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Dopamine D1-Dependent Trafficking of Striatal *N*-Methyl-D-aspartate Glutamate Receptors Requires Fyn Protein Tyrosine Kinase but Not DARPP-32

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ABSTRACT

Interactions between dopaminergic and glutamatergic systems in the striatum are thought to underlie both the symptoms and adverse effects of treatment of Parkinson's disease. We have previously reported that activation of the dopamine D1 receptor triggers a rapid redistribution of striatal *N*-methyl-D-aspartate (NMDA) receptors between intracellular and postsynaptic subcellular compartments. To unravel the signaling pathways underlying this trafficking, we studied mice with targeted disruptions of either the gene that encodes the dopamine- and cAMP-regulated phosphoprotein (DARPP-32), a potent and selective inhibitor of protein phosphatase-1, or the protein tyrosine kinase Fyn. In striatal tissue from DARPP-32-depleted mice, basal tyrosine and serine phosphorylation of striatal NMDA receptor subunits NR1, NR2A, and NR2B was normal, and activation of dopamine D1 receptors with the agonist SKF-82958 [(±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetra-hydro-1*H*-benzazepine] produced redistribution of NMDA receptors from vesicular compartments (P3 and LP2) to synaptosomal membranes (LP1). In the Fyn knockout mice, basal tyrosine phosphorylation of NR2A and NR2B was drastically reduced, whereas serine phosphorylation of these NMDA subunits was unchanged. In the Fyn knockout mice, the dopamine D1 receptor agonist failed to induce subcellular redistribution of NMDA receptors. In addition, Fyn-depleted mice lesioned with 6-hydroxydopamine also failed to exhibit L-DOPA-induced behavioral sensitization, but this may be caused, at least in part, by resistance of these mice to the neurotoxic lesion. These findings suggest a novel mechanism for the trafficking of striatal NMDA receptors by signaling pathways that are independent of DARPP-32 but require Fyn protein tyrosine kinase. Strategies that prevent NMDA receptor subcellular redistribution through inhibition of Fyn kinase may prove useful in the treatment of Parkinson's disease.

The striatum is the principal input structure of the mammalian basal ganglia, receiving both dopaminergic input from the substantia nigra pars compacta and glutamatergic input from the cerebral cortex and thalamus. Interactions between the two transmitter systems play a key role in the regulation of movement (Di Chiara et al., 1994; Smith et al., 1994, 1998). In Parkinson's disease, the primary disturbance is a loss of dopaminergic innervation, but this loss elicits secondary alterations in glutamate receptors, especially those of the *N*-methyl-D-aspartate (NMDA) class (Chase and Oh, 2000). Altered striatal NMDA receptor functions make important contributions, both to symptoms of the disease and to the response to treatment with dopaminergic drugs (Calabresi et al., 2000a; Nash and Brotchie, 2002; Oh and Chase, 2002).

NMDA glutamate receptors are heteromeric ligand-gated ion channels assembled from three subunit families: NR1, consisting of eight splice isoforms produced from a single gene; NR2A–NR2D, encoded by four distinct genes; and NR3A and NR3B subunits, which are prominent in developing brain and spinal motor neurons, respectively (Mori and Mishina, 1995; Sucher et al., 1995; Dingeldine et al., 1999; Nishi et al., 2001). In the adult rat and human striatum, the most abundant receptor subunit mRNAs are NR1 (the NR1a isoform), NR2A, and NR2B (Standaert et al., 1994; Kosinski et al., 1998).

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; DARPP-32, dopamine- and cAMP-regulated phosphoprotein; SKF-82958, (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetra-hydro-1*H*-benzazepine; 6-OHDA, 6-hydroxydopamine; PAGE, polyacrylamide gel electrophoresis; ANOVA, analysis of variance; PKA, protein kinase A

Recently, we described an NMDA receptor-trafficking mechanism that plays an important role in striatal dopamine-glutamate interactions. In a rat model of Parkinson's disease, we found that dopamine depletion leads to a redistribution of striatal NMDA receptor proteins, resulting in a loss of NR1/NR2B containing receptors from neuronal membranes. Although treatment with levodopa restores NMDA receptor composition, it is accompanied by hyperphosphorylation of the proteins (Dunah et al., 2000). In vitro, treatment of striatal tissue with a dopamine D1 receptor agonist produces rapid alteration in the distribution of NR1, NR2A, and NR2B, with accumulation of these proteins in synaptosomal membranes and enhanced tyrosine phosphorylation of NR2A and NR2B. These effects are blocked by protein tyrosine kinase inhibition and mimicked by phosphatase inhibition, suggesting that tyrosine phosphorylation plays an essential role in the trafficking mechanism (Dunah and Standaert, 2001). Subsequent studies have confirmed the existence of dynamic trafficking processes for synaptic NMDA receptors in several different neural systems, although the mechanisms responsible for these processes remain for the most part uncertain (Carroll and Zukin, 2002; Wenthold et al., 2003).

The Src family of nonreceptor protein tyrosine kinases are highly expressed in neurons and are believed to play a key role in tyrosine phosphorylation of NMDA receptors (Sala and Sheng, 1999). Recent studies have revealed that Fyn, a member of the Src family, is colocalized with NMDA receptors at the postsynaptic density (Tezuka et al., 1999) and directly phosphorylates NMDA subunits (Suzuki and Okumura-Noji, 1995), resulting in enhanced channel activity of the receptors (Köhr and Seeburg, 1996). A particularly prominent target of Fyn is Tyr 1472 of NR2B, which is located in the cytoplasmic tail of this protein (Tezuka et al., 1999; Nakazawa et al., 2001). Genetic inactivation of the gene that encodes Fyn in mice leads to impairment of hippocampal long-term potentiation (Grant et al., 1992) and deficits in learning (Yagi, 1999). Based on these observations, we hypothesized that Fyn might be the tyrosine kinase responsible for dopamine D1-dependent redistribution of striatal NMDA receptors.

The dopamine and cAMP regulated phosphoprotein (DARPP-32) is found at high levels in striatal neurons and is known to be a key molecular mediator of phosphorylation cascades (Greengard et al., 1999). When phosphorylated through the action of dopamine, DARPP-32 is a potent and selective inhibitor of protein phosphatase-1. This in turn leads to actions on a variety of downstream targets, including NMDA receptors, where it enhances serine phosphorylation of the NR1 subunit, leading to enhanced channel function (Blank et al., 1997; Snyder et al., 1998). Genetic inactivation of DARPP-32 blocks dopamine D1-induced serine phosphorylation of NR1 (Snyder et al., 1998), reduces dopamine D1-dependent enhancement of NMDA currents in dissociated striatal neurons (Flores-Hernandez et al., 2002), and prevents D1-dependent induction of long-term potentiation and depression in striatal slices (Calabresi et al., 2000b). These findings lead us to hypothesize that the DARPP-32-regulated serine phosphorylation of NMDA receptor complexes might also regulate D1-dependent trafficking in striatal neurons.

