The trafficking and phosphorylation of the NR1 and NR2 subunits of the N-methyl-D-aspartate-type glutamate receptor complex were studied in cultured rat hippocampal neurons. Surface expression was examined by modifying surface receptors via treatment of intact neurons with either the protease chymotrypsin or the cross-linking reagent bis(sulfosuccinimidyl)suberate, followed by quantification of anti-NR1 and anti-NR2B Western blot immunostaining. These studies revealed that only 40–50% of total NR1 immunoreactivity is found at the cell surface, as compared to more than 90% of total NR2B immunoreactivity. Metabolic labeling of the cultures with $^{32}$P revealed that NR2 subunits are highly phosphorylated under basal conditions, whereas basal phosphorylation of NR1 subunits is barely detectable. Following stimulation of the cultures with glutamate/glycine or phorbol esters, NR1 phosphorylation was found to be enhanced by 3–5-fold, whereas phosphorylation of NR2 subunits was enhanced by less than 2-fold. To determine whether the difference in the basal NR1 versus NR2 phosphorylation could be due to tyrosine phosphorylation of NR2, phosphoamino acid analyses of NR2 were performed. These analyses revealed phosphorylation on serine but not on threonine or tyrosine; immunoprecipitation and deglycosylation experiments using anti-phosphotyrosine antibodies confirmed that NR2 subunits in the primary hippocampal cultures are not detectably phosphorylated on tyrosine residues. These results demonstrate that the NR1 and NR2 subunits, which assemble into heteromeric complexes to form functional N-methyl-D-aspartate receptors, are trafficked in neurons with differential efficiency to the plasma membrane and exhibit different levels of basal versus stimulated serine phosphorylation.

Fast excitatory synaptic transmission in the mammalian brain is mediated by two classes of receptor; both classes contain intrinsic ion channels and are responsive to the neurotransmitter glutamate. These receptor classes are defined by the specific agonists that activate them: N-methyl-D-aspartate (NMDA)$^1$ and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). A third class of mammalian glutamate-gated ion channels, the kainate receptors, has been characterized and cloned, but the functional significance of these receptors in brain circuits is unclear (1).

The molecular structure of glutamate receptors has been the subject of intensive study. NMDA receptors are comprised of NR1 subunits, which exist as a number of splice variants derived from a single gene product, and NR2 subunits, which are four different gene products referred to as NR2A–D. AMPA receptors are comprised of the GluR1–4 subunits, four different gene products that may undergo alternative splicing and RNA editing (2). Both NMDA receptors (3) and AMPA receptors (4, 5) are believed to exist as heteropentameric assemblies of the aforementioned subunits. The nature of these complexes, however, is quite different for the two classes of glutamate receptor. Whereas the four AMPA receptor subunits are very similar in both size and primary sequence (6), the NR1 subunits of the NMDA receptor share less than 20% primary sequence identity with the NR2 subunits and are 120 kDa in size compared to 180 kDa for the NR2 subunits (7). This divergence in the structure of the subunits of the NMDA receptor suggests the possibility that the different subunits may be posttranslationally regulated in very different manners, with potentially interesting implications for NMDA receptor function. A number of important questions remain unanswered about the posttranslational regulation of NMDA receptor subunits. Although it is known that both the NR1 (8, 9) and NR2 subunits (10–12) can be phosphorylated, it is not known whether the level of NR subunit phosphorylation can be altered by physiological stimulation. Moreover, although immunohistochemical studies of NMDA receptor subunits have revealed staining in both synaptic and intracellular regions (13, 14), quantitative analyses of the surface expression of the various subunits have not been performed. In the present study, we examined the cell surface trafficking of the NR1 and NR2 subunits in cultured hippocampal neurons and also quantified NMDA receptor subunit phosphorylation under basal conditions and following stimulation. We report that the NR1 and NR2 subunits of the NMDA receptor differ with regard to both their level of surface expression and their basal versus stimulated levels of phosphorylation.

