Association of Protein Phosphatase 1γ_1 with Spinophilin Suppresses Phosphatase Activity in a Parkinson Disease Model*

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Sustained nigrostriatal dopamine depletion increases the serine/threonine phosphorylation of multiple striatal proteins that play a role in corticostriatal synaptic plasticity, including Thr^{286} phosphorylation of calcium/calmodulin-dependent protein kinase IIα (CaMKIIα). Mechanisms underlying these changes are unclear, but protein phosphatases play a critical role in the acute modulation of striatal protein phosphorylation. Here we show that dopamine depletion for periods ranging from 3 weeks to 10 months significantly reduces the total activity of protein phosphatase (PP) 1, but not of PP2A, in whole lysates of rat striatum, as measured using multiple substrates, including Thr^{286}-autophosphorylated CaMKIIα. Striatal PP1 activity is partially inhibited by a fragment of the PP1-binding protein neurabin-I, Nb-(146–493), because of the selective inhibition of the PP1γ1 isoform. The fraction of PP1 activity that is insensitive to Nb-(146–493) was unaffected by dopamine depletion, demonstrating that dopamine depletion specifically reduces the activity of PP1 isoforms that are sensitive to Nb-(146–493) (i.e. PP1γ1). However, total striatal levels of PP1γ1 or any other PP1 isoform were unaffected by dopamine depletion, and our previous studies showed that total levels of the PP1 regulatory/targeting proteins DARPP-32, spinophilin, and neurabin were also unchanged. Rather, co-immunoprecipitation experiments demonstrated that dopamine depletion increases the association of PP1γ1 with spinophilin in striatal extracts. In combination, these data demonstrate that striatal dopamine depletion inhibits a specific synaptic phosphatase by increasing PP1γ1 interaction with spinophilin, perhaps contributing to hyperphosphorylation of synaptic proteins and disruptions of synaptic plasticity and/or dendritic morphology.

Calcium/calmodulin-dependent protein kinase II (CaMKII)[3] and protein phosphatase 1 (PP1) are critical for synaptic plasticity. Autophosphorylation of CaMKII at Thr^{286} is required for normal long term potentiation and hippocampus-based learning and memory (reviewed in Ref. 1). PP1 selectively dephosphorylates CaMKII that is associated with postsynaptic densities, whereas soluble CaMKII is primarily dephosphorylated by PP2A (2, 3). CaMKII has been shown to inhibit some forms of PP2A (4), but inhibition of PP1 via cAMP-dependent pathways promotes autophosphorylation at Thr^{286} and long term potentiation induction (5, 6). Spinophilin and neurabin are similar F-actin-binding proteins that selectively target the PP1γ1 isoform to dendritic spines, modulating spine morphology and synaptic plasticity (7–16). Thus, coordinated regulation of CaMKII and PP1 is critical for normal synaptic physiology.

Loss of nigrostriatal dopamine inputs in Parkinson disease or in parkinsonian animal models results in morphological alterations in striatal medium spiny neurons (MSNs) (17–22), which constitute >90% of the total striatal neuron population, and impairment of multiple forms of corticostriatal synaptic plasticity (23–26). Symptoms of Parkinson disease initially respond to dopamine replacement therapy, but as the disease progresses this approach generates debilitating side effects and/or loses efficacy. Studies of animal models, such as the 6-hydroxydopamine (6-OHDA) lesioned rat, have provided innumerable insights about terminal consequences of striatal dopamine depletion and the mechanisms underlying striatal deficits in Parkinson disease, resulting in improved therapeutic strategies (reviewed in Ref. 27).

Dopamine depletion increases Thr^{286} autophosphorylation of CaMKII, and this increase is reversed by dopamine replacement using levodopa (28, 29). Moreover, CaMKII inhibitors normalize dopamine depletion-induced alterations in both synaptic plasticity and behavior (29, 30), suggesting that altered regulation of CaMKII plays a critical role in the parkinsonian phenotype. The enhanced phosphorylation of CaMKII at Thr^{286} is sustained at similar levels for up to 18 months after inducing dopamine depletion (28). However, increased phosphorylation of a downstream CaMKII target, Ser^{831} in the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate-type glutamate receptor GluR1 subunit, is detected 9–18 months, but not 3–6 weeks, after dopamine depletion (28). These data suggest complex interactions between the effects of long term dopamine depletion and aging that may have additional effects on striatal function.

Striatal dopamine depletion could increase CaMKII autophosphorylation by at least two potentially linked mechanisms. First, increased corticostriatal glutamatergic drive (21, 31) might activate N-methyl-D-aspartic acid receptors or voltage-

[3] The abbreviations used are: CaMKII, calcium/calmodulin-dependent protein kinase II; 6-OHDA, 6-hydroxydopamine; GST, glutathione S-transferase; MSN, medium spiny neuron; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; HRP, horseradish peroxidase.

gated Ca^{2+} channels, enhancing postsynaptic Ca^{2+} influx and CaMKII autophosphorylation. Second, reduced protein phosphatase activity(ies) might allow increased phosphorylation of CaMKII and other proteins. Many acute effects of dopamine are thought to require inhibition of striatal PP1 by the Thr^{34}-phosphorylated form of DARPP-32 (32, 33), although reduced PP1 activity has not been directly demonstrated. Regulation of PP1 localization by spinophilin and neurabin also is critical for normal synaptic plasticity and dendritic spine morphology (9, 10, 13, 15, 16, 34, 35).