In the current study, we have used mice with targeted disruptions of the genes that encode Fyn kinase or DARPP-32 to explore the roles of these two important proteins in the signaling pathways underlying dopamine D1 receptor-dependent trafficking of striatal NMDA glutamate receptors. Our observations demonstrate a crucial role for Fyn kinase, but DARPP-32 does not seem to be required for this process.

Materials and Methods

Antibodies and Pharmacological Agents. The subunit-specific monoclonal NR1 (Luo et al., 1997), polyclonal NR2A (Wang et al., 1995), and monoclonal NR2B (Wang et al., 1995) antibodies were generous gifts from Dr. Barry B. Wolfe (Georgetown University, Washington, DC). The following antibodies were obtained from commercial sources: polyclonal GluR2/3 (Chemicon International, Temecula, CA); monoclonal anti-phosphotyrosine (BD Biosciences Transduction Laboratories, Lexington, KY); monoclonal anti-phosphoserine (Sigma-Aldrich, St. Louis, MO); and horseradish peroxidase-linked goat anti-rabbit and horseradish peroxidase-linked goat anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA). The drugs used for treatment of striatal tissues were purchased from the following sources: SKF-82958 (Sigma/RBI, Natick, MA); sodium orthovanadate (Acros Organics, Fairlawn, NJ); and hydrogen peroxide (Fisher Scientific Co., Fairlawn, NJ).

Experimental Animals. Experimental protocols involving the use of vertebrate animals were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and met the guidelines set by the National Institutes of Health. Male homozygous Fyn-deficient mice from 40 to 50 days of age were purchased from The Jackson Laboratory (Bar Harbor, Maine). For controls for experiments involving the Fyn mice, we used age-matched hybrid B6129SF2/J F2 mice derived from parental strains C57BL/6J and 129S1/SvImJ. The DARPP-32 knockout male mice at about 12 weeks of age were produced by Dr. Allen Fienberg (The Rockefeller University, New York, NY) as previously reported (Fienberg et al., 1998). These mice have been backcrossed with the C57BL/6 strain. We used homozygous DARPP-null mice, with the wild-type littermates serving as control mice for these experiments.

Preparation and Treatment of Mouse Brain Tissues. The brains of mice were removed after decapitation. The striata were dissected and cross-chopped into slices at 200 μM using a McIlwain mechanical tissue chopper in Kreb's buffer (118 mM NaCl, 4.7 mM KCl, 2 mM CaCl, 1.2 mM $MgSO_4$, and 1.2 mM KH_2PO_4) that had been equilibrated with 95% O_2/5% CO_2. Striatal tissues were incubated in the absence (control) or presence (treatment) of SKF-82958 (50 μM) or pervanadate (sodium orthovanadate) in Kreb's buffer at 37°C for 10 min with gentle agitation. Tissues were collected by centrifugation at 700*g* at 4°C and subjected to subcellular biochemical fractionation. A stock solution of the pervanadate was prepared as described previously (Dunah et al., 1998).

Subcellular Fractionation. Biochemical fractionation was performed as described previously (Dunah and Standaert, 2001). Briefly, Dounce homogenates of the pellets in ice-cold TEVP buffer (10 mM Tris-HCl, pH 7.4, 5 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, and 1 mM EGTA) containing 320 mM sucrose were centrifuged at 1000*g* to remove nuclei and large debris (P1). The supernatant (S1) was centrifuged at 10,000*g* to obtain a crude synaptosomal fraction (P2) and subsequently lysed hypo-osmotically and centrifuged at 25,000*g* to pellet a synaptosomal membrane fraction (LP1). The resulting supernatant (LS1) was then centrifuged at 165,000*g* to obtain a synaptic vesicle-enriched fraction (LP2). Concurrently, the supernatant (S2) above the crude synaptosomal fraction (P2) was centrifuged at 165,000*g* to obtain a cytosolic fraction (S3) and light membrane/microsome-enriched fraction (P3). Crossover contamination was avoided by briefly rinsing pellets after each centrifugation.

Unilateral 6-Hydroxydopamine Lesions. The Fyn knockout mice were pretreated with desipramine hydrochloride (25 mg/kg, i.p.) and anesthetized with Avertin (2% 2,2,2-tribromoethanol and 1% amyl alcohol; 10 ml/kg, i.p.). The mice were positioned in a stereotaxic frame and nigrostriatal lesions were produced by injecting 10.5 μg of 6-hydroxydopamine (6-OHDA) (3.5 μg/μl in normal saline containing 0.05% ascorbic acid) delivered by a micro-infusion pump (0.5 μl/min) into the left dorsal striatum at the following coordinates: 0.5 mm anterior, 2.0 mm lateral, and 2.2 mm ventral from bregma (Franklin and Paxinos, 1997). Mice were maintained in cages with free access to food and water and housed on a 12-h light/dark cycle before rotational testing.

Long-Term L-DOPA Treatment and Behavioral Analysis. Extended levodopa (L-DOPA) treatment was performed according to the description of Fredduzzi et al. (2002). Briefly, treatment was started 7 days after lesioning with 6-OHDA and consisted of L-DOPA methyl ester (2.0 mg/kg) and benserazide (2.0 mg/kg) in saline, or saline-injected i.p. daily for 6 days. The dose of L-DOPA methyl ester was increased to 6.0 mg/kg. Contralateral rotations were recorded every other day using an automated rotometer system (San Diego Instruments) in a 60-min testing period immediately after injection with L-DOPA or saline.

Protein Solubilization for Phosphorylation Studies. The striatal extracts from subcellular fractionation were solubilized using 1% SDS in TEVP buffer and centrifuged at 15,000*g* for 5 min in a microcentrifuge. Protein concentrations in the supernatants were determined with a protein assay kit (Bio-Rad, Hercules, CA) and used for Western blot and immunoprecipitation studies.

Precoupling Antibodies to Protein A-Sepharose. The monoclonal anti-phosphotyrosine (PY20) antibody was 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 in 100 mM sodium borate, pH 8.0, with gentle rotation. The beads were washed with 100 mM sodium borate, pH 8.0, and used for immunoprecipitation.

Immunoprecipitation. The solubilized proteins were diluted 20-fold with immunoprecipitation buffer (150 mM NaCl, 50 mM Na_3SO_4, pH 7.2, 1% sodium deoxycholate, 2 mM EDTA, and 1% Triton X-100). The diluted samples were incubated with 50 μl of the anti-phosphotyrosine antibody-coupled protein A-Sepharose beads for each 100 μg of protein for 3 h in a cold room with gentle rotation. The immunoprecipitates were washed three times using ice-cold radioimmunoprecipitation assay buffer after brief centrifugations and resuspended in a suitable volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 50 mM dithiothreitol, and 7.5% glycerol). Samples were resolved on SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted for NR1, NR2A, and NR2B subunits.