MATERIALS AND METHODS

Animals and Supplies—Timed-pregnant Sprague-Dawley rats were obtained from Bantin and Kingman. Culture plates were from Falcon. Minimal essential medium (without glutamine) and heat-inactivated

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The abbreviations used are: NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; BS3, bis(sulfosuccinimidyl)suberate; SS, saline solution; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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Surface Expression of NMDA Receptor Subunits

fetal calf serum were from Life Technologies, Inc. Mitochondrial antigens were from Collaborative Research. Chymotrypsin and papain were from Worthington. BS5 was from Pierce. Monoclonal anti-NR1 was from Pharmingen. Monoclonal anti-NR2B was from Transduction Labs. Monoclonal anti-phosphotyrosine 4G10 was obtained from Upstate Biotechnology, Inc. Polyclonal anti-NR1 and anti-NR2B antibodies were gifts from Robert Wenthold (National Institutes of Health). Anti-actin monoclonal antibodies and microcystin-LR were from Boehringer Mannheim. Anti-β-tubulin monoclonal antibodies were from Sigma. Goat anti-rat brain calcium/calmodulin-dependent protein kinase II (α subunit) antibodies were developed by Bethyl Laboratories. Donkey antirabbit and donkey anti-mouse horseradish peroxidase secondary antibodies were from Amersham Corp.; swine anti-goat was from Boehringer Mannheim. Cellulose plates were from Schleicher & Schuell. ReadySafe liquid scintillation fluid was obtained from Beckman Instruments. TPA was obtained from Calbiochem. The Renaissance chemiluminescence kit was obtained from DuPont NEN. Radiolabeled orthophosphate was from ICN. Brain-derived neurotrophic factor and neurotrophin-3 were gifts from Amgen. All other reagents were from Sigma.

Cell Cultures—Primary cultures of rat hippocampal neurons were prepared from rat pups at 24–48 h of postnatal life. Hippocampi were dissected out into room temperature osmotically balanced saline solution (in mM: NaCl 5.3, KCl 1.7, NaHPO4, KH2PO4, 10 mM Hepes, 35 mM glucose, 44 mM sucrose, and 0.242 g/liter phenol red, pH 7.3, 325 mosm), cut into five to six pieces, and incubated for 1 h with 10 ml of 20% fetal calf serum in complete medium (minimal essential medium, 5% heat-inactivated fetal calf serum, 1 ml/ml serum extender, 21 mM glucose, 10 mM L-glutamine, 5-fluoro-2-deoxyuridine, and 25 μg/ml uridine). Cultures were then disassociated into complete medium via 15–20 passes through a Pasteur pipette and plated onto sterile, poly-L-lysine-coated 35-mm culture plates at an approximate density of 2 hippocampi per dish. Cultures were fed with almost complete changes of medium on postculture days 1, 4, and 7 and typically used for experiments on culture day 9 or 10. Cultures prepared via this method are of high density (roughly 1–2 million cells/plate) and have a very high neuron:glial ratio because they are cultured in the presence of a mitotic inhibitor (5-fluoro-2-deoxyuridine) from the very first day. Prior to use in experiments, cultures were washed with room temperature SS three times and then exposed to the various treatments described below.

Proteolysis—Following a wash incubation of 20 min at 37°C, cultures were incubated with 1 mg/ml chymotrypsin in SS for 10 min with agitation at 37°C. The SS was then aspirated, and the plates were washed three times in ice-cold harvest buffer (SS containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μM leupeptin, 50 mM ethylamine, 50 mM sodium orthovanadate, 50 mM NaF, 1 mM microcystin-LR, and 1 mM EDTA) for 15 min at room temperature. Following a wash incubation of 20 min at 37°C, cultures were incubated with 1 mg/ml BS5 in SS for 10 min with agitation at 37°C (these conditions were optimized in preliminary experiments). The SS was then aspirated, and the plates were washed three times in ice-cold harvest buffer (ethanolamine is present in the harvest buffer to quench any unreacted BS5). After the third wash, 1 ml of fresh, ice-cold harvest buffer was added, and the cells were scraped, transferred into a 1.5 ml snap-cap vial, briefly sonicated, and then frozen.

