

Alterations in Subunit Expression, Composition, and Phosphorylation of Striatal *N*-Methyl-D-Aspartate Glutamate Receptors in a Rat 6-Hydroxydopamine Model of Parkinson's Disease

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ABSTRACT

Recent evidence has linked striatal *N*-methyl-D-aspartate (NMDA) receptor function to the adverse effects of long-term dopaminergic treatment in Parkinson's disease. We have studied the abundance, composition, and phosphorylation of NMDA receptor subunits (NRs) in the rat 6-hydroxydopamine lesion model of parkinsonism. In lesioned striatum, the abundance of NR1 and NR2B in striatal membranes was decreased to 68 ± 3.2 and $62 \pm 4.4\%$, respectively, relative to the unlesioned striata, whereas the abundance of NR2A was unchanged. Coimmunoprecipitation of NMDA receptors under nondenaturing conditions revealed that these changes reflected a selective depletion of receptors composed of NR1/NR2B, without alteration in receptors composed of NR1/NR2A. However, the abundance and composition of striatal NMDA receptors in extracts containing both cytoplasmic and mem-

brane proteins were not altered in lesioned rats, suggesting that the changes in the membrane fraction resulted from intracellular redistribution of receptors. The phosphorylation of NR1 protein at serine 890 and serine 896, but not at serine 897, and the tyrosine phosphorylation of NR2B but not NR2A were decreased in the membrane fraction of the lesioned striatum. Chronic treatment of lesioned rats with L-dopa normalized the alterations in the abundance and subunit composition of the NMDA receptors in striatal membranes, and produced striking hyperphosphorylation, both of NR1 at serine residues, and NR2A and NR2B at tyrosine residues. These findings suggest that the adverse motor effects of chronic L-dopa therapy may result from alterations in regulatory phosphorylation sites on NMDA receptors.

Parkinson's disease is characterized by loss of dopaminergic neurons innervating the striatum. The most effective therapy is replacement of dopamine with L-dopa or dopamine receptor agonists, but virtually all patients treated for extended periods develop motor complications: "wearing off," the abrupt loss of effectiveness of the medication at the end of the dosing interval, and "dyskinesias," abnormal involuntary choreiform movements. These symptoms are often disabling (Standaert and Stern, 1993). An animal model for Parkinson's disease is the unilateral, 6-hydroxydopamine (6-OHDA)-lesioned rat (Ungerstedt and Arbuthnott, 1970). These animals respond to dopamine agonists by circling, and

with chronic treatment they exhibit responses of increasing amplitude and decreasing duration, analogous to the motor effects observed in human patients (Papa et al., 1995).

N-Methyl-D-aspartate (NMDA) glutamate receptors play a key role in the regulation of movement by the striatum, and are thought to be promising therapeutic targets for treatment of Parkinson's disease. NMDA-binding sites are very abundant in the striatum (Albin et al., 1992). In rodents, injection of NMDA agonists into the striatum causes contralateral rotation, whereas bilateral injection causes parkinsonism (Thanos et al., 1992; Klockgether and Turski, 1993). NMDA antagonists potentiate the immediate effects of dopamine on striatal function (Klockgether and Turski, 1990; Morelli et al., 1992; Papa et al., 1993; Kaur and Starr, 1997) and are highly effective in attenuating the motor effects of chronic dopaminergic therapy (Papa et al., 1995; Marin et al., 1996; Blanchet et al., 1997). Recent evidence suggests that

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ABBREVIATIONS: 6-OHDA, 6-hydroxydopamine; NMDA, *N*-methyl-D-aspartate; NR, NMDA receptor subunit; RT, room temperature; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

the basis for this effect is that chronic dopaminergic treatment modifies the properties of striatal NMDA receptors (Chase et al., 1998).

NMDA receptors are heteromeric assemblies of NMDA receptor subunits (NRs) NR1 and NR2; seven isoforms of NR1 are produced by alternative splicing of a single gene, whereas four distinct genes encode NR2A, NR2B, NR2C, and NR2D (Dingledine et al., 1999). In the rat striatum, the mRNAs for NR1, NR2A, and NR2B are abundant, whereas the relative levels of NR2C and NR2D mRNA are low (Standaert et al., 1994). Both the abundance as well as the subunit composition of receptor complexes probably have an important influence on NMDA receptor function. Protein phosphorylation is an additional means of modulating the properties of NRs. The NR1 subunit has at least three distinct serine phosphorylation sites in the carboxy tail region, which affect the interaction with calmodulin as well as subcellular distribution (Hisatsune et al., 1997; Tingley et al., 1997). The NR2s are tyrosine phosphorylated, and modulation of NR2 phosphorylation has been observed in several models of synaptic plasticity, including taste learning (Lau and Huganir, 1995; Rostas et al., 1996; Rosenblum et al., 1997; Dunah et al., 1998b).

Only limited direct studies of striatal NMDA receptors in Parkinson's disease models have been reported. Ligand-binding studies with [^3H]glutamate have produced inconclusive results, suggesting relatively small changes in binding to NMDA receptor sites (Wüllner et al., 1993; O'Dell and Marshall, 1996; Ulas and Cotman, 1996), whereas with [^3H]MK801 a bilateral reduction in binding, greater on the lesioned side, was observed (Porter et al., 1994). An *in situ* hybridization study demonstrated a small-magnitude up-regulation of the NR2A (Ulas and Cotman, 1996). Recently, two reports (Menegoz et al., 1996; Oh et al., 1998) described increased tyrosine phosphorylation of NMDA receptors after nigrostriatal denervation and chronic L-dopa treatment.

In the current study, biochemical methods were used to study the subunit abundance, composition, and the serine and tyrosine phosphorylation of NRs present in the normal rat striatum, and to determine how these properties are altered in the rat unilateral 6-OHDA model of parkinsonism and by chronic treatment with L-dopa. NR proteins are known to be present in the cytoplasm as well as in association with the cell membrane of neurons (Petralia et al., 1994) and trafficking of receptor proteins from the cytoplasm to the cell surface is a potentially important means of functional regulation (Shi et al., 1999). Therefore, we analyzed separately the receptors present in total striatal homogenate and those associated with neuronal membranes.

Experimental Procedures

Materials

Protein A-Sepharose, dimethylpimelimidate, and benserazide were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium deoxycholate (S285-100, lot 951636), L-glutamate, L-glycine, sodium fluoride, and Triton X-100 were obtained from Fisher Scientific (Pittsburgh, PA). L-Dopa and 6-OHDA were from Research Biochemicals (Natick, MA). The chemiluminescence detection system (Super Signal) was from Pierce (Rockford, IL). The subunit-specific monoclonal NR1 (Luo et al., 1997), polyclonal NR2A (Wang et al., 1995), and monoclonal NR2B (Wang et al., 1995) antibodies were

developed in the laboratory of Dr. Barry B. Wolfe, Georgetown University. The phosphoserine specific polyclonal NR1 antibodies (Tingley et al., 1997) were produced in the laboratory of Dr. Richard L. Huganir, Johns Hopkins University. The polyclonal α -Actinin-2 antibody (Wyszynski et al., 1997) was a generous gift from Dr. Morgan Sheng, Harvard Medical School. The following antibodies were obtained from commercial sources: polyclonal anti-GluR2/3 (Chemicon International, Temecula, CA); monoclonal PSD-95 (K28/43) (Upstate Biotechnology, Lake Placid, NY); monoclonal antiphosphotyrosine (PY20) and recombinant antiphosphotyrosine monoclonal (RC20) (Transduction Laboratories, Lexington, KY); monoclonal tyrosine hydroxylase antibody and nonimmune purified rabbit immunoglobulins (Sigma Chemical Co.); horseradish peroxidase-linked goat anti-rabbit and horseradish peroxidase-linked goat anti-mouse (Jackson ImmunoResearch, West Grove, PA).