Here we report that chronic striatal dopamine depletion selectively decreases the activity of the $PP1\gamma_1$ isoform, apparently by enhancing its association with spinophilin. This reduction in PP1 activity may allow enhanced phosphorylation of multiple striatal proteins, including CaMKII, thereby playing a key role in mediating changes in synaptic morphology and/or function following dopamine depletion.

EXPERIMENTAL PROCEDURES

6-OHDA Lesion Surgery—Male Sprague-Dawley rats (Harlan; Indianapolis, IN) were housed under a 12:12 light:dark cycle with food and water freely available. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), under the oversight of the Institutional Animal Care and Use Committee. Rats (3 months old) underwent unilateral 6-OHDA lesion surgery, or received sham (vehicle) injections, as detailed previously (28). At the times indicated after surgery, rats were lightly anesthetized with isoflurane, decapitated, and the brains removed. Data reported here were obtained from six batches of 6–8 rats sacrificed at times ranging from 3 weeks to 11 months following lesion surgery to assess the long term consequences of dopamine depletion. Rats in which a 6-OHDA lesion induced a >90% loss of tyrosine hydroxylase in the lesioned striatum relative to the intact contralateral striatum were considered to be completely dopamine-depleted. Rats with <90% loss of tyrosine hydroxylase in the lesioned hemisphere were considered to have a partial dopamine depletion, and data obtained from these animals were not included in quantitative analyses. The 6-OHDA lesioned rat is a well established model of essentially complete dopamine depletion and is well suited to the study of long term effects of striatal dopamine depletion. As such, it has proven to be a reliable animal model of Parkinson disease (27).

Antibodies—The following primary antibodies were used for immunoblotting (dilutions indicated) and/or for immunoprecipitation: whole serum and affinity-purified sheep and rabbit anti-$PP1\gamma_1$ and anti-$PP1\beta$ (7, 36, 37); mouse anti-tyrosine hydroxylase (ImmunoStar, 1:1000); mouse anti-PP2Ac (BD Transduction Laboratories, 1:4000); rabbit antibody to spinophilin (8) purified using protein A-Sepharose (Upstate); and mouse anti-spinophilin (BD Transduction Laboratories, 1:1000). Secondary antibodies were from Promega (goat anti-mouse HRP, 1:2000; goat anti-rabbit HRP, 1:4000), AlphaQuest (rabbit anti-goat HRP, 1:4000), or Santa Cruz Biotechnology (goat anti-mouse HRP or donkey anti-sheep HRP preadsorbed with human and/or mouse IgG, 1:5000).

Immunoblots—Dorsolateral striatum was isolated, processed for immunoblotting, and immunoblotted as described previously (28).

Tissue Homogenates for Phosphatase Activity Assays—Punches (1.15-mm inner diameter) of dorsolateral striatum were removed from both hemispheres of 1.0-mm-thick coronal slices, at the level of the crossing of the anterior commissure. Punches were immediately homogenized using a Kontes tissue homogenizer in ice-cold 7 mM Tris-HCl, pH 7.5, containing 0.2 mM EDTA, 0.2 mM EGTA, 320 mM sucrose, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μM pepstatin, 50 mM sodium fluoride, 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate. Homogenates were stored at -80 °C and analyzed within 1–2 weeks.

Protein Phosphatase Activity Assays—Phosphatase activities were assayed using [^{32}P]phosphorylase *a*, [^{32}P]casein, or Thr^{286}-autophosphorylated CaMKIIα substrates, as described previously (3). Briefly, extracts (10 μl of 0.1–0.2 mg/ml extract) were incubated at 30 °C in 50 mM Tris-HCl, pH 7.5, 0.1 M sodium chloride, 20 mM magnesium acetate, 2 mg/ml bovine serum albumin, 0.2 mM EGTA, 1 mM dithiothreitol for 30 min ([^{32}P]phosphorylase *a* and [^{32}P]casein) or 45 min (Thr^{286}-autophosphorylated CaMKIIα) in a final volume of 50 μl. Caffeine (5 mM) was included with [^{32}P]phosphorylase *a*, and assay blanks were incubated without extract. Trichloroacetic acid (50 μl of 40% w/v) was added to terminate the reaction and samples were centrifuged (10,000 $\times$ *g*, 10 min). Supernatants (80-μl aliquots) were analyzed in a liquid scintillation counter to quantify ^{32}P released from the protein substrate.

Where indicated, diluted extracts were preincubated with the following inhibitors for $\sim$10 min on ice prior to initiating assays by addition of substrate. Nb-(146–453) and Nb-(146–493) were prepared as glutathione *S*-transferase (GST) fusion proteins and were characterized previously (12, 38). Nb-(146–453) does not contain the PP1 binding domain (residues 457–460) or the $PP1\gamma_1$ selectivity domain (residues 473–479) that are present in Nb-(146–493). Inhibitor-2 (Calbiochem) and okadaic acid (LC Laboratories) were also used. PP2A activity was defined as the activity inhibited by 2.5 nM okadaic acid. PP1 activity was defined as the difference in activities measured using 2.5 nM and 2.5 μM okadaic acid. $PP1\gamma_1$ activity was defined as the phosphatase activity inhibited by Nb-(146–493).