Cross-linking—Following a wash incubation of 20 min at 37°C, cultures were incubated with 1 mg/ml BS5 in SS for 10 min with agitation at 37°C (these conditions were optimized in preliminary experiments). Proteins were then blotted onto nitrocellulose for 4 h at 60 V. Blots were blocked for 30 min with 5% nonfat dry milk in TBS-buffered saline (TBS, 50 mM NaCl, 10 mM Tris, pH 7.4) with 0.1% Tween 20. Membranes were incubated with primary antibodies in 2% milk-TBS with 0.1% Tween 20 (“wash buffer”) for 1 h at room temperature. Blots were then washed three times (10 min each) in wash buffer and incubated for 1 h at room temperature with an appropriate horseradish peroxidase-linked secondary antibody at a dilution of 1:2000. Following this more washes with wash buffer, blots were developed via a 1-min incubation with the Renaissance chemiluminescence reagent, followed by exposure to sheets of Kodak X-Omat film for varied lengths of time. Films were developed such that all bands resulting from a given blot exposure were in the most linear range of intensity, as determined from preliminary experiments in which standard curves were constructed by plotting the relative absorbance of the immunoreactivity for increasing concentrations of lysed membranes versus the amount of membranes loaded per lane. Reductions in immunoreactivity in experimental samples were determined from these standard curves.

Metabolic Labeling and Immunoprecipitation—Hippocampal cultures were preincubated for 2 h in phosphate-free minimal essential medium and then labeled with [32P]orthophosphate (1 mCi) in 1.0 ml phosphate-free minimal essential medium for 2 h at 37°C. The cultures were washed and incubated for 15 min more at 37°C with either SS (for control cultures) or SS containing glutamate/glycine or TPA (for stimulated cultures). Following this incubation, the supernatants were removed, and the beads were washed five times with solubilization buffer. After the final wash, the beads were resuspended in 1 ml of SDS sample buffer with agitation for 5 min, and briefly centrifuged; the supernatants were loaded on 9% SDS-PAGE gels as described. Incorporation of 32P was quantified by exposing dried gels to film for varying lengths of time and then performing densitometric scanning as described. Western blots were performed on all immunoprecipitated samples from the metabolically labeled cultures to verify that equal amounts of NR1 and NR2 protein were being precipitated under all conditions.

Enzymatic Deglycosylation—To remove N-linked glycosyl residues from the NMDA receptor subunits, lysate or immunoprecipitated samples were denatured via boiling for 5 min in the presence of 0.5% SDS and 250 mM β-mercaptoethanol. The samples were then dilute five times in a buffer containing 50 mM Heps, pH 7.4, 50 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 100 μM leupeptin, 1 μM microcystin-LR, and 1 mM EDTA. The diluted samples were incubated for 12 h at 35°C in the absence or presence of 10 units/ml peptide-N-glycosidase F. The samples were then prepared for SDS-PAGE as described.

Phosphoamino Acid Analysis—Two-dimensional phospho- amino acid analyses were performed according to a method described previously (15). Briefly, excised bands from gels containing 32P-labeled samples were rehydrated, digested overnight at 37°C with trypsin, lyophilized, and analyzed as described for 1D gels. The phosphatidylstep and resuspension in a small volume of buffer, samples were loaded onto cellulose plates along with phosphoamino acid standards and separated in two dimensions as described in the aforementioned reference. The cellulose plates were dried and sprayed with ninhydrin to visualize standards and then exposed to Kodak X-Omat film for varying lengths of time to visualize the location of 32P-containing species.