Methods

Experimental protocols involving the use of vertebrate animals were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and conformed to the National Institutes of Health guidelines.

Unilateral 6-OHDA Lesions. Adult male Sprague-Dawley rats weighing 200 to 250 g were treated with imipramine (25 mg/kg *i.p.*) and anesthetized with sodium pentobarbital (50 mg/kg *i.p.*). The rats were positioned in a stereotaxic frame and nigrostriatal lesions were produced by injecting 16 μg of 6-OHDA dissolved in 4 μl of water containing 0.02% ascorbate into the left medial forebrain bundle with a 10- μl Hamilton syringe. The stereotaxic coordinates were anterior 1.6 mm from the interaural line, 2.2 mm lateral, and 7.7 mm below the surface of dura with the atlas of Paxinos and Watson (1986). Sham lesions were performed with the same procedure, with injection of 4 μl of saline. The rats were maintained in cages with free access to food and water and housed on a 12-h light/dark cycle for 14 days before rotational testing.

Behavioral Screening. To determine the success of nigrostriatal denervation, rats were injected with apomorphine (0.25 mg/kg *i.p.*) in saline containing 0.1% sodium *meta*-bisulfite 14 days after the lesion and placed in an automated rotameter (Omnitech Electronics, Columbus, OH). Complete rotations ipsilateral and contralateral to the side of lesion were recorded for 60 min. Rats that exhibited >300 rotations contralateral to the side of lesion were used in this study. To study the effects of the lesion, rats were sacrificed by rapid decapitation 48 h after behavior screening. The brains were removed, the left striatum and right striatum dissected separately, and frozen on dry ice. Tissue samples were stored at -80°C .

Chronic L-dopa Treatment. Additional groups of rats that met the above-mentioned behavioral criteria were treated with repeated injections of L-dopa, as previously described (Papa et al., 1995). Treatment was started 24 h after testing with apomorphine, and consisted of L-dopa methyl ester (25 mg/kg) and benserazide (6.25 mg/kg) in saline, or saline injected *i.p.* twice daily for 21 consecutive days. These rats were sacrificed by rapid decapitation 12 h after the final injection, and the left and right striata were dissected and frozen at -80°C .

Nondenaturing Conditions of Protein Solubilization for Subunit Composition Studies. The immunoprecipitation and immunoblot methods used in this investigation had been characterized and used in previous studies (Wang et al., 1995; Luo et al., 1997; Dunah et al., 1998a). Two different methods of protein extraction were used in this study. Rat brain tissues were homogenized with a polytron (Brinkmann, Dallas, TX) in ice-cold Tris-EDTA buffer (10 mM Tris-HCl, 5 mM EDTA, pH 7.4) and centrifuged at 700g at 4°C . The pellet containing mainly nuclei and large debris was discarded. The resulting supernatant was centrifuged at 10,000g at 4°C and the pellet (P2 fraction) was used as total striatal protein homogenate. Membrane enriched proteins were prepared by homogenizing the P2 fraction in ice-cold Tris-EDTA buffer containing 320 mM sucrose. The sample was centrifuged at 25,000g at 4°C to pellet the synapto-

somal membrane fraction. Extracts prepared by either method were solubilized by the addition of one-tenth volume of 10% sodium deoxycholate in 500 mM Tris-HCl, pH 9.0, and incubated at 36°C for 30 min. A one-tenth volume of a buffer containing 1% Triton X-100, and 500 mM Tris-HCl, pH 9.0, was added and the samples were dialyzed against binding buffer (50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100) overnight in cold room. Samples were centrifuged at 37,000g at 4°C. The supernatants were used for immunoblot and immunoprecipitation.

Denaturing Conditions of Protein Solubilization for Phosphorylation Studies. The total striatal homogenate and synaptosomal striatal membrane fractions were prepared as described above with TEVP buffer (10 mM Tris-HCl, pH 7.4, 5 mM NaF, 1 mM Na_3VO_4 , 1 mM EDTA, 1 mM EGTA). The resultant pellets were solubilized with 2% SDS in TEVP buffer and centrifuged at 15,000g. The supernatants were used for immunoblot and immunoprecipitation studies.

Precoupling Antibodies to Protein A-Sepharose. The monoclonal NR1 and the monoclonal antiphosphotyrosine (PY20) antibodies were incubated with protein A-Sepharose beads at a concentration of 20 μg of antibody per 50 μl of hydrated protein A-Sepharose beads for 2 h at room temperature (RT) in 100 mM sodium borate, pH 8.0, with gentle rotation. To maximize immunoprecipitation efficiency, the NR1 antibody was chemically coupled to the protein A-Sepharose with dimethylpimelimidate in 200 mM sodium borate, pH 9, for 30 min at RT. Nonspecific sites on the beads were blocked with 200 mM ethanolamine, pH 8.0, for 2 h at RT. The beads were washed with 100 mM sodium borate, pH 8.0, and used for immunoprecipitation. This covalent coupling procedure was not necessary for the antiphosphotyrosine (PY20) antibody.

Immunoprecipitation. Solubilized protein samples were diluted 20-fold with immunoprecipitation buffer (150 mM NaCl, 50 mM Na_2SO_4 , pH 7.2, 1% sodium deoxycholate, 2 mM EDTA, 1% Triton X-100). The diluted samples were incubated with 50 μl of protein A-Sepharose/antibody-coupled beads for each 200 μg of soluble protein for 3 h in cold room with gentle rotation. The immunoprecipitation pellets were washed three times with ice-cold immunoprecipitation buffer after brief centrifugation. For the determination of NR composition, the supernatants were transferred to separate tubes and aliquots were taken and diluted with loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 50 mM DTT, 7.5% glycerol). The pellets were resuspended in a suitable volume of loading buffer so that their fractional loads are directly comparable with diluted samples from the supernatants. The serine phosphorylation of NR1 subunit was studied with previously characterized antibodies that specifically recognize the NR1 protein only when it is phosphorylated at serine residues 890, 896, and 897 (Tingley et al., 1997).