Immunoprecipitations—For phosphatase activity assays (Fig. 5*A*), native $PP1\gamma_1$ and $PP1\beta$ holoenzymes were isolated from extracts of whole striatum by immunoprecipitation. Briefly, freshly dissected whole striatal tissue was homogenized in 2 mM Tris-HCl, pH 7.5, containing 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 40 mg/liter soybean trypsin inhibitor, 10 mg/liter leupeptin, and 0.5% Triton X-100). Homogenates were centrifuged (10,000 $\times$ *g*, 10 min, 4 °C), and supernatants were diluted to 1 mg/ml in IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.5% Triton X-100). Samples were precleared using gammabind-G-Sepharose (Amersham Biosciences) and then incubated at 4 °C for 1 h with sheep antisera to $PP1\gamma_1$ or $PP1\beta$ (5 μl each). After addition of gammabind-G-Sepharose (30 μl of a 50:50 slurry), incubation was continued overnight at 4 °C. Immunoprecipitates were sedimented, washed five times

with 1 ml of IP buffer, suspended in homogenization buffer lacking Triton X-100, and immediately assayed.

For co-immunoprecipitations, intact (control) and lesioned (dopamine depleted) dorsolateral striatal punches (see above) were homogenized separately in the same buffer containing phosphatase inhibitors (50 mM sodium fluoride, 50 mM β-glycerophosphate, 20 mM sodium pyrophosphate). Aliquots (0.5 ml) of diluted/precleared soluble extracts were immunoprecipitated using rabbit antibodies to PP1γ_1 (5 μl of affinity-purified), spinophilin (5 μl of protein A-purified), or control IgG in IP Buffer containing the same phosphatase inhibitors. Washed immunoprecipitates and aliquots of the input and immune supernatants were mixed with sample buffer and analyzed by immunoblotting using enhanced chemiluminescence detection. X-ray films exposed in the linear range were scanned and quantified using Image J (rsb.info.nih.gov). PP1γ_1 and spinophilin signals in immune complexes were normalized to the corresponding input signal and then expressed as a ratio between the dopamine-depleted (lesioned) and intact hemispheres.

Statistical Analyses—Statistical comparisons were made by paired or unpaired Student's *t* test or Wilcoxon signed rank test, as appropriate.

RESULTS

Phosphorylation of protein phosphatase catalytic or regulatory subunits can regulate their activity and/or localization. Therefore, we developed conditions that limit dephosphorylation of putative regulatory sites following homogenization of striatal samples but allow for detection of phosphatase activities in diluted extracts. Homogenization in the presence of organic phosphatase inhibitors such as microcystin LR is most effective in blocking protein dephosphorylation, but no phosphatase activity was detected when striatal homogenates containing microcystin LR were diluted (data not shown), presumably because this inhibitor is effectively irreversible. However, inclusion of a mixture of inorganic phosphatase inhibitors in the homogenization buffer (see "Experimental Procedures") allowed the detection of PP1 and PP2A activity if striatal homogenates were diluted 10-fold immediately prior to the assay.

Total Phosphatase Activity Is Decreased Following Dopamine Depletion—We measured phosphatase activities in extracts of dorsolateral striatum ipsilateral or contralateral to 6-OHDA lesion of the substantia nigra. Extracts were prepared from different batches of animals 3–4 weeks or 10–11 months after lesion surgery to assess the consequences of long term dopamine depletion. Using [^{32}P]phosphorylase *a* as a model substrate, activity in the dopamine-depleted striatum was significantly decreased by ≈18% when measured 3–4 weeks after lesion surgery (Fig. 1*A*) or by ≈14% when measured 10–11 months after surgery (data not shown; $n = 13$, $p = 0.04$). In contrast, there was no significant difference in total phosphatase activities at either time point if [^{32}P]casein was used as the model substrate (Fig. 1*B* and data not shown).

Dopamine Depletion Selectively Decreases PP1 Activity—The choice of exogenous protein substrate affects the specific phosphatases that can be detected in assays of whole tissue homogenates. Generally, [^{32}P]phosphorylase *a* is considered a PP1-
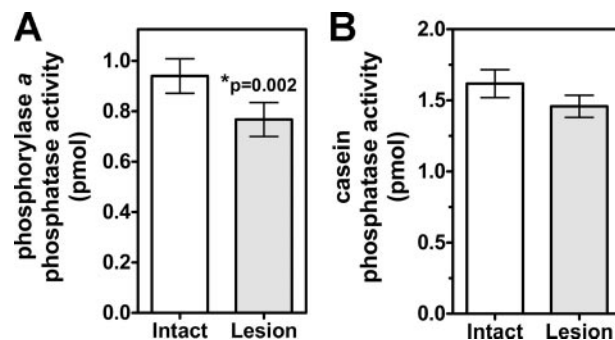