Gel Electrophoresis, Quantitative Immunoblotting, and Statistical Analysis. SDS-polyacrylamide gel electrophoresis and transfer of separated proteins to polyvinylidene difluoride membrane were performed as described previously (Wang et al., 1995; Dunah et al., 1996; Luo et al., 1996). For protein separation, 7.5 and 12.5% polyacrylamide gels were employed, and the concentration of antibodies used for immunoblotting was 1 to 2 μg/ml. For quantification of proteins in subcellular fractionation experiments, equal amounts (10 μg) of protein from each fraction were loaded into each gel lane. Bands were visualized on film by enhanced chemiluminescence and their net intensities were quantified using computer-assisted densitometry (Kodak 1-D System; Eastman Kodak, Rochester, NY). The net intensities of the bands were expressed as a percentage of that in control striatum. The resulting values were used to calculate group means and reported as mean ± S.E.M. Differences between groups were analyzed using analysis of variance (ANOVA) with post hoc tests (Scheffé's). For all analyses, statistical significance was taken to be $p < 0.05$.

Results

Basal Protein Expression and Phosphorylation of NMDA Subunits in DARPP-32-Depleted Mice. The basal levels of NMDA receptor proteins were determined by immunoblot analysis of striatal homogenates dissected from wild-type and DARPP-32-depleted mice (Fig. 1A). The abundance of NR1, NR2A, and NR2B subunits was similar in the wild-type (lane 1) and DARPP-32-depleted (lane 2) mice. The tyrosine (Fig. 1B) and serine (Fig. 1C) phosphorylation levels of NMDA receptors were examined by precipitating striatal protein extracts from wild-type and DARPP-32-depleted mice with anti-phosphotyrosine and anti-phosphoserine antibodies, respectively. Samples of the input and pellet were probed for NMDA subunits (Fig. 1, B and C). Both NR2A and NR2B subunits were tyrosine-phosphorylated in the DARPP-32-depleted mice (Fig. 1B, lane 4), and quantitative analysis revealed no difference in the abundance of these phosphoproteins between the knockout and wild-type mice (lanes 2 and 4). Consistent with previous reports (Lau and Huganir, 1995; Dunah et al., 2000), tyrosine phosphorylation of NR1 was not detected in either the wild-type or DARPP knockout animals (data not shown). Serine phosphorylation of the NR1, NR2A, and NR2B subunits was detected in both the DARPP-32-depleted mice (Fig. 1C, lane 4) and wild-type mice (Fig. 1C, lane 2), and no significant differences in the abundance of these serine phosphoproteins were observed in the basal state.

The distribution of NMDA receptors in distinct rat striatal compartments was examined by a biochemical fractionation method, as described previously (Dunah and Standaert, 2001). Using this approach, we found that in the DARPP-32-depleted mice, the NR1, NR2A, and NR2B subunits were detectable only in membrane-associated compartments, specifically the light membrane (Fig. 2A P3, lane 5), synaptosomal membrane (LP1, lane 7), and synaptic vesicle-enriched

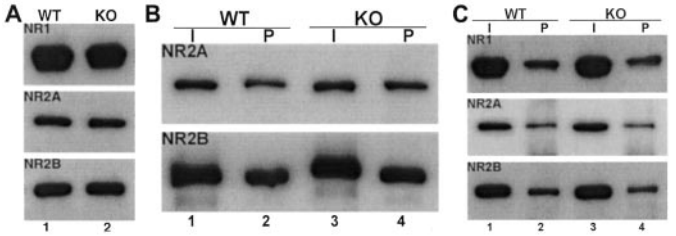

Fig. 1. The basal protein expression and phosphorylation of NMDA receptors are normal in DARPP-32-depleted mice. A, NMDA receptor proteins. Ten micrograms of total protein from each sample was loaded in each lane. The blots were probed with anti-NR1, anti-NR2A, and anti-NR2B antibodies. In this and most subsequent experiments, quantitative analysis of the band intensities was studied in striatal tissue from 15 animals of each genotype pooled to form three independent samples (experiments in which a different number of animals was used are indicated in the corresponding legend). This analysis revealed no significant differences in the abundance of the proteins between the wild-type and knockout mice. B, tyrosine phosphorylation. Samples from wild-type (lanes 1 and 2) and DARPP-32 knockout striata (lanes 3 and 4) were solubilized and immunoprecipitated using anti-phosphotyrosine antibody. The inputs (I; lanes 1 and 3; 5 μg) and pellets (P; lanes 2 and 4; 40 μg) were separated on SDS-PAGE gels and the blots were probed with anti-NR1, anti-NR2A, and anti-NR2B. C, serine phosphorylation. The samples above (B) were precipitated with anti-phosphoserine antibody, and blots were probed for NR1, NR2A, and NR2B subunits. No significant differences in the abundance of tyrosine- or serine-phosphorylated NR1, NR2A, or NR2B proteins were detected.

(LP2, lane 9) fractions. A similar pattern of NMDA receptor distribution was observed in wild-type mice (data not shown).

The tyrosine and serine phosphorylation of NMDA receptors in the different subcellular compartments was studied by immunoprecipitating protein extracts from each fraction with anti-phosphotyrosine (Fig. 2B) and anti-phosphoserine (Fig. 2C) antibodies. Consistent with data from previous studies using rat striatum (Dunah and Standaert, 2001), in both the wild-type and DARPP knockout mice, the NR2A and NR2B subunits in the synaptosomal membrane fraction (LP1, lane 10), and not those in the light membrane (P3, lane 8) and synaptic vesicle-enriched fractions (LP2, lane 12), were tyrosine-phosphorylated (Fig. 2B). The same pattern of distribution was observed for serine phosphorylation; serine-phosphorylated NR1, NR2A, and NR2B was present in the LP1 fraction, but not in P3 or LP2 (Fig. 2C).