Gel Electrophoresis, Quantitative Immunoblotting, and Statistical Analysis. SDS-polyacrylamide gel electrophoresis (PAGE) and transfer of separated proteins to polyvinylidene difluoride (PVDF) were performed as previously described (Wang et al., 1995; Dunah et al., 1996; Luo et al., 1996). In all experiments, 7.5% polyacrylamide gels were used for protein separation, and the concentration of antibodies used for immunoblotting was 1 to 2 $\mu\text{g}/\text{ml}$. Protein concentration was determined with the BioRad protein assay kit. Bands were visualized on film by enhanced chemiluminescence (SuperSignal) and their net intensities were quantified with computer-assisted densitometry (Kodak 1-D System; Kodak, Rochester, NY). For the analyses of NMDA receptor subunit abundance, samples from sham and 6-OHDA-lesioned rats were loaded onto a single gel, and the intensities of the bands were expressed as a percentage of the lesioned striatum of the sham-operated rat. For the phosphorylation and subunit composition studies, the primary analyses were conducted by running samples from the lesioned and unlesioned sides of each rat on the same gel, and expressing the lesioned intensity as a percentage of that found on the unlesioned side. These values were then used to calculate group means, and reported as means \pm S.E. Differences between groups were analyzed with

ANOVA with post hoc tests (Scheffe's). For all analyses, statistical significance was taken to be $P < .05$.

Results

Characterization of 6-OHDA Nigrostriatal Lesions.

The extent of denervation of the nigrostriatal system induced by stereotactic unilateral 6-OHDA injection was determined by behavioral and biochemical methods. All rats were selected for study with a threshold criterion of 300 ipsilateral rotations per hour after administration of 0.25 mg of apomorphine s.c. The rats studied averaged 398.4 ± 24 contralateral rotations in an hour with negligible ipsilateral rotations, whereas apomorphine did not elicit rotational behavior in the sham-operated animals. Striatal protein extracts from a subset of sham- and 6-OHDA-lesioned rats ($n = 5$) were examined for tyrosine hydroxylase immunoreactivity, a marker for dopaminergic neurons (Fig. 1). In sham-operated rats, tyrosine hydroxylase immunoreactivity in the lesioned striatum was $92.3 \pm 1.6\%$ of that on the unlesioned side (Fig. 1A). In contrast, tyrosine hydroxylase immunoreactivity in the lesioned striatum was reduced to $3.6 \pm 1.8\%$ of that on the unlesioned side in 6-OHDA-lesioned rats (Fig. 1B). The extent of tyrosine hydroxylase depletion was not altered by chronic treatment with saline (Fig. 1C) or L-dopa (Fig. 1D).

Unilateral Nigrostriatal Denervation: Effect on Abundance, Subunit Composition, and Phosphorylation of NMDA Receptors in Total Striatal Protein Homogenate. We first examined the NMDA receptor subunit proteins in total striatal homogenates (membrane and cytoplasmic compartments) extracted as described in Methods. For each rat, the lesioned and unlesioned striatum were studied in parallel on the same gel, and the results are expressed as ratio of the lesioned to the unlesioned side. There were no significant differences in the abundance of NR1 ($102 \pm 2.9\%$), NR2A ($106 \pm 2.7\%$), and NR2B ($100 \pm 3.2\%$) in the striatum of 6-OHDA-lesioned rats (Fig. 2, A and B). Similarly, sham-operated rats showed no difference in the amounts of NR1, NR2A, and NR2B between the lesioned and unlesioned sides (Fig. 2, A and B). Immunoblots that were probed with antibodies to the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor subunits GluR2/3 and the postsynaptic proteins α -Actinin-2 and PSD-95 revealed that these proteins also were not altered in total striatal homogenates (Fig. 2, A and B).

The subunit composition of NMDA receptors in the rat total striatal homogenate was investigated by immunoprecipitating NMDA receptors from the lesioned and unlesioned striatum with anti-NR1 antibody (Fig. 2, C and D). In all of the experiments described, immunoprecipitation by the anti-NR1 antibody was found to be complete, as demonstrated by the absence of detectable NR1 in the supernatants (Fig. 2, C and D). The anti-NR1 antibody coimmunoprecipitated NR2A and NR2B (Fig. 2, C and D). In rats with unilateral 6-OHDA lesions, the amounts of NR1 ($99 \pm 3.8\%$), NR2A ($101 \pm 4.1\%$), and NR2B ($104 \pm 4.4\%$) precipitated from the lesioned striatum were not different from the unlesioned striatum (Fig. 2D). Similar results were obtained in sham-operated rats (Fig. 2C).

Tyrosine phosphorylation of total striatal NRs was examined by immunoprecipitation with a phosphotyrosine (PY20) antibody. The immunoblots were probed with anti-NR2A,

anti-NR2B, and a recombinant antiphosphotyrosine monoclonal (RC20) antibody (Fig. 2, E and F). Tyrosine phosphorylated NR2A and NR2B were detected in the rat striatal extracts, and the abundance of these phosphoproteins was the same in the lesioned and unlesioned striatum of both 6-OHDA-lesioned and sham-operated rats (Fig. 2, E and F). Total phosphotyrosine proteins were also similar in the two groups of rats (PY; Fig. 2, E and F). The specificity of precipitation of phosphotyrosine proteins was demonstrated by the ability of phosphotyrosine but not phosphoserine to inhibit the precipitation of phosphotyrosine proteins (Fig. 3).

Unilateral Nigrostriatal Denervation: Effect on Abundance, Subunit Composition, and Phosphorylation of NRs in Striatal Membranes. In contrast to the results obtained studying total striatal protein extracts,

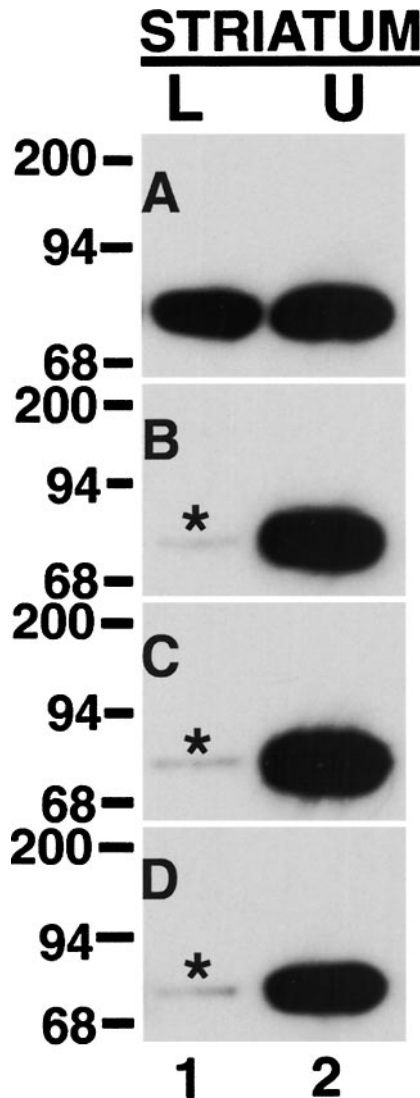


Fig. 1. Striatal tyrosine hydroxylase immunoreactivity is decreased in rats with unilateral nigrostriatal dopaminergic lesions. Solubilized proteins from either sham-operated rats (A), or 6-OHDA (B)-lesioned rats, and 6-OHDA-lesioned rats that were treated with saline (C) and L-dopa (D) were resolved on SDS-PAGE. The blots were probed with anti-tyrosine hydroxylase antibody. Lanes 1 and 2 contain samples from the lesioned striatum (L) and unlesioned striatum (U), respectively. Each lane contains 5 μ g of protein. The positions and sizes of molecular weight markers are indicated in kilodaltons. Immunoreactivity was reduced in each of the lesioned striata (*).