FIGURE 1. **Total striatal phosphatase activity is decreased following dopamine depletion.** Dorsolateral striatal tissue ipsilateral (*Lesion*) or contralateral (*Intact*) to the 6-OHDA lesion was collected 3–4 weeks after surgery, and whole extracts were assayed using [^{32}P]phosphorylase *a* or [^{32}P]casein as model substrates. *A*, total [^{32}P]phosphorylase *a* phosphatase activity was decreased by ≈18% in the lesioned hemisphere ($n = 8$, paired *t* test). *B*, there was no significant difference in the total [^{32}P]casein phosphatase activity between striatal hemispheres ($n = 8$).
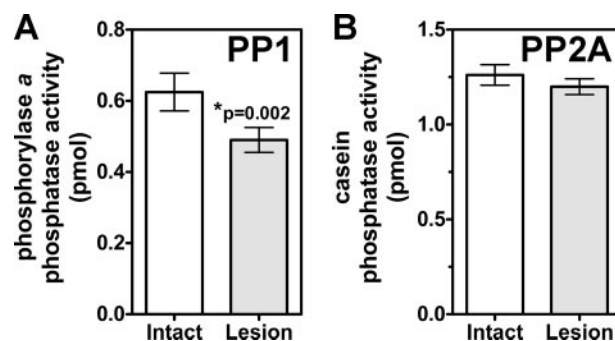

FIGURE 2. **Dopamine depletion selectively decreases PP1 activity.** Phosphatase activities in extracts of rat dorsolateral striatal tissue ipsilateral (*Lesion*) or contralateral (*Intact*) to the 6-OHDA lesion collected 3–4 weeks after surgery were assayed using [^{32}P]phosphorylase *a* or [^{32}P]casein in the presence of either 0, 2.5 nM, or 2.5 μM okadaic acid. *A*, dopamine depletion significantly decreased by ≈22% the PP1 activity measured using [^{32}P]phosphorylase *a* (difference in activities measured in the presence of 2.5 nM and 2.5 μM okadaic acid) ($n = 8$, paired *t* test). *B*, there was no significant difference in PP2A activity measured using [^{32}P]casein (difference in activities measured in the absence and presence of 2.5 nM okadaic acid) ($n = 8$).

selective substrate but also detects PP2A activity, whereas [^{32}P]casein is an effective PP2A substrate (reviewed in Ref. 39). To more specifically compare PP1 and PP2A activities in control and dopamine-depleted striatal extracts, we measured phosphatase activities toward [^{32}P]phosphorylase *a* and [^{32}P]casein in the presence of 0, 2.5 nM, or 2.5 μM okadaic acid. Okadaic acid selectively inhibits PP2A-like enzymes at low nanomolar concentrations, but will also inhibit PP1 at micromolar concentrations (39). Our previous studies defined PP2A activity as the activity inhibited by 2.5 nM okadaic acid and PP1 activity as the difference in activities measured at 2.5 nM and 2.5 μM okadaic acid (3). PP1 activity detected using [^{32}P]phosphorylase *a* was significantly decreased by 22% at 3–4 weeks following lesion surgery (Fig. 2*A*) and by ≈16% at 10–11 months after surgery (data not shown; $n = 13$, $p = 0.014$). However, PP2A activity detected using either [^{32}P]phosphorylase *a* or [^{32}P]casein substrate was not significantly different between the striatal hemispheres when measured 3–4 weeks (Fig. 2*B*) or 10–12 months (data not shown; $n = 13$) after lesion surgery.
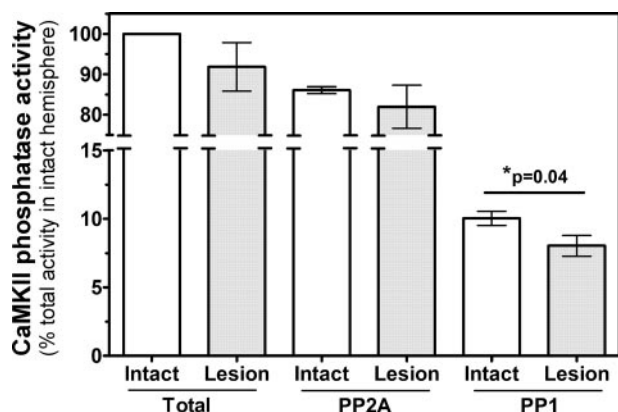
FIGURE 3. **Dopamine depletion decreases PP1-mediated dephosphorylation of $[^{32}P\text{-Thr}^{286}]$CaMKII$\alpha$.** Phosphatase activities in extracts of rat dorsolateral striatal tissue ipsilateral (*Lesion*) or contralateral (*Intact*) to the 6-OHDA lesion collected 3–4 weeks after surgery were assayed using $[^{32}P\text{-Thr}^{286}]$CaMKII$\alpha$ in the presence of 0, 2.5 nM, or 2.5 μM okadaic acid. The graph plots contributions of PP1 and PP2A (calculated as under "Experimental Procedures") as the percentage of total activity in the intact hemisphere. Dopamine depletion had no significant effect on the PP2A-mediated dephosphorylation of $[^{32}P\text{-Thr}^{286}]$CaMKII$\alpha$ but significantly decreased PP1-mediated dephosphorylation by $\approx$19% ($n = 8$).

Dopamine Depletion Inhibits PP1-mediated Dephosphorylation of CaMKIIα—To ascertain whether dopamine depletion affects dephosphorylation of a physiologically relevant substrate, we assayed phosphatase activity toward exogenous Thr^{286}-autophosphorylated CaMKIIα in the presence of various concentrations of okadaic acid. As reported previously in whole forebrain extracts (3), $\sim$80% of the total activity could be assigned to PP2A based on the sensitivity to 2.5 nM okadaic acid. However, this PP2A activity was unaffected by dopamine depletion. In contrast, PP1 activity toward $[^{32}P\text{-Thr}^{286}]$CaMKII$\alpha$ (defined as the difference in activities at 2.5 nM and 2.5 μM okadaic acid) was significantly decreased by $\approx$19% after dopamine-depletion surgery (Fig. 3).