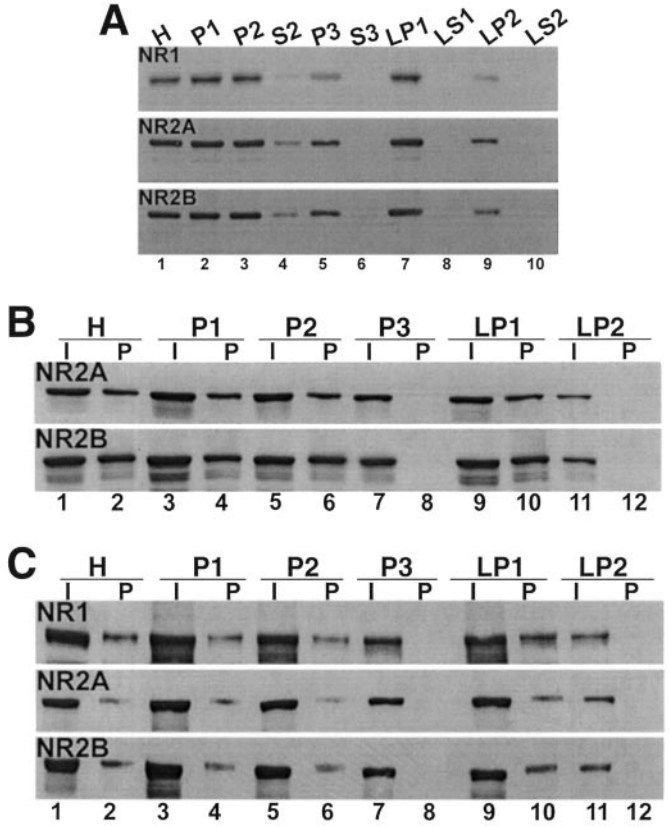

Fig. 2. DARPP-32-depleted mice exhibit normal subcellular distribution of NMDA subunits. A, Striata from DARPP-32-deficient mice were dissected, separated into different biochemical fractions as described under *Materials and Methods*, and resolved on SDS-polyacrylamide gels. The blots were probed with anti-NR1, anti-NR2A, and anti-NR2B antibodies. H, total homogenate; P1, nuclei and large debris; P2, crude synaptosomal fraction; P3, light membrane fraction; LP1, synaptosomal membrane fraction; LP2, synaptic vesicle-enriched fraction; S2, S3, LS1, and LS2 are supernatants from P2, P3, LP1, and LP2, respectively. The NMDA receptors are found in membrane-containing subcellular fractions (P3, LP1, and LP2) but are not present in purely cytosolic fractions (S3 and LS2). B, tyrosine phosphorylation. Samples from membrane-associated fractions were precipitated with anti-phosphotyrosine antibody, and the inputs (I; lanes 1, 3, 5, 7, 9, and 11) and pellets (P; lanes 2, 4, 6, 8, 10, and 12) separated on SDS-PAGE gels and probed with anti-NR1, anti-NR2A, and anti-NR2B antibodies. C, serine phosphorylation. The samples above (B) were precipitated with anti-phosphoserine antibody and blots were probed for NR1, NR2A, and NR2B subunits. Tyrosine-phosphorylated NR2A and NR2B and serine-phosphorylated NR1, NR2A, and NR2B subunits were detected in LP1 (lane 10) but not in P3 (lane 8) and LP2 (lane 12) fractions.

Targeted Disruption of the DARPP-32 Gene Does Not Impair D1-Dependent Redistribution of Striatal NMDA Receptors or Alter the Response to Phosphatase Inhibition. Striatal tissues from wild-type and DARPP-32-deficient mice were treated with a full agonist of the dopamine D1 receptor (SKF-82958) according to protocols described previously (Snyder et al., 1998; Dunah and Standaert, 2001). We found that SKF-82958 produced identical effects on the subcellular distribution of NMDA receptors in both the wild-type and DARPP-32-depleted mice (Fig. 3). In both the DARPP-32-depleted and wild-type mice, we observed reductions in the relative abundance of NR1, NR2A, and NR2B subunits in the P3 (light membrane) (Fig. 3A, lanes 6 and 8) and LP2 (synaptic vesicle enriched) (Fig. 3A, lanes 14 and 16) fractions, and an increase in these subunits in the LP1 (synaptosomal membrane) fraction (Fig. 3A, lanes 10 and 12). When the magnitude of redistribution in the wild-type and knockout mice was compared quantitatively, no significant differences were detected (Fig. 3B).

We examined the effect of SKF-82958 on tyrosine and serine phosphorylation of NMDA receptors in the synaptosomal membrane (LP1) compartment of the wild-type and DARPP knockout mice (Fig. 4). Treatment with this D1 agonist resulted in a significant increase in tyrosine phosphorylation of NR2A and NR2B subunits in the LP1 fraction in both the wild-type and DARPP-32-depleted mice (Fig. 4, A, lanes 4 and 8, and B). SKF-82958 also produced a marked increase in serine phosphorylation of the NR1 subunit in the LP1 fraction of wild-type mice (Fig. 4, C, lane 4, and D), but

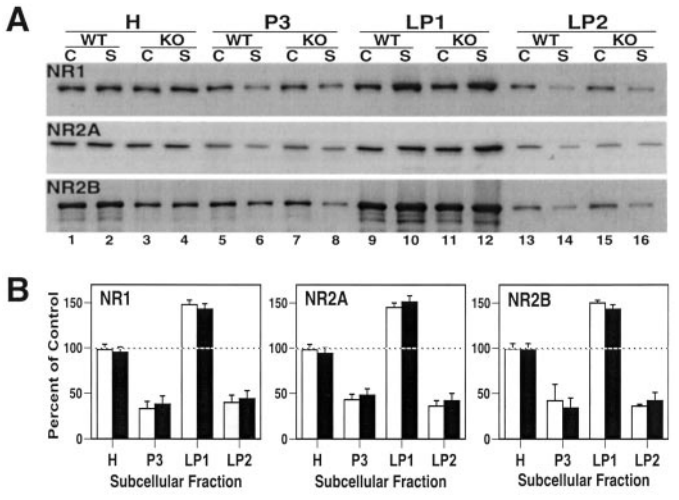

Fig. 3. NMDA receptor redistribution in DARPP-32-depleted mice in response to treatment with the dopamine D1 receptor agonist SKF-82958. A, striatal samples from wild-type (WT) and DARPP-32 knockout (KO) mice were incubated for 10 min under control conditions (C) or with 50 μM SKF-82958 (S) and subjected to biochemical fractionation. Fractions H, P3, LP1, and LP2 were resolved by SDS-PAGE, and blots were probed with NR1, NR2A, and NR2B antibodies. In both the wild-type and DARPP-32 knockout mice, this treatment produced a decrease in NR1, NR2A, and NR2B in P3 (lanes 6 and 8) and LP2 (lanes 14 and16), and an increase in these subunits in LP1 (lanes 10 and 12). B, densitometric analysis from five replicate experiments, with pooled tissue from three mice in each group. The abundance of NMDA receptors in the different fractions from wild-type (white bars) and DARPP-32-depleted (black bars) mice after treatment with the dopamine D1 receptor agonist SKF-82958 is expressed as mean percentage of abundance ± S.E.M. in the treated striata, relative to control tissue. The values observed in the DARPP-32-depleted mice were not significantly different from the wild-type strain ($p > 0.05$ for each comparison, ANOVA with post hoc tests).

in the DARPP-32-deficient mice this treatment had no effect on the serine phosphorylation of NR1 in LP1 (Fig. 4, B, lane 8, and D).