analysis of NRs present in striatal membrane fractions revealed striking alterations in subunit abundance, composition, and phosphorylation. In 6-OHDA-lesioned rats, the abundance of NR1 and NR2B, but not NR2A was reduced in the lesioned striatum (NR1, $68 \pm 3.2\%$; NR2B, $62 \pm 4.4\%$) relative to the unlesioned striatum (Fig. 4, A and B). There were no significant changes in the relative abundance of GluR2/3, α -Actinin-2 and PSD-95 (Fig. 4, A and B). The abundance of NR1, NR2A NR2B, GluR2/3, α -2-Actinin, and PSD-95 were similar on the lesioned and unlesioned sides of the sham-operated rats (Fig. 4, A and B). The abundance of NR1, NR2A, and NR2B in the cortex, thalamus, and mid-brain of the 6-OHDA-lesioned rats also was examined and no difference between the lesioned and unlesioned sides in these regions was found (data not shown).

The alterations observed in the abundance of NRs in striatal membranes were reflected in their composition and phosphorylation determined by immunoprecipitation. In 6-OHDA-lesioned rats, the anti-NR1 antibody precipitated $70 \pm 3.8\%$ of NR1, $61 \pm 4.6\%$ of NR2B, and $98 \pm 4.4\%$ of NR2A from the lesioned striatum relative to the unlesioned side (Fig. 4D). The amounts of these NRs precipitated from the lesioned striatum of sham-operated rats were not different from the unlesioned side (Fig. 4C). The lesioned striatum also exhibited a reduction in tyrosine phosphorylated NR2B to $75 \pm 3.8\%$ of that present on the unlesioned side, whereas tyrosine phosphorylated NR2A was unchanged (Figs. 4F and 5). No alterations in tyrosine phosphorylated NR2A and NR2B were found in the striatum of sham operated rats (Figs. 4E and 5). Because of the possibility that the 6-OHDA lesions might produce bilateral effects, we compared subunit composition and phosphorylation in the unlesioned striatum of the 6-OHDA animals to the unlesioned striatum of the sham-operated rats, and found these samples were not different from one another (data not shown).

Serine phosphorylation of NR1 was studied by immunoprecipitating protein extracts with anti-NR1, and probing the blots with three phosphoserine specific NR1 antibodies, NR1-S890, NR1-S896, and NR1-S897. Although several bands were observed in the extracts of membrane protein (Fig. 6, lanes 1 and 6), after immunoprecipitation a single band corresponding to the predicted size of NR1 (~ 120 kDa) was observed with each of the antibodies (Fig. 6, lanes 2–5). These bands were taken to represent serine phosphorylated NR1 (Tingley et al., 1997) and analyzed quantitatively. Each of the antibodies also recognized some very high-molecular-weight species in the immunoprecipitated material, migrating at >200 kDa. Although it is possible that these represent aggregates of NR1 with other proteins, because the significance of the high-molecular-weight signals was uncertain, they were not included in the analysis. The phosphorylation of NR1 at serine 890 and serine 896 were reduced to 73 ± 2.8 and $71 \pm 3.2\%$, respectively, relative to the unlesioned striatum of the same rat (Fig. 6B, NR1-Ser890 and NR1-Ser896; Fig. 5). The phosphorylation of NR1 at serine 897 was not altered on the lesioned side (Fig. 6B, NR1-Ser890 and NR1-Ser896; Fig. 5). Also, no changes in serine phosphorylation of NR1 were observed in sham-operated rats (Fig. 6A, NR1-Ser897; Fig. 5).

Effect of Chronic L-dopa Treatment on Abundance, Subunit Composition, and Phosphorylation of NMDA Receptors in Striatal Membranes. Treatment of unilat-