Phosphatase Isoform Levels Are Unchanged by Dopamine Depletion—The decreased phosphatase activity in dopamine-depleted striatum may be due to changes in the levels of specific phosphatase catalytic subunit isoforms, perhaps because of degeneration of dopaminergic terminals and/or the dendritic spines of MSNs. Therefore, aliquots of striatal extracts used in activity assays were immunoblotted for various PP1 catalytic subunit isoforms (α, β, and γ_1) and for the PP2A catalytic subunit. These proteins are highly expressed in MSNs but are also present in presynaptic terminals. Despite the substantial degeneration of terminals, as reflected by the loss of >90% of tyrosine hydroxylase, there were no significant differences in levels of PP1 and PP2A catalytic subunit isoforms between intact and lesioned hemispheres (Fig. 4). This may reflect relatively low expression of these proteins in dopaminergic terminals and/or the small contribution of the terminals to total striatal tissue.

Nb-(146–493) Is a Novel, Selective Inhibitor of PP1γ_1 in Striatal Extracts—Differential subcellular targeting of PP1 isoforms in neurons suggests that these proteins have different biological roles (7, 14). We sought to develop an assay that differentiates the contributions of PP1 isoforms to the total PP1 activity in extracts. Nb-(146–493) is a GST fusion protein that
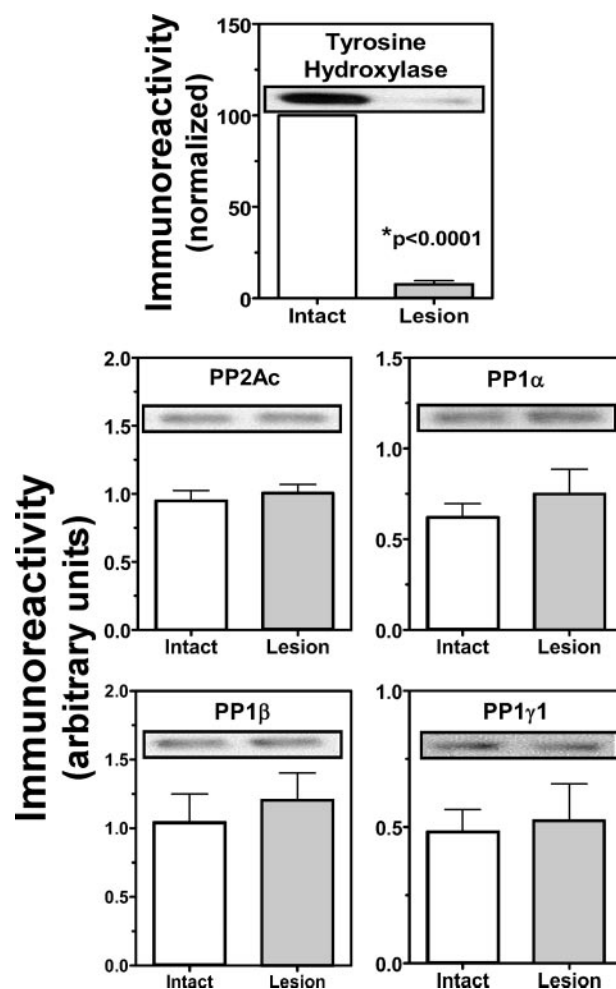

FIGURE 4. **PP1 catalytic subunit isoform expression is unaffected by dopamine depletion.** Whole dorsolateral striatal extracts ipsilateral (*Lesion*) or contralateral (*Intact*) to the 6-OHDA lesion collected 10–11 months after surgery were immunoblotted for tyrosine hydroxylase, PP2Ac, and various isoforms of PP1 (α, β, and γ_1). The efficacy of the 6-OHDA lesion was confirmed by the $\approx$90% decrease of tyrosine hydroxylase immunoreactivity ($n = 11$), but there was no significant difference in the levels of PP2Ac, PP1α, PP1β, or PP1γ_1 between intact and lesioned hemispheres ($n = 9$–11, $p > 0.240$).

inhibits isolated brain PP1γ_1 catalytic subunit $\approx$20-fold more potently than it inhibits isolated brain PP1β catalytic subunit (12). To determine whether this selectivity might be useful in discriminating PP1 isoform activities in tissue extracts, we investigated the effects of Nb-(146–493) (1 μM) on the activities of striatal PP1γ_1 and PP1β holoenzyme complexes isolated by immunoprecipitation. Immunoblots of the isolated complexes confirmed the isoform specificity of the immunoprecipitations (Fig. 5A). Both isoform complexes displayed activity toward $[^{32}P]$phosphorylase *a* in the presence of 2.5 nM okadaic acid, which could be completely blocked by 2.5 μM okadaic acid, consistent with the specific immunoprecipitation of active PP1. Significantly, Nb-(146–493) (1 μM) almost completely blocked activity in PP1γ_1 immunoprecipitates but had no significant effect on PP1β activity (Fig. 5A). Thus, Nb-(146–493) is a highly selective inhibitor of striatal PP1γ_1 holoenzymes over PP1β holoenzymes.