We also determined whether inhibition of endogenous protein phosphatase activity would alter the subcellular distribution and phosphorylation of NMDA receptors in the DARPP-32-depleted mice. For these studies, striatal tissues were treated with pervanadate, a protein tyrosine phosphatase inhibitor, before fractionation and phosphorylation analysis. We found that in both the DARPP-32-depleted and the wild-type mice, pervanadate treatment increased NR1, NR2A, and NR2B in the LP1 fraction and produced a corresponding reduction in these proteins in the P3 and LP2 fractions (data not shown). In the presence of pervanadate, tyrosine phosphorylation of NR2A and NR2B subunits was markedly increased in the synaptosomal membrane (LP1) compartments of wild-type mice, and a similar increase in tyrosine-phosphorylated NR2A and NR2B was seen in the DARPP-32-deficient mice. Unlike the effect observed with SKF-82958, the serine phosphorylation of the NR1 subunit was not changed in either the wild-type or DARPP-32-depleted mice by the pervanadate intervention (data not shown).

Basal Protein Expression and Phosphorylation of NMDA Receptors in Mice with Deletion of the Gene for the Protein Tyrosine Kinase Fyn. Immunoblot analysis of whole striatal homogenates from wild-type (Fig. 5A, lane 1) and Fyn knockout mice (Fig. 5A, lane 2) revealed that the protein content of NMDA receptor subunits NR1, NR2A, and NR2B in the striata of these mice was identical. In the Fyn knockout mice, the basal content of tyrosine-phosphorylated NR2A and NR2B was significantly reduced relative to the wild-type mice (NR2A, 28 ± 4%; NR2B, 17 ± 7% of wild type) (Fig. 5B, compare lanes 2 and 4), whereas serine phosphorylation of the NMDA subunits was similar in the wild-type and Fyn knockout mice (Fig. 5C, compare lanes 2 and 4).

Disruption of the Gene for Fyn Tyrosine Kinase Inhibits Dopamine D1 Receptor-Induced Subcellular Redistribution of NMDA Receptors. Treatment of striatal tissues from the Fyn knockout mice with the dopamine D1 receptor agonist SKF-82958 failed to alter the subcellular distribution of NMDA receptors (Fig. 6). After D1 agonist treatment, the abundance of NR1, NR2A, and NRB subunits in LP1, P3, and LP2 was identical to that in untreated tissues, whereas the agonist induced redistribution in the control mice (Fig. 6B). Furthermore, the deletion of Fyn blocked SKF-82958-induced enhancement of tyrosine-phosphorylated NR2A and NR2B in the synaptosomal membrane (LP1) fraction (Fig. 7, A, lanes 4 and 8, and B). However, disruption of the Fyn gene did not prevent the increase in serine phosphorylation of the NR1 subunit induced by SFK-82958 in the LP1 compartment (Fig. 7, C, lanes 4 and 8, and D).

Effect of Depletion of Fyn Protein on L-DOPA-Induced Behavioral Sensitization in a 6-OHDA-Lesioned Mouse Model. The effect of deletion of the Fyn tyrosine kinase gene on L-DOPA-mediated behavioral sensitization was investigated using unilateral 6-OHDA lesions of the nigrostriatal pathway (Franklin and Paxinos, 1997). After lesioning, the wild-type and Fyn-depleted mice were treated with L-DOPA daily for 21 days, and the rotational response was recorded using an automated rotometer (Fig. 8). The

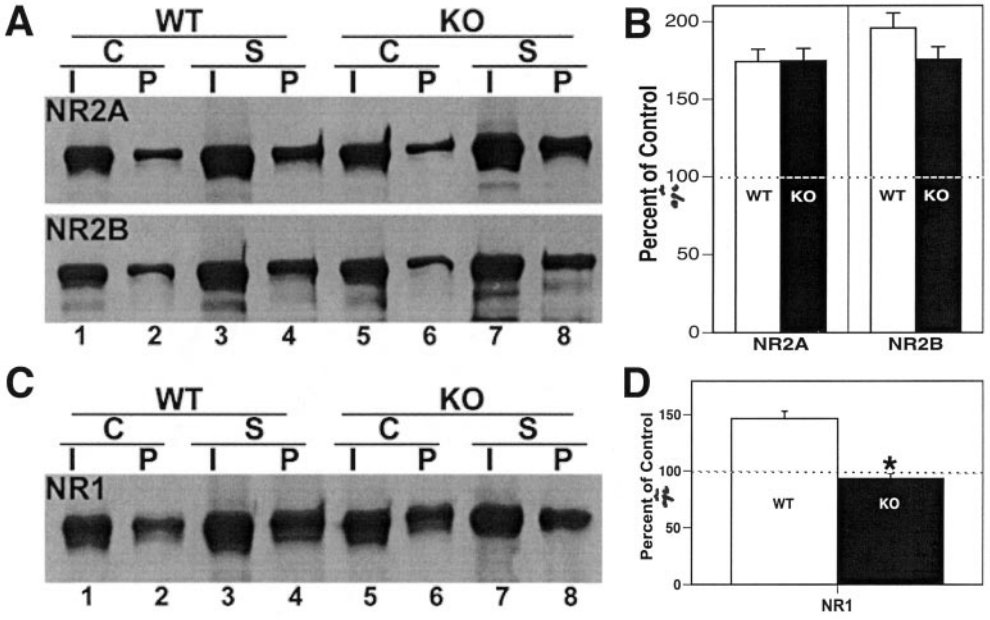

Fig. 4. The dopamine D1 receptor agonist SKF-82958 increased tyrosine but not serine phosphorylation of striatal NMDA receptors in DARPP-32-depleted mice. The synaptosomal membrane fraction (LP1) from control (C) and SKF-82958-treated (S) striatal tissues of both wild-type (WT) and DARPP-32-depleted (KO) mice were immunoprecipitated with either anti-phosphotyrosine (A) or anti-phosphoserine (C) antibodies. The resulting blots were probed with anti-NR1, anti-NR2A, and anti-NR2B. The input (I) and pellet (P) samples are indicated across the top of the figure. B and D, densitometry of tyrosine- (B) and serine- (D) phosphorylated NMDA receptors in wild-type (white bars) and DARPP-32-depleted (black bars) mice treated with SKF-82958. Values on the ordinate represent phosphorylation of NMDA receptor subunits in the stimulated groups as percentage of control. Data are means ± S.E.M. obtained from three groups of mice. SKF-82958 increased tyrosine-phosphorylated NR2A and NR2B subunits in both wild-type and DARPP-32-depleted mice. Serine-phosphorylated NR1 was increased in the wild-type mice but not altered in the DARPP-32-deficient mice after SKF-82958 treatment. Asterisk indicates a significant ($p < 0.05$, Mann-Whitney test) difference between the effect in the wild-type mice and that in the DARPP-32 knockout mice.

wild-type mice showed features of behavioral sensitization with an increase in the amplitude of the behavioral response after repeated treatment. In contrast, the Fyn-depleted mice failed to develop rotational behavior in response to L-DOPA administration (Fig. 8A). As a measure of the effectiveness of the 6-OHDA lesions in these animals, we analyzed tyrosine hydroxylase immunoreactivity in the lesioned and unlesioned striata of both wild-type and Fyn knockout mice by immunoblot (Fig. 8, B and C) at the conclusion of the experiment. In the wild-type animals, tyrosine hydroxylase was reduced by nearly 90%, whereas in the Fyn knockout animals, the same treatment with 6-OHDA produced less than 20% depletion of tyrosine hydroxylase. Thus, the differences in behavioral response to L-DOPA in these animals probably reflects the striking neuroprotective effect of the Fyn deletion in this model and cannot be taken as evidence for an alteration in striatal dopamine/glutamate interactions.