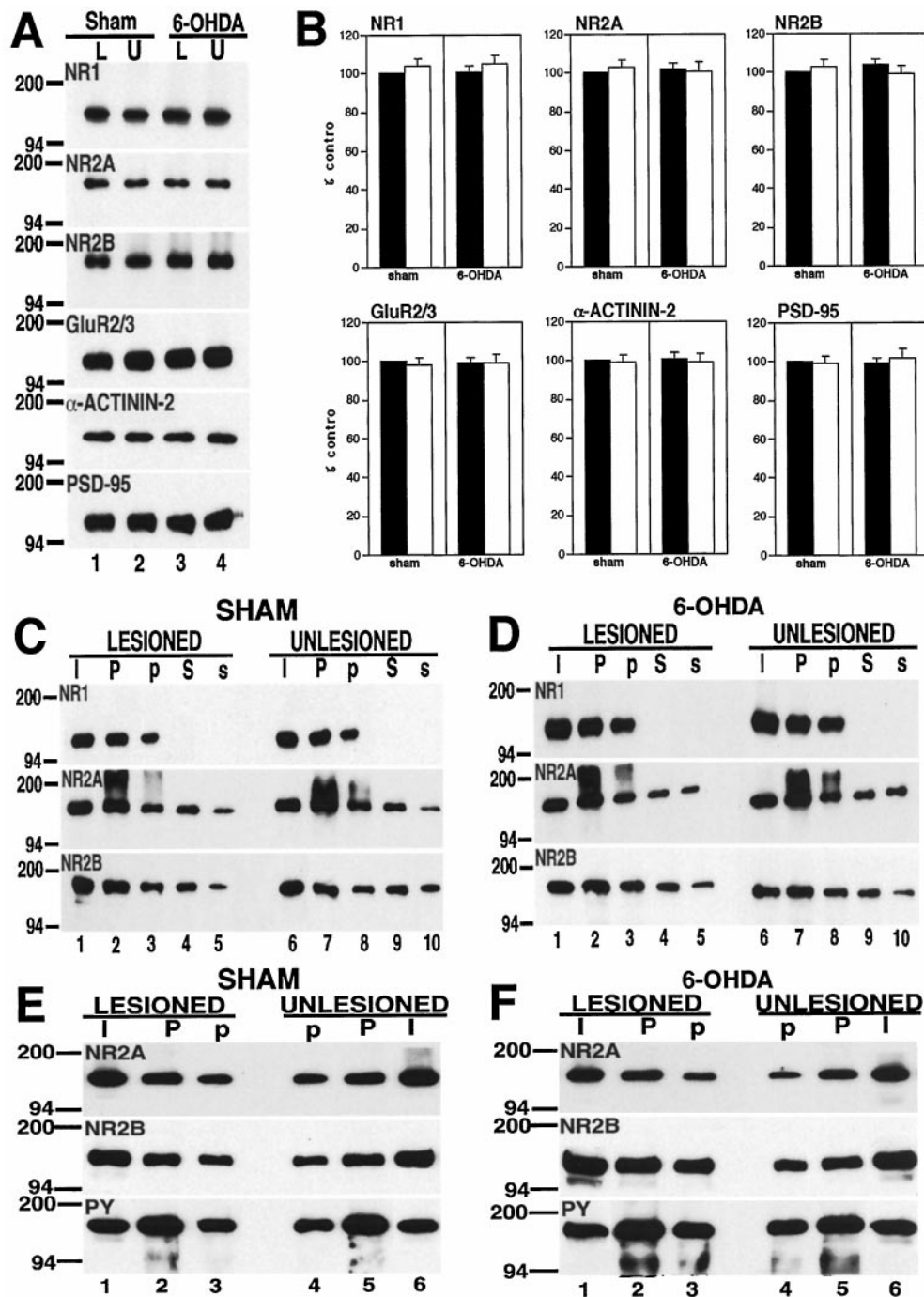


Fig. 2. The subunit abundance, composition, and phosphorylation of NMDA receptor subunits in total striatal protein homogenates are not altered in rats with unilateral nigrostriatal ablation. Abundance: A, total protein homogenates from sham-operated rats (lanes 1 and 2) and 6-OHDA-lesioned rats (lanes 3 and 4) were solubilized and separated on SDS-PAGE. The blots were probed with antibodies specific for NR1, NR2A, NR2B, GluR2/3, α -Actinin-2, and PSD-95. The lesioned striatum (L) and unlesioned striatum (U) are indicated on top of the figure. Each lane contains equal amounts (5 μ g) of total protein. B, densitometric quantification of the relative abundance of NR1, NR2A, NR2B, GluR2/3, α -Actinin-2, and PSD-95 in the lesioned striatum (■) and unlesioned striatum (□) of sham-operated and 6-OHDA-lesioned rats. None of the proteins studied are significantly altered in the total striatal extracts. Values on the ordinate represent intensity of the signal relative to the unlesioned side of the sham-operated rats. Data are means \pm S.E. obtained from four rats. Composition: C and D, total protein homogenates from the lesioned striatum (lanes 1–5) and unlesioned striatum (lanes 6–10) of sham-operated rats (C) and 6-OHDA-lesioned rats (D) were solubilized with nondenaturing conditions. The soluble proteins were immunoprecipitated with anti-NR1 antibody. Samples from input (I; lanes 1 and 6; 5 μ g), immunoprecipitation pellet (P and p; 5 μ g and 2.5 μ g of protein, respectively), and supernatant (S and s; 5 μ g and 2.5 μ g of protein, respectively) were resolved on SDS-PAGE. The blots were probed with anti-NR1, anti-NR2A, and anti-NR2B antibodies. Phosphorylation: E and F, total protein homogenates from the lesioned striatum (lanes 1–3) and unlesioned (lanes 4–6) of sham-operated (E) and 6-OHDA-lesioned rats (F) were solubilized and immunoprecipitated with anti-phosphotyrosine (PY) antibody. The pellets were separated on SDS-PAGE and the blots were probed with anti-NR2A, anti-NR2B, and anti-phosphotyrosine (PY) antibodies. Lanes 1 and 6 contain 2.5 μ g of protein from the input. Lanes 2 to 5 contain samples from immunoprecipitation pellets (P and p; 20 μ g and 10 μ g of protein, respectively).

eral 6-OHDA-lesioned rats with L-dopa or saline was initiated 2 days after behavioral testing with apomorphine, and continued for 21 days. Consequently, the rats were studied 38 days after unilateral 6-OHDA lesion, in contrast to the 16-day postlesion period used in the above-mentioned experiments. In lesioned rats treated with saline, the reductions in NR1 and NR2B observed at 16 days persisted (NR1, $69 \pm 3.6\%$; NR2B, $64 \pm 4.2\%$) (Fig. 7, A and B). However, chronic treatment of lesioned rats with L-dopa normalized the abundance of NR1 ($98 \pm 3.3\%$) and NR2B ($103 \pm 4.3\%$) in the lesioned striatum (Fig. 7, A and B). The abundance of NR2A GluR2/3, α -2-Actinin and PSD-95 were not altered by treatment with L-dopa or saline (Fig. 7, A and B). To control for the possibility that L-dopa treatment might produce bilateral effects, we compared the unlesioned striatum of L-dopa treated rats to the unlesioned striatum of the saline treated rats, and found that the subunit abundance in these sets of samples were not different (data not shown).

Comparable results were obtained from analysis of the subunit composition of NMDA receptors following immunoprecipitation with anti-NR1. In saline-treated rats, there were reductions of NR1 ($68 \pm 2.8\%$) and the coimmunoprecipitated NR2B ($64 \pm 4.2\%$), whereas the amount of NR2A precipitated remained unchanged ($100 \pm 2.8\%$) (Fig. 7C). Subsequent chronic treatment with L-dopa resulted in the normalization of the precipitated NR1 ($109 \pm 3.7\%$) and NR2B ($111 \pm 3.5\%$).

Both tyrosine and serine phosphorylation of striatal NMDA receptor subunits was altered by chronic treatment with L-dopa. In rats treated with saline, there were reductions in tyrosine phosphorylated NR2B ($77 \pm 3.8\%$ of the unlesioned striatum) and also in the phosphorylation of NR1 at serine 890 ($69 \pm 3.4\%$) and at serine 896 ($71 \pm 2.8\%$) (Figs. 5 and 6C) comparable to those observed at 16 days after the lesion. Interestingly, chronic treatment with L-dopa increased tyrosine phosphorylated NR2A ($120 \pm 3.7\%$) and

tyrosine phosphorylated NR2B ($176 \pm 4.2\%$) in the lesioned striatum relative to the unlesioned striatum (Figs. 5 and 7D). Furthermore, the phosphorylation of NR1 at serine 890, serine 896, and serine 897 in the lesioned striatum of these rats also was increased to 110 ± 2.7 , 120 ± 3.1 , and 114 ± 2.7 , respectively, of the unlesioned striatum (Figs. 5D and 6).

Discussion

In rats with unilateral 6-OHDA lesions of the nigrostriatal pathway, we have found that there is a reduction in the abundance of NR1 and NR2B in the membrane fractions of lesioned striatum, whereas the abundance of NR2A is not altered. These alterations appear to arise from a selective decrease in the number of NMDA receptors composed of NR1/NR2B. The phosphorylation state of the NMDA subunits also is modified: there is a decrease in the serine phosphorylation of NR1 at residues 890 and 896, whereas the tyrosine phosphorylation of NR2B but not NR2A is altered. Interestingly, these changes are seen only in NMDA receptors present on striatal membranes and not receptors found in total striatal homogenate. This finding implies a redistribution of receptor subunits from the membrane to the cytoplasmic compartment. Finally, chronic treatment with L-dopa results in normalization of the abundance and composition of striatal NMDA receptors in the membrane fraction, but produces marked increases in the phosphorylation of NR1 at serine 890, serine 896, and serine 897, and tyrosine phosphorylation of NR2A and NR2B.