RESULTS

Surface Expression of NR1 and NR2B Subunits—Western blots of lysate from hippocampal cultures were probed with commercially available antibodies raised against the NMDA receptor subunits NR1 and NR2B. NR2A and NR2B are the most prevalent NR2 subunits in hippocampal neurons, but NR2A does not appear in high levels until late in development (16, 17). Thus, NR2B should be the major NR2 subunit found in these hippocampal cultures, which are prepared from neonatal rats. Blots probed with a monoclonal anti-NR1 antibody revealed a tight cluster of bands at 120 kDa. These species of slightly different sizes probably represent alternatively spliced forms of NR1; because these bands were too compacted to allow individual quantitation, the 120-kDa anti-NR1 staining was considered in the present study as a single broad band. Blots of the same samples probed with a monoclonal anti-NR2B antibody revealed a sharp band of 180 kDa.
When intact hippocampal cultures were treated before harvest with 1 ng/ml chymotrypsin for 10 min to proteolyze externally accessible proteins, the major bands for both anti-NR1 and anti-NR2B Western blot immunostaining of the resulting lysates were reduced (Fig. 1). Longer treatments or higher concentrations of chymotrypsin did not result in significantly larger losses of immunoreactivity. Along with the reductions in immunoreactivity of the major bands came the appearance of breakdown products of approximately 65 kDa for the NR1 subunit and approximately 130 and 85 kDa for the NR2B subunit. It is likely that this pattern of immunostaining represents cleavage of surface-exposed NMDA receptor subunits by chymotrypsin, because preliminary experiments revealed that intracellular proteins were not affected by the chymotrypsin incubation. Western blot immunoreactivities for the cytoskeletal proteins actin and tubulin and the abundant intracellular enzyme calcium/calmodulin-dependent protein kinase II were unaltered by exposure of intact cells to chymotrypsin, as reported previously (18). All of these proteins were, however, readily degraded by chymotrypsin when the protease was simply added to a lysate preparation, supporting the conclusion previously (18). All of these proteins were, however, enzymatically deglycosylated overnight with peptide-N-glycosidase F; these experiments demonstrated that removal of N-linked glycosyl residues decreased the apparent size of some NMDA receptor complexes are disrupted when receptors are solubilized with Triton X-100. When the anti-NR1 precipitates were subjected to SDS-PAGE gels and autoradiography to detect $^{32}$P incorporation, a single major band of approximately 180 kDa was evident (Fig. 3). Because this band exactly comigrated with the major anti-NR2B immunoreactive band on Western blots, it presumably represents the phosphorylated NR2B subunit cross-linked together with associated proteins. Longer incubations or higher concentrations of BS$_3$ did not result in significantly larger reductions in the immunoreactivities of the major bands. As the chymotrypsin-treated samples, immunoreactivities for the intracellular proteins actin, tubulin, and calcium/calmodulin-dependent protein kinase II were unaffected by the BS$_3$ treatment, as reported previously (18), suggesting that only externally accessible proteins were cross-linked by this protocol. The reductions in immunostaining induced by the chymotrypsin and BS$_3$ treatments were quantified as described under “Materials and Methods.” The results of this quantification are shown in Fig. 2. For anti-NR1 immunoreactivity, the two surface treatments removed between 40 and 50% of the total 120-kDa immunoreactivity. For anti-NR2B immunoreactivity, the 180-kDa bands of the treated samples were reduced by more than 90% relative to control.

Stimulation of the cultures for 15 min with either 10 $\mu$M glutamate/1 $\mu$M glycine or with 2 $\mu$M of the phorbol ester TPA had no significant effect on the fraction of NR1 and NR2 subunits in the plasma membrane. The percentages of anti-NR1 immunoreactivity remaining in Western blots of stimulated cultures following cleavage of surface receptors with chymotrypsin were 52 $\pm$ 6% and 55 $\pm$ 8% for glutamate/glycine and TPA stimulation, respectively (n = 4); for anti-NR2B staining, the values were 6 $\pm$ 3% and 5 $\pm$ 3%, respectively (n = 4). Although the glutamate/glycine and TPA treatments had no acute effects on surface expression, both of these treatments were observed to result in substantial increases in NMDA receptor subunit phosphorylation (see below).
Surface Expression of NMDA Receptor Subunits

**FIG. 3. Phosphorylation of NMDA receptors in cultured hippocampal neurons.** Hippocampal cultures were labeled with [32P]orthophosphate for 2 h and then either harvested directly (CON) or stimulated for 15 min with 10 μM glutamate/1 μM glycine (GLU) or 2 μM TPA and then harvested. Samples were solubilized with a buffer containing 0.5% Triton X-100 and 0.1% sodium deoxycholate and then subjected to immunoprecipitation with an anti-NR1 antibody. The location of molecular weight markers (in thousands) is given on the left, while the arrows on the right indicate the positions of immunoprecipitated bands corresponding to the NR1 and NR2 subunits. Two independent samples are shown for each condition.