We extended these findings by comparing the effects of Nb-(146–493) and other phosphatase inhibitors on the activi-
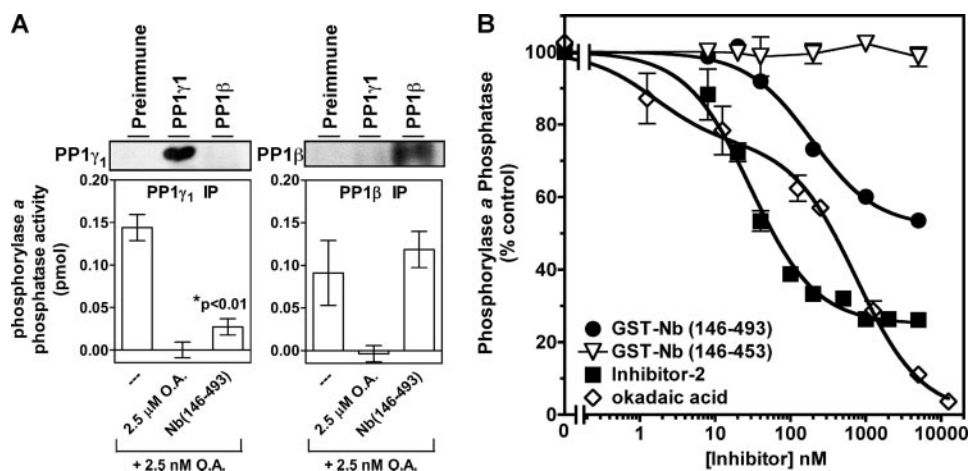
FIGURE 5. **Nb-(146–493) is a selective inhibitor of striatal PP1γ₁.** *A*, PP1γ₁ or PP1β were immunoprecipitated (*IP*) from whole striatal extracts. *Upper panels* show representative immunoblots confirming the immunoprecipitation specificity. *Lower panels* show activities in the immunoprecipitates measured using [³²P]phosphorylase *a* in the presence of 2.5 nM okadaic acid (*O.A.*) alone or with the addition of 2.5 μM okadaic acid or 1 μM Nb-(146–493). Nb-(146–493) inhibited immunoprecipitated PP1γ₁, but not immunoprecipitated PP1β. *B*, phosphatase activity in a control whole striatal extract was assayed using [³²P]phosphorylase *a* in the presence of indicated concentrations of okadaic acid (*open diamonds*), inhibitor-2 (*solid squares*), Nb-(146–493) (*solid circles*), or Nb-(146–453) (*open inverted triangles*). Each point is the mean ± S.E. of 2–6 observations, plotted as a percentage of total activity (without inhibitor). PP1 accounts for 75–80% of the total activity when estimated using okadaic acid or inhibitor-2 (see "Results"), but Nb-(146–493) inhibits only ≈48% of the total activity.

both hemispheres of control (sham-operated) animals revealed no significant difference in total protein phosphatase activities or Nb-(146–493)-sensitive PP1 activity (Fig. 6*B*). Moreover, there was no difference in Nb-(146–493)-sensitive PP1 activities between striatal hemispheres collected from rats with incomplete dopamine depletions, as defined by <90% loss of tyrosine hydroxylase (normalized Nb-(146-493)-sensitive PP1 activities of 45.3 ± 6.7 and 53.4 ± 7.3, respectively, $n = 5$, $p = 0.44$). However, Nb-(146–493)-sensitive PP1 activity was significantly reduced in dopamine-depleted striatum collected from a second batch of rats 3–4 weeks after 6-OHDA lesion surgery (18 ± 6% reduction, $p = 0.03$; data not shown). Thus, the selective loss of PP1γ₁ activity is manifest within 3–4 weeks of striatal dopamine depletion and is sustained for several months.

Dopamine Depletion Enhances PP1γ₁ Association with Spinophilin—PP1γ₁ is selectively associated with spinophilin in brain extracts, and spinophilin inhibits the activity of PP1γ₁ *in vitro* (8). We investigated the interaction of PP1γ₁ with spinophilin in control and dopamine-depleted dorsolateral striatal extracts by immunoprecipitation using anti-PP1γ₁ or control IgG. Similar amounts of PP1γ₁ were detected in immune complexes isolated from the two hemispheres, but no PP1γ₁ was detected in control precipitations (Fig. 7). Quantitative analyses revealed a lesion:intact ratio that averaged 1.2 ± 0.5 (mean ± S.E., $n = 7$ animals) (Fig. 7), consistent with our findings that total levels of PP1γ₁ are unaltered by dopamine depletion (28) (Fig. 4). Spinophilin was readily detected in PP1γ₁ immune complexes but not in the control IgG samples (Fig. 7), confirming the specificity of co-immunoprecipitation seen previously (8, 12). Notably, levels of spinophilin were markedly higher in PP1γ₁ immune complexes from dopamine-depleted tissue than in those from contralateral control tissue. The lesion:intact ratio for the amount of spinophilin in PP1γ₁ immune complexes was 3.4 ± 0.8 (mean ± S.E., $n = 7$), significantly greater than the expected value of 1.0 if there was no change in the association of spinophilin with PP1γ₁ ($p = 0.029$ by two-tailed, one sample *t* test) (Fig. 7).