Alterations in Subunit Expression and Composition of NMDA Receptors in 6-OHDA-Lesioned Rat. The abundance and composition of NMDA receptors present in total striatal extracts were not changed in the 6-OHDA-lesioned rats. These data are in accord with previous study of Menegoz et al. (1996) but differ from the study by Oh et al. (1998) in that the latter study reported a modest (25%) increase in NR2A with no change in NR2B in total striatal extract after 6-OHDA lesions. These differences may be related to the duration of survival because both our study and that of Menegoz et al. (1996) examined rats 14 days after lesioning, whereas Oh et al. (1998) studied rats at 21 days after lesioning.

In contrast to the results obtained in total striatal extracts, in striatal membrane fractions we observed a substantial reduction in the abundance of the NR1 and NR2B on the lesioned side, with no alteration of NR2A. The reduction in membrane associated NMDA receptor subunits, without alteration in total cellular content of the same subunits, suggests redistribution of NR1 and NR2B from the membrane to the cytoplasmic compartment in the lesioned striatum. The coimmunoprecipitation data confirm the alterations in the membrane associated NMDA subunits, and further suggest that the changes reflect a significant reduction in NMDA receptor complexes composed of NR1/NR2B, without any change in receptors composed of NR1/NR2A. Physiological regulation of the insertion of cytoplasmic receptors into synaptic sites has recently been demonstrated in other systems (Hall and Soderling, 1997; Shi et al., 1999), and may be an important mechanism for regulating the activity of ionotropic glutamate receptors in animal models of dopamine depletion.

Alterations in Phosphorylation of NMDA Subunits in 6-OHDA-Lesioned Rat. Tyrosine phosphorylation is a

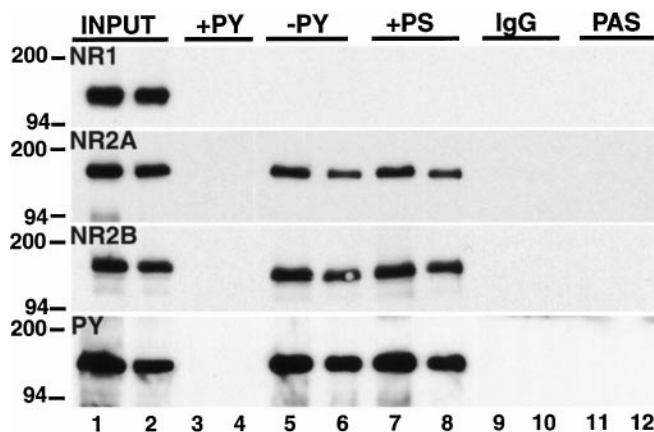


Fig. 3. Specificity of immunoprecipitation of tyrosine-phosphorylated proteins by antiphosphotyrosine antibody. Rat striatal membrane proteins were solubilized and immunoprecipitated with anti-phosphotyrosine antibody in the presence of 10 mM phosphotyrosine (+PY; lanes 3 and 4), absence of 10 mM phosphotyrosine (-PY; lanes 5 and 6), presence of 10 mM phosphoserine (+PS; lanes 7 and 8); and with protein A-Sepharose-coupled rabbit immunoglobulins (IgG; lanes 9 and 10) and protein A-Sepharose beads (PAS; lanes 11 and 12). The pellets were subjected to SDS-PAGE, and the blots were probed with anti-NR2A, anti-NR2B, and anti-phosphotyrosine (PY). Lanes 1 and 2 contain 2.5 and 1.25 μ g, respectively, of input. Lanes 3 to 12 contain 2-fold (40 and 20 μ g) load of protein from the immunoprecipitation pellet.