**FIG. 4. Quantitation of stimulation-induced increases in NMDA receptor subunit phosphorylation.** A, the control [32P] incorporation for either NR1 subunits (left) or NR2 subunits (right). Because all of the stimulated values are normalized to their matched control band within the same autoradiogram, these two control values are shown as equal in this bar graph, although NR2 basal phosphorylation was considerably higher than NR1 basal phosphorylation. B, the percentage of control [32P] incorporation observed following stimulation for 15 min with either 10 μM glutamate/1 μM glycine (GLU) or 2 μM TPA. Data are the means (bars, S.E.) for six independent determinations. C, deglycosylation of hippocampal culture extract that had been solubilized with 1% SDS. Western blots probed with either anti-NR2B (left) or anti-phosphotyrosine (right). The location of molecular weight markers (in thousands) is shown at left. Deglycosylation decreased the apparent size of all NR2B polypeptides in the extract but had no effect on the apparent size of the major 180-kDa phosphotyrosine-containing protein. This experiment was performed twice with identical results. B, immunoprecipitation with an anti-NR2B antibody of hippocampal culture extract that had been solubilized with 1% SDS. Western blots probed with either anti-NR2B (left) or anti-phosphotyrosine (right) are shown for the same three samples: T, total soluble extract; S, the supernatant remaining following immunoprecipitation; and P, the precipitate. Roughly one-half of total NR2B subunit immunoreactivity was precipitated by this protocol, but no anti-phosphotyrosine immunoreactivity was evident in the precipitate. C, phosphoamino acid analysis of basal NR2 phosphorylation. Excised bands were digested with trypsin, boiled in 6 M HCL, and then separated by two-dimensional electrophoresis as described under "Materials and Methods." All detectable incorporation of radiolabeled phosphate was on serine residues, with no incorporation on threonine or tyrosine residues evident.

**FIG. 5. NR2 subunits are phosphorylated on serine but not threonine or tyrosine residues.** A, deglycosylation of hippocampal culture extract followed by probing of Western blots with either anti-NR2B (left) or anti-phosphotyrosine (right). The location of molecular weight markers (in thousands) is given on the left. Deglycosylation decreased the apparent size of all NR2B polypeptides in the extract but had no effect on the apparent size of the major 180-kDa phosphotyrosine-containing protein. This experiment was performed twice with identical results. B, immunoprecipitation with an anti-NR2B antibody of hippocampal culture extract that had been solubilized with 1% SDS. Western blots probed with either anti-NR2B (left) or anti-phosphotyrosine (right) are shown for the same three samples: T, total soluble extract; S, the supernatant remaining following immunoprecipitation; and P, the precipitate. Roughly one-half of total NR2B subunit immunoreactivity was precipitated by this protocol, but no anti-phosphotyrosine immunoreactivity was evident in the precipitate. C, phosphoamino acid analysis of basal NR2 phosphorylation. Excised bands were digested with trypsin, boiled in 6 M HCL, and then separated by two-dimensional electrophoresis as described under "Materials and Methods." All detectable incorporation of radiolabeled phosphate was on serine residues, with no incorporation on threonine or tyrosine residues evident.

Evidence for Phosphorylation of NR2 Subunits on Serine but Not Threonine or Tyrosine—Phosphorylation sites for NR1 have been well characterized (8, 11), but much less is known about the sites of NR2 phosphorylation. Because the NR2 subunits have been reported to be the major tyrosine-phosphorylated species in rat brain postsynaptic density (10) and synaptic plasma membrane preparations (11, 12), a potential explanation for the much higher basal phosphorylation observed for NR2 relative to NR1 in the present study is that NR2 subunits are phosphorylated by basally active tyrosine kinases, whereas NR1 subunits are not. As shown in Fig. 5A, extracts of hippocampal culture lysate probed on Western blots with an anti-phosphotyrosine monoclonal antibody did, indeed, reveal a major band at 180 kDa, which approximately comigrated with anti-NR2B staining. When the extracts were boiled and deglycosylated, however, the major anti-NR2B band shifted to an apparent size of 165 kDa, whereas no change was evident in the anti-phosphotyrosine main band. This suggests that the two species are not the same.