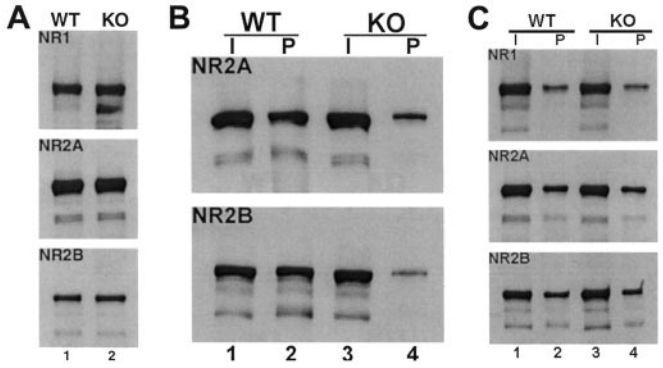

Fig. 5. The basal tyrosine phosphorylation of NMDA subunits are markedly reduced in Fyn protein tyrosine kinase-depleted mice. A, NMDA receptor proteins. Striatal homogenates from wild-type (WT) and Fyn knockout (KO) mice were resolved on SDS-polyacrylamide gels after loading 10 μg of total protein from each sample. The blots were probed for NR1, NR2A, and NR2B subunits. B, tyrosine phosphorylation. Striatal samples from wild-type (lanes 1 and 2) and Fyn knockout (lanes 3 and 4) were immunoprecipitated with anti-phosphotyrosine antibody and inputs (I; lanes 1 and 3; 5 μg) and pellets (P; lanes 2 and 4; 40 μg) were separated on SDS-PAGE gels and probed with anti-NR1, anti-NR2A, and anti-NR2B. C, serine phosphorylation. Striatal samples above (B) were precipitated with anti-phosphoserine antibody and probed for NR1, NR2A, and NR2B subunits. Targeted disruption of the Fyn protein kinase gene drastically decreased tyrosine but not serine phosphorylation of NMDA subunits. Protein levels of NMDA subunits are not altered in the Fyn knockout mice.

Discussion

Using mice with targeted disruption of DARPP-32 and Fyn, we have observed a differential effect of these two key regulators of NMDA receptor phosphorylation on D1-dependent NMDA receptor trafficking in the striatum. Knockout of DARPP-32 does not alter the basal abundance or phosphorylation of striatal NMDA subunits, and the effect of D1 activation on the subcellular distribution of NMDA receptor subunits is preserved. In contrast, the knockout of Fyn reduces basal tyrosine phosphorylation, but not serine phosphorylation, of NR1, NR2A, and NR2B subunits and prevents D1 agonist-induced subcellular redistribution of NMDA receptors. These findings suggest a mechanism for the trafficking of striatal NMDA receptors by signaling pathways that require Fyn protein tyrosine kinase but are independent of DARPP-32.

Role of Fyn in Striatal NMDA Receptor Regulation. Fyn is a member of the Src family of protein tyrosine kinases, which also includes Lyn, Lck, Yes, and Src itself (Ali and Salter, 2001). Considerable evidence supports the view that these tyrosine kinases are essential regulators of NMDA channel function. Enhanced tyrosine phosphorylation of NMDA receptors has been observed in response to a variety of physiological stimuli, including long-term potentiation (Rosenblum et al., 1996; Rostas et al., 1996), taste learning (Rosenblum et al., 1997), and transient ischemia (Takagi et al., 1997). In vitro, Src and Fyn potentiate NMDA currents and NMDA-mediated excitatory postsynaptic potentials (Köhr and Seeburg, 1996; Yu et al., 1997; Zheng et al., 1998). Fyn interacts with NMDA receptors by binding to the scaf-

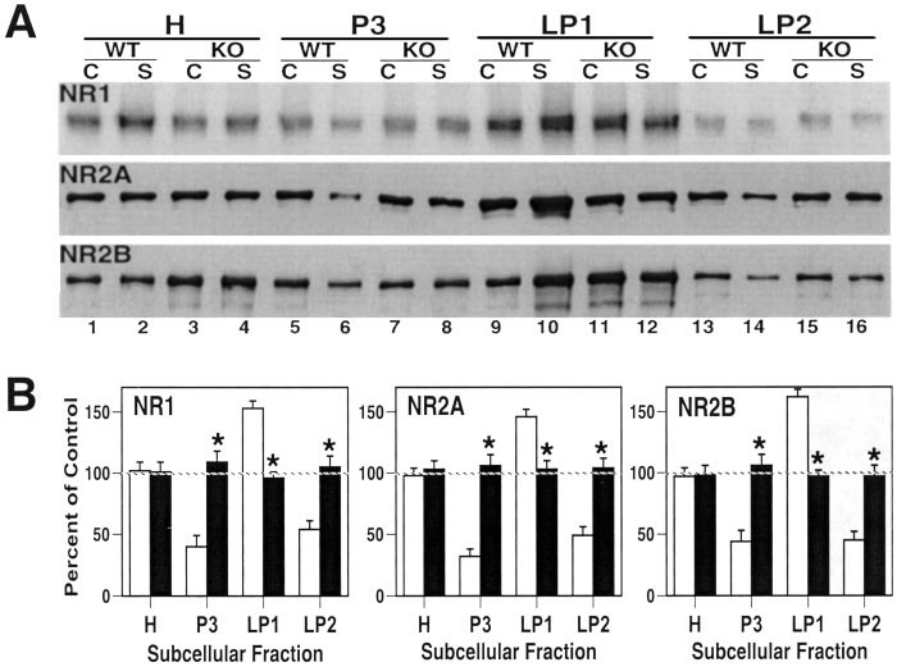

Fig. 6. Deletion of the Fyn protein tyrosine kinase gene blocked the dopamine D1 receptor-induced subcellular redistribution of NMDA receptors. A, striatal tissues from wild-type (WT) and Fyn knockout (KO) mice were incubated for 10 min under control conditions (C) or with 50 μM SKF-82958 (S) and fractionated. Fractions H, P3, LP1, and LP2 were electrophoresed on SDS-PAGE and blots were probed with NR1, NR2A, and NR2B antibodies. In wild-type mice, this treatment resulted in an increase in the abundance of all three proteins in LP1 (lanes 9 and 10) and a reduction in P3 (lanes 5 and 6) and LP2 (lanes 13 and 14). In the Fyn knockout mice, the abundance of the proteins in the different fractions was not altered by SKF-82958 treatment. B, densitometric analysis of striatal NMDA receptor subunits in wild-type (white bars) and Fyn knockout (black bars) mice treated with SKF-82958. Data are means ± S.E.M. obtained from five rats ($p < 0.05$, ANOVA). Asterisks indicate significant ($p < 0.05$, ANOVA with post hoc tests) differences between wild-type and Fyn knockout mice.