ties of PP1 holoenzymes present in whole striatal extracts using [³²P]phosphorylase *a* as a model substrate. About 20% of the activity detected was inhibited by nanomolar concentrations of okadaic acid, and the remaining activity was almost completely inhibited by micromolar concentrations of okadaic acid, suggesting that total PP1 activity accounted for about 80% of [³²P]phosphorylase *a* phosphatase activity (Fig. 5*B*). Consistent with this assignment, a PP1 inhibitor with no known isoform selectivity (inhibitor-2) blocked ≈75% of the total activity with an apparent EC₅₀% ≈30 nM (Fig. 5*B*). In contrast, Nb-(146–493) inhibited a maximum of only ≈48% of the total activity at the highest concentration tested (5 μM), with an apparent EC₅₀% of ≈190 nM. However, Nb-(146–453), a GST-Nb fusion protein lacking the PP1 binding domain, had no significant effect on striatal phosphatase activity. Thus, Nb-(146–493) only partially inhibits striatal PP1 activity, presumably because of the isoform selectivity of Nb-(146–493) toward endogenous holoenzymes (Fig. 5*A*). Nb-(146–493)-insensitive activity may be due to PP1β and PP2A holoenzymes, whereas PP1γ₁ holoenzyme complexes likely account for the Nb-(146–493)-sensitive activity.

Dopamine Depletion Selectively Decreases Striatal PP1γ₁ Activity—To determine the effect of dopamine depletion on the activity of different PP1 isoforms, [³²P]phosphorylase *a* phosphatase activity in dorsolateral striatal extracts from 6-OHDA-lesioned rats was assayed in the absence and presence of 1 μM Nb-(146–493) (Fig. 6). Dopamine depletion significantly reduced total phosphatase activity in the whole extract by ≈17% in extracts collected 10–11 months after 6-OHDA lesion surgery to this cohort of animals. However, there was no significant difference in Nb-(146–493)-insensitive activities in samples from the two hemispheres; thus, the Nb-(146–493)-sensitive PP1 activity was reduced by ≈27% (Fig. 6*A*). However, parallel analyses of PP1 activities in dorsolateral striatum from

Separate aliquots of the control and dopamine-depleted striatal extracts were also immunoprecipitated with antibodies to spinophilin. Spinophilin was not detected in control immunoprecipitates, but similar levels were detected in spinophilin immune complexes from extracts of control or dopamine-depleted striatum (lesion:intact ratio = 1.6 ± 0.3, $n = 7$) (Fig. 7), consistent with our findings that total levels of spinophilin are unaltered by dopamine depletion (28). PP1β was not detected in spinophilin immune complexes (data not shown), consistent with our previous data (8, 12). However, PP1γ₁ was present, and
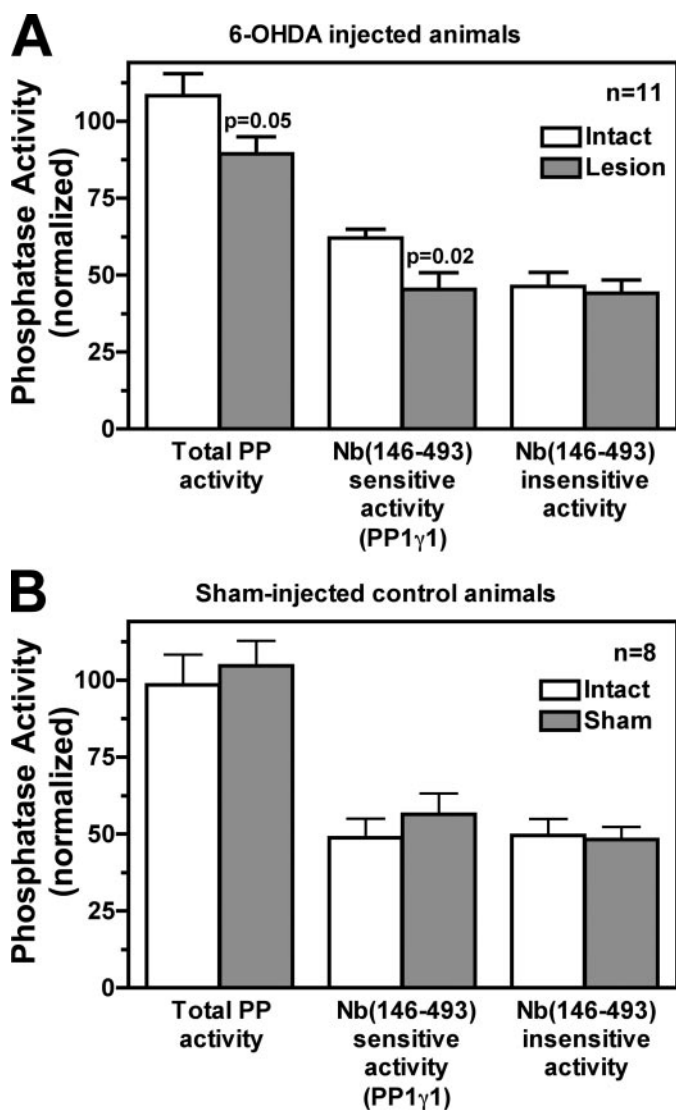
FIGURE 6. **Dopamine depletion selectively decreases striatal PP1γ₁ activity.** *A,* extracts of dorsolateral striatum ipsilateral (*Lesion*) or contralateral (*Intact*) to the 6-OHDA lesion collected 10–11 months following surgery were assayed using [³²P]phosphorylase *a* in the absence or presence of 1 μM Nb-(146–493). Dopamine depletion significantly reduces total protein phosphatase activity by ≈17% ($p = 0.04$), but activity that is insensitive to Nb-(146–493) was not significantly different between the two hemispheres. Thus, dopamine depletion significantly reduced Nb-(146–493)-sensitive PP1 activity (*i.e.* PP1γ₁) by ≈27% ($p = 0.02$). *B,* dorsolateral striatal extracts from both brain hemispheres of sham-operated rats were separately analyzed as in *A*. No significant differences in activities of any protein phosphatases were detected.