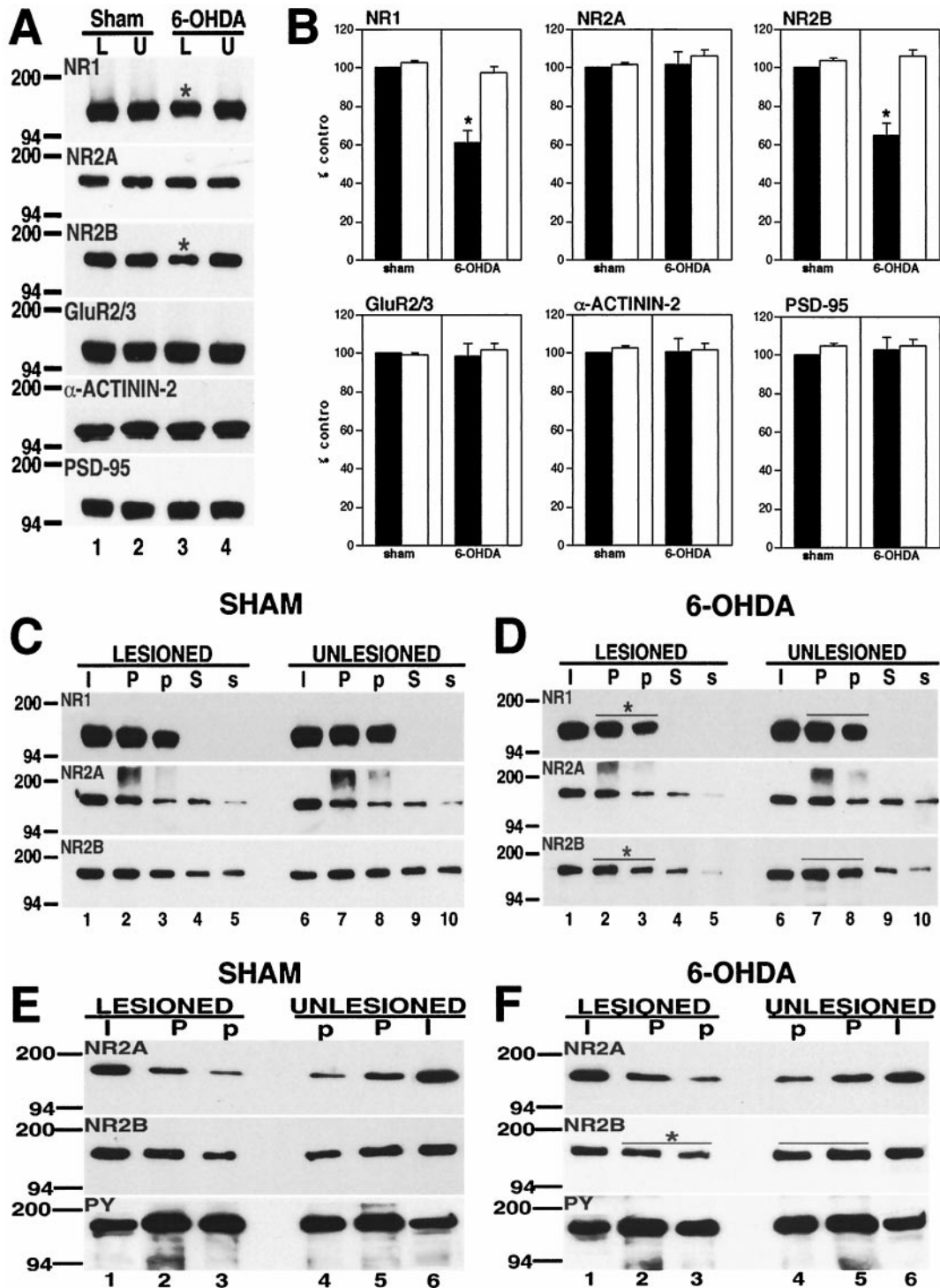


Fig. 4. NMDA receptor subunits NR1 and NR2B, but not NR2A are decreased in the striatal membrane protein extracts of rats with unilateral nigrostriatal depletion. Abundance: A, striatal membrane proteins from sham-operated rats (lanes 1 and 2) and 6-OHDA-lesioned rats (lanes 3 and 4) were separated on SDS-PAGE. The blots were probed with anti-NR1, anti-NR2A, anti-NR2B, anti-GluR2/3, anti- α -Actinin, and anti-PSD-95 antibodies. Each lane contains 5 μ g of protein. B, relative abundance of NR1, NR2A, NR2B, GluR2/3, α -2-Actinin, and PSD-95 in the lesioned striatum (■) and unlesioned striatum (□) of sham and 6-OHDA-lesioned rats. Values on the ordinate represent the amount of each protein as a percentage of the unlesioned side of the sham-operated animals. The abundance of NR1 and NR2B are reduced ($P < .05$), whereas the other proteins measured were unchanged. Data are means \pm S.E. values obtained from five rats. Values on the ordinate represent intensity of the signal on lesioned striatum relative to the unlesioned striatum obtained from five rats. Composition: C and D, membrane proteins from the lesioned striatum (lanes 1–5) and unlesioned striatum (lanes 6–10) of sham-operated rats (A) and 6-OHDA-lesioned rats (B) were extracted with nondenaturing conditions and immunoprecipitated with NR1 antibody. Lanes 1 and 6 contain 5 μ g of protein from the input (I). Lanes 2, 3, 7, and 8 contain samples from the immunoprecipitation pellet (P and p; 5 and 2.5 μ g of protein, respectively). Lanes 4, 5, 9, and 10 contain samples from the supernatant (S and s; 5 and 2.5 μ g of protein, respectively). The blots were probed with anti-NR1, anti-NR2A, and anti-NR2B antibodies. Phosphorylation: E and F, membrane proteins from the lesioned striatum (lanes 1–3) and unlesioned striatum (lanes 4–6) were immunoprecipitated with antiphosphotyrosine antibody. The blots were probed with antibodies against NR2A, NR2B, and phosphotyrosine (PY). Lanes 1 and 6 contain 2.5 μ g of the input. Lanes 2 to 5 contain samples from immunoprecipitation pellet (P and p; 20 and 10 μ g of protein, respectively). Asterisks indicate significant differences between the lanes marked with bars.