folding protein PSD-95, and this interaction promotes tyrosine phosphorylation of the NMDA receptors subunits (Tezuka et al., 1999). Recent mapping studies have demonstrated that multiple sites on both NR2A and NR2B are targets of Fyn kinase, although they also suggest that not all of the sites are accessible when the receptor proteins are assembled with components of the postsynaptic density (Cheung and Gurd, 2001; Nakazawa et al., 2001).

We have observed that mice with targeted disruption of the *Fyn* gene failed to exhibit D1-dependent redistribution of striatal NMDA receptors. In agreement with previous studies, we found that these mice have a markedly reduced level of tyrosine phosphorylation of NR2A and NR2B (Tezuka et al., 1999). Interestingly, the content of NR1, NR2A, and NR2B found in the LP1 (synaptosomal membrane), LP2 (synaptic vesicle-enriched), and P3 (light membrane) fractions of the untreated Fyn knockout mice was not different from the control strain. Although Fyn and tyrosine phosphorylation is required for D1-dependent redistribution, this finding suggests that there are alternate pathways for trafficking of NMDA receptors that maintain the normal basal subcellular distribution of NMDA subunits in the absence of Fyn.

Different mechanisms that may regulate the activity of Fyn and the tyrosine phosphorylation of NMDA channels in vivo have been proposed. Protein kinase C seems to have an important regulatory role in the activity of Src kinases (Lu et al., 1999). A second mechanism (that may be more relevant to D1 dopamine receptor action) involves the scaffolding protein RACK1, which inhibits phosphorylation of NR2B by Fyn (Yaka et al., 2002). Activation of the cAMP/PKA pathway leads to disruption of the interaction between Fyn, NMDA receptors, and RACK1, enhancing tyrosine phosphorylation of the subunits (Yaka et al., 2003). This provides a potential link between D1 receptor stimulation, cAMP formation, Fyn activation, and NMDA receptor redistribution. Moreover, a recent study has revealed that there are also direct protein-protein interactions between NMDA and dopamine D1 receptors (Lee et al., 2002). It is possible that Fyn kinase is important for the regulation of these interactions.

Role of DARPP-32 in Striatal NMDA Receptor Regulation. DARPP-32 is a regulatory protein that is abundantly expressed in striatal neurons and is involved in a myriad of interactions between neurotransmitter receptors and targets of serine and threonine phosphorylation (Greengard et al., 1999). The most thoroughly described activity of DARPP-32 is inhibition of protein phosphatase 1. This is regulated by protein kinase A, which phosphorylates threonine 34 to unmask the inhibitory effect. A second action of DARPP-32 is inhibition of PKA itself, which is modulated by the action of cyclin-dependent kinase 5 at threonine 75 (Bibb et al., 1999).

DARPP-32 has a well established role in the regulation of NMDA receptor function. In oocyte preparations, the presence of DARPP-32 is required to observe PKA-dependent potentiation of NMDA responses (Blank et al., 1997). DARPP-32 is also required for PKA-dependent serine phos-

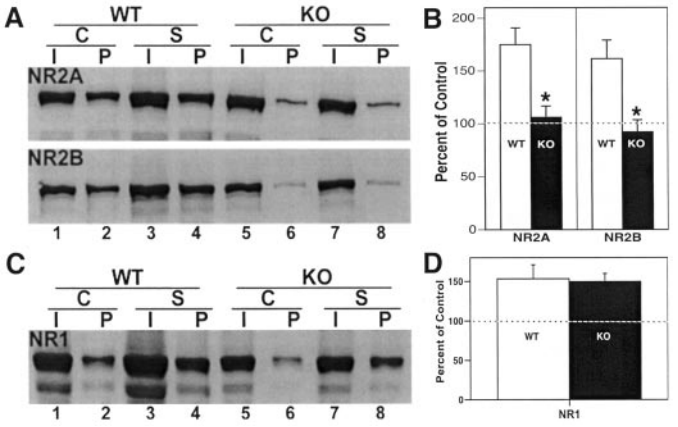

Fig. 7. The dopamine D1 receptor agonist SKF-82958 enhanced serine but not tyrosine phosphorylation of NMDA subunits in Fyn knockout mice. The fractionated synaptosomal membrane (LP1) samples from control (C) and SKF-82958-treated (S) striatal tissues of wild-type and Fyn knockout (KO) mice were precipitated with either anti-phosphotyrosine (A) or anti-phosphoserine (B) antibodies. The input (I) and pellets (P) were separated on SDS-PAGE and blots were probed with anti-NR1, anti-NR2A, and anti-NR2B. B and D, densitometric analysis of tyrosine- (B) and serine- (D) phosphorylated NMDA receptors in wild-type (WT; white bars) and Fyn knockout (KO; black bars) mice treated with SKF-82958. Phosphorylated NMDA receptor subunits, reported as percentage of control (untreated samples), are shown on the ordinate. Data are means ± S.E.M. obtained from three groups of mice. In the Fyn knockout mice, tyrosine-phosphorylated NR2A and NR2B subunits were not altered with SKF-82958 treatment, but they did exhibit a normal enhancement of serine phosphorylation of the NR1 subunit. Asterisks indicate significant differences between the wild-type and Fyn knockout mice ($p < 0.05$, Mann-Whitney test).

