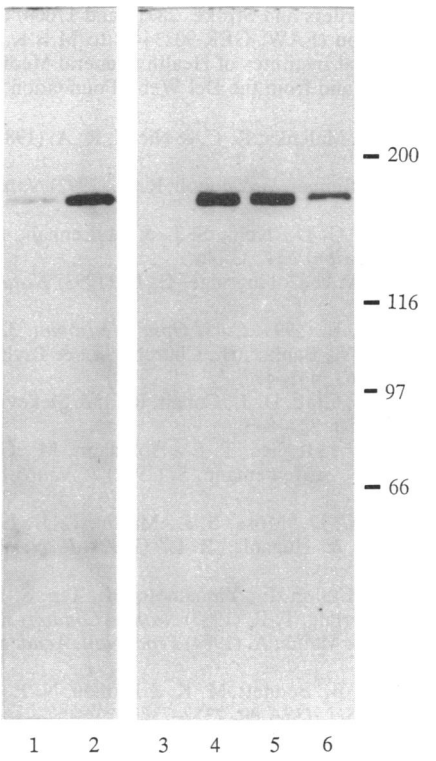

FIG. 4. Enrichment of PSD-gp180 in the PSD fraction. Fifty micrograms of rat forebrain homogenate and synaptosomes (lanes 1 and 2, respectively), 5 μg of synaptosomes (lane 3), 5 μg of crude PSD fraction (lane 4), PSD fraction extracted twice with 0.5% Triton X-100 (lane 5), and PSD fraction extracted once with Triton X-100 followed by 3% sarkosyl (lane 6) were electrophoresed in a SDS/6% gel and transferred to nitrocellulose. The blots were probed with mouse polyclonal anti-NR2B antibody as described in Fig. 2. Forebrain homogenate, synaptosomes, and detergent-extracted PSD fractions were prepared as described (18). Positions of molecular size standards (in kilodaltons) are at right.

The concentration of NR2B in the PSD fraction is higher than that of NR2A, which is also expressed in the forebrain (22). NR2A would be expected to comigrate on SDS gels with NR2B because they have nearly identical molecular masses. However, our peptide sequences (Table 1) did not match any unique to NR2A, indicating that it is absent or present in considerably lower quantity than NR2B. In addition, more than two-thirds of the protein migrating at 180 kDa was immunoprecipitated by a specific anti-NR2B antibody. Nevertheless, the small amount of tyrosine-phosphorylated protein remaining at the 180-kDa position of SDS gels after immunoprecipitation of NR2B may be NR2A. The relatively high concentration of NR2B in the PSD fraction indicates either that it is expressed at higher levels than NR2A or that it binds more avidly to PSD proteins and, therefore, remains in the PSD fraction during the detergent extraction that removes other synaptic membrane proteins. If the latter is true, we suggest that NR2B may anchor NMDA receptors in the postsynaptic membrane through its association with PSD proteins.

Two potential regulatory roles for tyrosine phosphorylation of NR2B, which are not mutually exclusive, are suggested by studies of other membrane receptors. Agrin, a protein released from motor neuron terminals, induces clustering of acetylcholine receptor subunits at postsynaptic sites (39). Agrin also induces tyrosine phosphorylation of the β subunit of the chicken acetylcholine receptor (40). The tyrosine phosphorylation precedes receptor clustering; furthermore, agents that block the tyrosine phosphorylation also block agrin-induced receptor clustering, indicating that tyrosine phosphorylation is a critical part of the clustering mechanism in chicken muscle. Thus, by analogy, it is possible that tyrosine phosphorylation of NR2B may catalyze clustering of NMDA receptors.

An intriguing possibility is that the tyrosine phosphorylation may permit interaction of the NMDA receptor with proteins that contain src-homology-2 domains (termed SH2 domains; ref. 41), creating an assembly point for cytosolic signal transduction complexes (42). For example, it was recently demonstrated that binding of growth factors to receptor tyrosine kinases results in the formation of a complex between autophosphorylated tyrosine residues in the cytoplasmic tales of the receptors and SH2 domains contained in a protein termed Grb2 (43). Formation of this complex leads to activation of the Ras GTP-binding protein. Activated Ras then catalyzes, through a protein kinase cascade, activation of mitogen-activated protein kinase. Formation of such complexes might permit the NMDA receptor to participate in similar signal transduction pathways and could underlie the recent observation that activation of NMDA receptors results in phosphorylation and activation of mitogen-activated protein kinase in hippocampal neurons (44).

It will be important to localize in the NR2B sequence the sites of tyrosine phosphorylation and the domains of interaction with PSD proteins. These studies may help to clarify the functional significance of the unusually long C-terminal domains of the type 2 NMDA receptor subunits.

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