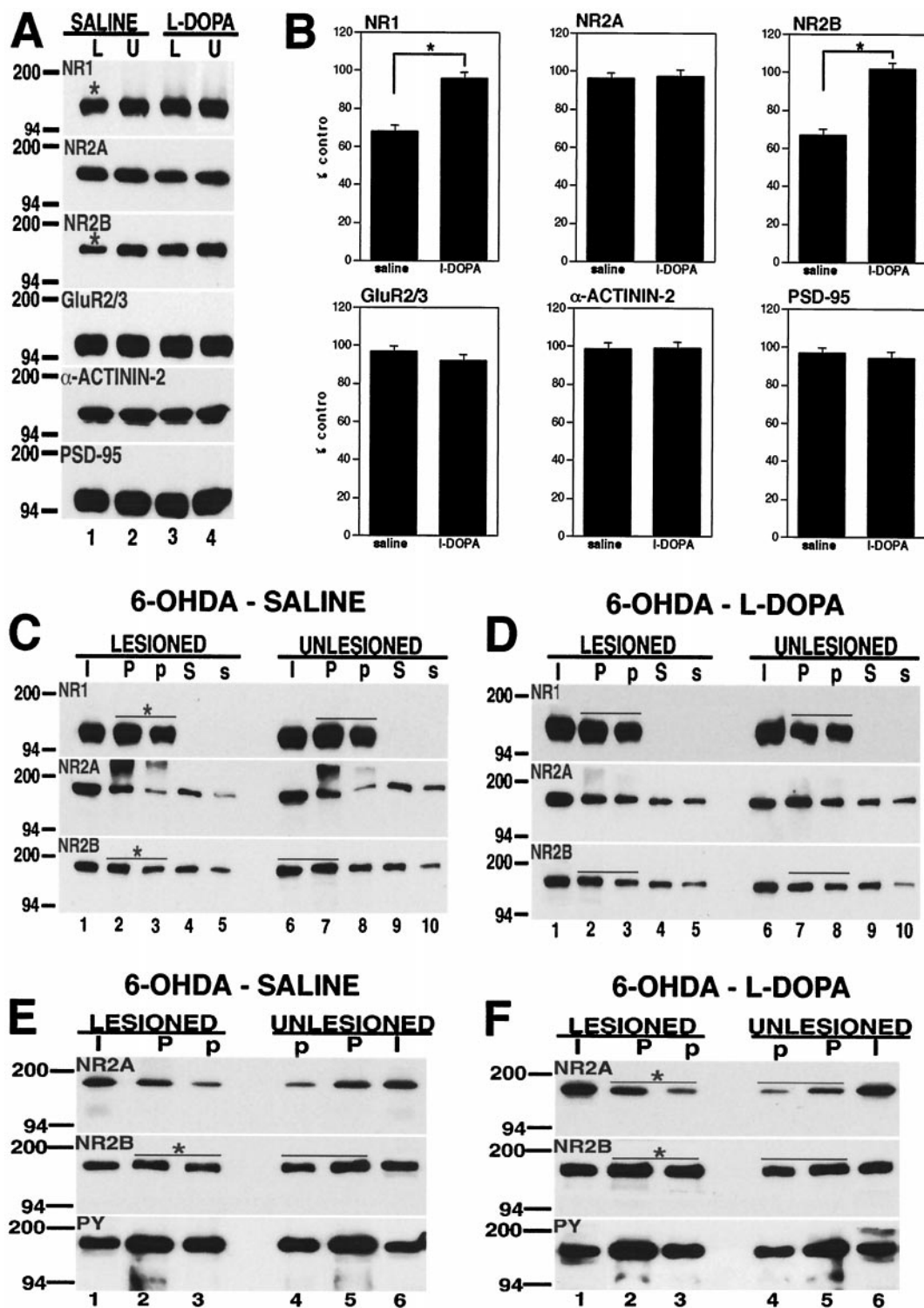


Fig. 7. Chronic treatment of unilateral nigrostriatal-lesioned rats with L-dopa normalized the subunit expression of NR1 and NR2B, and increased the tyrosine phosphorylation of NR2A and NR2B in striatal membranes. Abundance: A and B, membrane protein extracts from the striatum of 6-OHDA-lesioned rats treated with saline (lanes 1 and 2) and L-dopa (lanes 3 and 4) were separated on SDS-PAGE by loading 5 μ g of protein per lane. The blots were probed for NR1, NR2A, NR2B, GluR2/3, α -Actinin-2, and PSD-95. B, densitometric analysis of the relative amounts of NR1, NR2A, NR2B, GluR2/3, α -Actinin-2, and PSD-95 from the lesioned striatum of saline- and L-dopa-treated rats. Values on the ordinate represent the amount of each protein as percentage of unlesioned striatum. Data are means \pm S.E. obtained from five rats. Subunit composition: C and D, striatal membrane protein extracts from 6-OHDA-lesioned rats treated with saline (C) and L-dopa (D) were precipitated with NR1 antibody. Samples from input (I; lanes 1 and 6; 5 μ g), immunoprecipitation pellet (P and p; 5 and 2.5 μ g of protein, respectively), and supernatant (S and s; 5 and 2.5 μ g of protein, respectively) were subjected to electrophoresis. The blots were probed with anti-NR1, anti-NR2A, and anti-NR2B antibodies. Phosphorylation: E and F, membrane proteins from the striatum of 6-OHDA-lesioned rats treated with saline (E) and L-dopa (F) were precipitated with anti-phosphotyrosine antibody. The blots were probed with anti-NR2A, anti-NR2B, and anti-phosphotyrosine (PY) antibodies. Lanes 1 and 6 contain 2.5 μ g of the input. Lanes 2 to 5 contain samples from immunoprecipitation pellet (P and p; 20 and 10 μ g of protein, respectively). Asterisks indicate significant differences between the lanes marked by bars.

In the striatal membrane preparations, there was a 25% reduction in tyrosine phosphorylated NR2B but also an even greater decrease in the amount of total NR2B protein (38%), suggesting there is a modest increase in the proportion of NR2B that is tyrosine phosphorylated in the striatal membranes. Although differences in the preparation and analysis of samples make it difficult to resolve the minor disparities in results of all these studies, collectively the data seem to support a modest increase in NR2B phosphorylation, particularly in the membrane fraction, in the 6-OHDA-lesioned striatum, whereas NR2A tyrosine phosphorylation is unchanged.

Although NR1 is not tyrosine phosphorylated in vivo (Lau and Huganir, 1995), serine phosphorylation of the carboxy tail of NR1 appears to be an important means for modulation of NMDA channel activity. We studied the phosphorylation of NR1 at three distinct sites, serine residues 890, 896, and 897. In the 6-OHDA-lesioned rat, we found a marked reduction in the phosphorylation of NR1 at serine 890 and serine 896, whereas serine 897 was unchanged. Interestingly, in vitro studies indicate that serine 890 and serine 896 are phosphorylated by protein kinase C, whereas serine 897 is a substrate for protein kinase A. The functional importance of the phosphorylation of NR1 at these potential regulator sites is not entirely defined. In vitro, phosphorylation at serine 890, but not serine 896 and serine 897 resulted in the redistribution of the NR1 receptors in transfected fibroblasts (Tingley et al., 1997). It will be important to determine whether the modulation of phosphorylation at one or more of these sites could account for the redistribution of receptor subunits we have observed in vivo.

Effects of Chronic L-dopa Treatment on NMDA Subunits in 6-OHDA-Lesioned Rats. Unilateral 6-OHDA-lesioned rats have been used as experimental models for investigating the effects of chronic L-dopa treatment in human Parkinson's disease. The treatment paradigm used in this study is essentially the one developed by Chase and colleagues, consisting of L-dopa injections twice a day for 21 consecutive days. This treatment paradigm induces alterations in motor response, which include enhanced amplitude of rotation and shortened duration of action, thought to be analogous to the wearing off and fluctuations observed in patients (Papa et al., 1995). Previous work has revealed that low doses of NMDA receptor antagonists can prevent development of the behavioral alterations, and reduce severity once they have developed (Papa et al., 1995; Chase et al., 1998).

In this study, chronic treatment of unilateral 6-OHDA-lesioned rats with L-dopa restored the abundance of NR1 and NR2B in striatal membranes to the level observed in the unlesioned striatum. Chronic treatment of sham-operated rats with L-dopa had no effect on the striatal NMDA receptor subunits. These results are compatible with an earlier report in which total striatal extracts were studied (Oh et al., 1998). Furthermore, we observed marked increases in the tyrosine phosphorylation of striatal NR2A and NR2B after L-dopa treatment, consistent with the results of Oh et al. (1998). Similar increase in tyrosine phosphorylation of NR2B have been seen in other animal models of neural plasticity, including long-term potentiation in the rat dentate gyrus (Rostas et al., 1996) and taste learning in the insular cortex (Rosenblum et al., 1997). Chronic treatment with L-dopa also produced an

increase in the serine phosphorylation of NR1 at residues 890, 896, and 897. The phosphorylation of NR1 protein at these three serine residues suggests that this dopaminergic treatment may involve the activation of both protein kinase A and protein kinase C pathways. A potential mechanism for this effect is suggested by the report of Snyder et al. (1998) showing that agonists of the dopamine D₁ receptor increased the phosphorylation of NR1 by regulating a phosphoprotein (DARPP-32) that selectively inhibits the protein phosphatase-1.

NMDA Receptor Properties in 6-OHDA-Lesioned Rats. The alterations in NMDA subunit abundance, composition, and phosphorylation observed in the lesioned striata may have important effects on the functional properties of striatal receptor channels. In particular, the reduction in the proportion of NMDA receptors composed of NR1 and NR2B, relative to receptors containing NR1 and NR2A would be expected to result in a population of receptors with high sensitivity for competitive NMDA antagonists, and reduced affinity for glutamate (Laurie and Seeburg, 1994; Lynch et al., 1994). The relative enrichment of NR1/NR2A receptors also would be predicted to lead to a corresponding increase in receptors with fast deactivation kinetics, and reduced affinity of the receptors for the noncompetitive polyamine antagonists such as ifenprodil and haloperidol (Williams et al., 1994; Lynch and Gallagher, 1996).

The precise role of phosphorylation in modulating the properties of NMDA channels is still unclear. Electrophysiological studies of spinal dorsal horn neurons have demonstrated that protein tyrosine kinases and protein tyrosine phosphatase inhibitors potentiate NMDA receptor currents (Wang and Salter, 1994; Yu et al., 1997). Also, phosphorylation of NMDA receptors at serine and threonine residues has been suggested to regulate the subcellular redistribution and targeting of intracellular NMDA receptors to the synaptic membrane (Raman et al., 1996; Hisatsune et al., 1997; Tingley et al., 1997). Our data suggest that the principal alteration of NMDA receptors induced by chronic L-dopa treatment in the rat 6-OHDA model is hyperphosphorylation of NR1, NR2A, and NR2B. If these modifications of the receptor subunits in fact alter the channel conductance or synaptic localization of NMDA channels, they may underlie the behavioral sensitization produced by L-dopa, and account for the ability of NMDA antagonists to reverse this sensitization. Further studies identifying the specific effects of the several NR1 and NR2 phosphorylation sites, and the underlying regulatory mechanisms, may lead to new approaches to the therapy of Parkinson's disease.

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