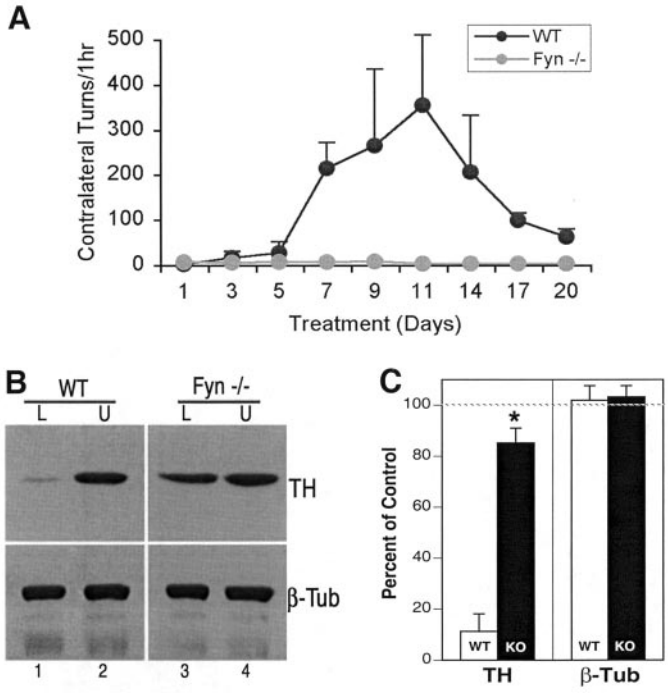

Fig. 8. The Fyn-depleted mice lesioned with 6-OHDA fail to show L-DOPA-induced behavioral sensitization. A, the 6-OHDA-lesioned wild-type (WT) and Fyn knockout (Fyn−/−) mice were treated daily for 21 days with either L-DOPA or saline. Complete contralateral rotations to the side of the lesion were recorded using an automated rotometer for a 60-min testing period immediately after injection with L-DOPA. In contrast to the wild-type mice, the Fyn-depleted mice do not exhibit L-DOPA-induced behavioral sensitization. B, protein homogenates from the lesioned (L; lanes 1 and 3) and unlesioned (U; lanes 2 and 4) striata from the wild-type (lanes 1 and 2) and Fyn knockout (Fyn−/−; lanes 3 and 4) mice in A were resolved on SDS-polyacrylamide gel. The blots were probed with anti-tyrosine hydroxylase (TH) and anti-β-tubulin (β-Tub) antibodies. C, values on the ordinate represent the relative levels of tyrosine hydroxylase (TH) and β-tubulin (β-Tub) (loading control) given as percentage of control (unlesioned striatum). Data are means ± S.E.M. obtained from four mice of each gene type. The reduction in immunoreactivity of striatal tyrosine hydroxylase is less in Fyn-depleted (black bars) compared with wild-type (white bars) mice ($p < 0.05$, ANOVA).

phorylation of NR1 (Snyder et al., 1998). Knockout of DARPP-32 prevents the induction of striatal long-term potentiation and long-term depression, forms of plasticity thought to underlie motor learning (Calabresi et al., 2000b). In a recent electrophysiological study, Flores-Hernandez et al. (2002) demonstrated that knockout of DARPP-32 reduces (but does not eliminate) D1 enhancement of NMDA currents in dissociated striatal neurons.

In mice with targeted disruption of DARPP-32, we found that the basal protein distribution and tyrosine and serine phosphorylation of NR1, NR2A, and NR2B subunits were unaltered. In both the DARPP-32 knockout animals and littermate control mice, activation of D1 receptors with the agonist SKF-82958 produced a rapid redistribution of NMDA receptor subunits, with increased abundance in the LP1 (synaptosomal membrane) fraction, and reduced abundance in the P3 (light membrane) and LP2 (synaptic vesicle-enriched) fractions. This was accompanied by enhanced tyrosine phosphorylation of NR2A and NR2B proteins in both wild-type and knockout mice, but D1-stimulated serine phosphorylation of NR1, NR2A, and NR2B subunits was markedly inhibited in the mice lacking DARPP-32. These observations demonstrate that D1-dependent trafficking of striatal NMDA receptors can proceed in the absence of DARPP-32.

Subcellular Trafficking of Striatal NMDA Receptors in Parkinson's Disease. Our original observations of the altered subcellular distribution of NMDA receptors in the rat 6-OHDA model of Parkinson's disease led us to consider the role of NMDA receptor trafficking in striatal dopamine/glutamate interactions (Dunah et al., 2000). Subsequent work in striatal tissue supported a role for tyrosine phosphorylation in this process (Dunah and Standaert, 2001). We have also observed that the types of NMDA receptors present in subcellular fractions of the rat striatum are distinct (Dunah and Standaert, 2003).

In the last several years, substantial evidence has accumulated demonstrating the importance of trafficking in the regulation of NMDA receptor systems, but a clear model that accounts for the phenomenon has not yet emerged (Carroll and Zukin, 2002; Wenthold et al., 2003). One important element seems to be subunit coassembly, which occurs in the endoplasmic reticulum. NR1 is produced in excess but is retained by ER retention signaling sequences unless coassembled with NR2 subunits (Ehlers et al., 1995). A mechanism for transport of subunits along dendrites that requires the kinesin KIF17 has been described previously (Setou et al., 2000), and in developing neurons, mobile NMDA receptor "transport packets" have been visualized (Washbourne et al., 2002). It is presumed that NMDA receptors are inserted near synapses by exocytosis, and a recent study has implicated proteins that are homologs of the yeast exocyst complex in this process (Sans et al., 2003). In the membrane, NMDA receptors undergo lateral diffusion (Tovar and Westbrook, 2002), which, as was recently emphasized, may be a more significant process than membrane insertion or removal (Choquet and Triller, 2003). Endocytosis of NMDA receptors from synaptic sites has also been clearly demonstrated and seems to be regulated by agonist and coagonist (glycine or D-serine) binding, tyrosine dephosphorylation and clathrin, and PSD-95 interactions; interestingly, though, it does not require ion flux through NMDA channels (Roche et al., 2001; Vissel et al., 2001; Nong et al., 2003).

We have demonstrated a crucial role for Fyn kinase in trafficking of NMDA receptors in striatal neurons, but the site of action is not certain. Fyn seems to participate in the component that is regulated by D1 activation but is not required for D1-independent trafficking. It is possible that phosphorylation by Fyn promotes exocytosis of NMDA-containing packets. It is also possible that Fyn-dependent phosphorylation stabilizes NMDA receptors in synaptic sites or prevents endocytosis of synaptic receptors. The ability of pervanadate, a phosphatase inhibitor, to mimic the effects of D1 activation suggests that there is also a constitutively active phosphatase present. A candidate for this is striatal-enriched protein tyrosine phosphatase, a tyrosine phosphatase found abundantly in striatal neurons that dephosphorylates NMDA receptors and is itself regulated by D1 receptor and PKA activation (Paul et al., 2000; Pelkey et al., 2002). We also attempted to directly examine the effect of Fyn kinase inhibition on levodopa sensitization using 6-OHDA lesions in the knockout animals. These experiments reveal a previously unsuspected neuroprotective effect of Fyn deletion; this finding may be important in understanding dopaminergic injury, but it precludes meaningful analysis of sensitization in this model. Pharmacological and genetic approaches that produce more selective inhibition of striatal Fyn may lead to useful therapeutic approaches in Parkinson's disease.

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