This point was further examined via the immunoprecipitation studies shown in Fig. 5B. Hippocampal extract was solubilized with 1% SDS to achieve full solubilization and then diluted five times with buffer containing 1% Triton X-100, according to the method of Lau and Huganir (11). An immunoprecipitation was then performed using an anti-NR2A/B antibody (14) and protein A-Sepharose beads. Following this protocol, roughly one-half of total anti-NR2B immunostaining was precipitated. Immunostaining of the 180-kDa major band of anti-phosphotyrosine immunostaining, however, was not al-
tered. Moreover, no anti-phosphotyrosine staining was detected in the precipitated samples, even upon overexposure of the blots.

Phosphoamino acid analyses of the NR2 phosphorylation under basal conditions revealed phosphorylation on serine residues but no detectable phosphorylation on threonine or tyrosine residues (Fig. 5C). This finding correlates with the lack of NR2 tyrosine phosphorylation observed using the anti-phosphotyrosine antibody and suggests that the NR2 subunits in these primary hippocampal cultures are not tyrosine-phosphorylated. We tried a number of manipulations to attempt to induce tyrosine phosphorylation of the NR2 subunits, including exposure of the cultures for varying lengths of time to glutamate/glycine, TPA, epidermal growth factor, brain-derived neurotrophic factor, and neurotrophin-3. We also attempted to induce integrin-mediated tyrosine kinase activation (19) by plating cultures on different substrata, such as laminin or fibronectin. All of these treatments were followed by harvest of the cultures, solubilization, immunoprecipitation with anti-NR2A/B antibody, and probing of Western blots with anti-phosphotyrosine antibody as described. None of these treatments was found to lead to detectable tyrosine phosphorylation of NR2 (data not shown).

**DISCUSSION**

The data presented here demonstrate that the NR1 and NR2 subunits of the NMDA receptor are regulated in quite different ways. In terms of cell surface expression, almost all NR2B subunits are found in the plasma membrane, as compared to less than one-half of total NR1 subunits. This observed difference between the subunits is unlikely to be an artifact due simply to differences in the availability or conformation of NR1 versus NR2B extracellular domains, because two very different techniques (proteolytic cleavage with chymotrypsin and cross-linking with the small, membrane-impermeant molecule BS5) yielded strikingly similar estimates of NR1 versus NR2B surface expression. Given that recent studies in nonneuronal cells have demonstrated that NR1 subunits require coexpression with NR2 subunits to be targeted efficiently to the cell surface (20), our data suggest that neurons possess a large intracellular pool of NR1 subunits that await heteromeric assembly with NR2 subunits before they can be expressed in the plasma membrane.

The large discrepancy between NR1 and NR2B surface expression reported here represents a more quantitative description of observations made in electron microscopic immunohistochemical studies of brain slices that staining for NR2A/B subunits (14) seems to be concentrated more specifically with postsynaptic densities than staining for NR1 subunits (13), which is observed frequently in what seem to be intracereellar regions. As for a more detailed mechanistic explanation as to why NR2 subunits are trafficked so much more efficiently than NR1 subunits to the plasma membrane, it has been found recently that NR2 subunits contain specialized motifs, termed tSKV domains, which facilitate interactions with various membrane proteins including the postsynaptic density protein PSD-95 (21). Such interactions may explain, at least in part, the efficiency with which NR2 subunits are trafficked to the plasma membrane.

Protein kinase C phosphorylation of the NR1 subunit expressed in quail fibroblasts has been shown to modulate receptor clustering (9). In the present study, we did not find any evidence for an acute change in NR1 surface expression following large increases in NR1 phosphorylation induced by stimulation with either glutamate/glycine or TPA. These data suggest that although protein kinase C phosphorylation can regulate the distribution of NR1 subunits within the plasma membrane, it has no acute effect on the trafficking of NR1 subunits to and from the plasma membrane. The present data also reveal that the surface expression of NR2B subunits is not rapidly malleable in response to stimulation; the lack of an acute effect of stimulation on NMDA receptor surface expression mirrors previous observations made on the surface expression of the AMPA receptor subunits GluR1–3 (18).

It has been shown previously that phosphorylation of the NR1 subunit in neurons can be enhanced by stimulation with phorbol esters (8). The present results confirm and extend this finding by showing that NR1 phosphorylation in neurons is increased by both phorbol esters and by stimulation of NMDA receptors with their endogenous agonist, glutamate. It was also shown in the present study that stimulation with either phorbol esters or glutamate leads to an increase in NR2 subunit phosphorylation. Direct phosphorylation of NMDA receptors following NMDA receptor activation provides a potential feedback mechanism for NMDA receptors to regulate their own activity. In almost every case where a physiological effect of kinase activation or phosphatase inhibition on NMDA receptor function has been reported, the effect has been to increase the size of currents mediated by the receptor (22). Thus, phosphorylation of NMDA receptors in response to NMDA receptor activation, as observed in the present study, provides a potential positive feedback loop that might be expected to produce transient increases in synaptic strength of the type commonly reported at hippocampal synapses in response to brief bursts of synaptic activity (23).

Basal phosphorylation of NR1 under the culture conditions examined in the present study is quite low, and phosphorylation is markedly enhanced by stimulation. The situation is different for the NR2 subunits, which exhibit a high degree of basal phosphorylation and more modest increases in response to stimulation. Direct evidence for serine/threonine phosphorylation of NR2 subunits has not been reported previously, but the NR2B subunit has been shown to be a major phosphotyrosine-containing protein in brain tissue (10–12). Thus, it might seem likely that tyrosine phosphorylation would account for the high basal NR2 phosphorylation observed in the present study. Such a scenario would also account for the absence of this basal phosphorylation in the NR1 subunits, which have been shown to be phosphorylated on serine but not on tyrosine (8, 11).

It was found in the present study that the major phosphotyrosine-containing protein in hippocampal culture extract is approximately 180 kDa in size, as reported previously for rat brain postsynaptic density (10) and synaptic plasma membrane preparations (11, 12). However, this species in the hippocampal culture extract does not represent tyrosine-phosphorylated NR2 subunits, based on the following three lines of evidence: (i) the 180-kDa major anti-phosphotyrosine band does not shift in size with enzymatic deglycosylation, as the NR2 subunits do; (ii) NR2 subunits immunoprecipitated from hippocampal culture extract do not exhibit any anti-phosphotyrosine immunoreactivity; and (iii) phosphoamino acid analysis of NR2 reveals phosphorylation only on serine residues. Thus, in our primary hippocampal cultures, NR2 subunits are not detectably phosphorylated on tyrosine.

We attempted to induce NR2 tyrosine phosphorylation in the hippocampal cultures via a variety of treatments, as described under “Results.” None of these treatments resulted in detectable NR2 tyrosine phosphorylation. Thus, at the present time, it is not certain what factor or factors present in the brain but absent from primary hippocampal cultures are responsible for inducing tyrosine phosphorylation of NR2 subunits. The lack of NR2 tyrosine phosphorylation in the cultures studied here may
simply reflect the fact that these cultures are prepared from neonatal rats; it is known that the tyrosine phosphorylation levels of many proteins in rat brain are very low at birth and reach high levels only after the third or fourth week of postnatal life (24). At any rate, the phosphorylation of NR2 subunits exclusively on serine residues observed in the present study is of interest because it may help direct future studies aimed at identifying NR2 phosphorylation sites. There are a multitude of consensus phosphorylation sites for various kinases located on both serine and threonine residues within the C-terminal tail region of the NR2 subunits (7), but in the present study, we found evidence only for phosphorylation on serine.

In conclusion, the present data demonstrate that NMDA receptor NR1 and NR2 subunits are regulated differentially in hippocampal neurons in terms of both their cell surface trafficking and their basal versus stimulated phosphorylation state. These regulatory mechanisms allow neurons tight posttranslational control over the formation and function of NMDA receptors. Multi-subunit receptors like NMDA receptors in which each subunit can be regulated distinctly are susceptible to more levels of regulation than are single-subunit receptors or multi-subunit receptors composed of subunits that are closely homologous to one another. Given that both underactivation (25) and overactivation (26) of NMDA receptors can lead to neuronal dysfunction and death, it is perhaps not surprising that neuronal NMDA receptors are subject to multiple layers of regulation to constrain their activity within narrowly defined parameters.

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