increased levels were detected in spinophilin immune complexes isolated from dopamine-depleted striatum than from control tissue (lesion:intact ratio = 5.7 ± 2.8, *n* = 7) (Fig. 7). This represents a significant increase over an expected value of 1.0 if this protein interaction is unaffected by dopamine depletion ($p = 0.031$ by Wilcoxon signed rank test) (Note: this test was used because these data did not pass the Shapiro-Wilk normality test.). In combination, these data show that dopamine depletion induced by the 6-OHDA lesion increases the interaction of spinophilin and PP1γ₁ in the dorsolateral striatum.

DISCUSSION

Dopamine depletion has diverse effects on corticostriatal synaptic transmission and striatum-based behaviors in Parkin-son disease and in parkinsonian animal models. The intracellular mechanisms underlying these effects are poorly understood, although altered protein phosphorylation in striatal MSNs is likely to be involved. Previous studies have linked changes in striatal protein phosphorylation following dopamine depletion to alterations in protein kinase activities (*e.g.* Refs. 29, 30, 40). However, protein phosphatases are believed to mediate many acute effects of dopamine in the striatum. It is widely accepted that dopamine acutely modulates PP1 activity and/or localization via phosphorylation/dephosphorylation of regulatory and targeting proteins such as DARPP-32 and spinophilin (33), although to the best of our knowledge changes in PP1 activity have not been directly demonstrated. We show here that chronic dopamine depletion significantly decreases striatal PP1 activity apparently because of regulation of the interaction between the PP1γ₁ isoform and spinophilin. The decrease in PP1γ₁ activity is observed within a few weeks of inducing dopamine depletion by 6-OHDA lesion surgery and is maintained for at least 11 months, with no evidence for progressive changes in PP1 activity over this time period. Decreased activity of this synaptically targeted PP1 isoform may be important in mediating chronic effects of dopamine depletion at corticostriatal synapses.

Dopamine Depletion Selectively Decreases PP1 Activity—Initial studies using model substrates showed that there was a significant reduction in phosphatase activity toward [³²P]phosphorylase *a* but not toward [³²P]casein (Fig. 1). Various concentrations of okadaic acid were used to confirm that PP1 activity was significantly decreased following dopamine depletion but that PP2A activity was unaffected (Fig. 2). Similar reductions in total PP1 activity were detected 3–4 weeks and 10–11 months after 6-OHDA lesion surgery (see "Results"). Moreover, PP1 activity toward the Thr²⁸⁶ autophosphorylation site in CaMKIIα (a physiologically relevant substrate in postsynaptic densities) was significantly decreased by dopamine depletion. Because PP1 is the major phosphatase responsible for dephosphorylation of PSD-associated CaMKII (3), the reduced PP1 activity may contribute to the enhanced autophosphorylation of striatal CaMKIIα at Thr²⁸⁶ following dopamine depletion (28, 29). Phosphorylation of a variety of synaptic receptors and other proteins is enhanced following dopamine depletion (see below), so it will be interesting to determine whether decreased PP1 activity also plays an important role in mediating these changes.

PP1γ₁ Is Selectively Modulated Following Dopamine Depletion—The three major PP1 catalytic subunit isoforms present in the striatum (α, β, and γ₁) are not known to differ in their fundamental enzymatic properties, but they are enriched in distinct subcellular compartments (7, 14, 41, 42). In particular, interactions of PP1γ₁ with the actin-binding proteins spinophilin and neurabin are thought to be responsible for the selective localization of PP1γ₁ in dendritic spines and at the PSD (8, 11, 14). Thus, PP1γ₁ is poised to efficiently dephosphorylate synaptic substrates, such as CaMKII, and also to respond to activation of D₁- and D₂-like dopamine receptors that are localized on dendritic spine heads and necks.

In a new approach to selectively assess the contribution of PP1γ₁ to total PP1 activity, we exploited a PP1-binding frag-

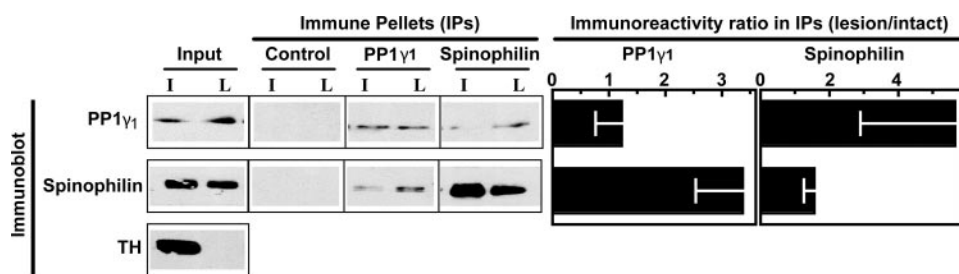## Inhibition of Striatal PP1 Following Dopamine Depletion

FIGURE 7. **Dopamine depletion increases the association of PP1γ_1 with spinophilin.** Extracts of dorsolateral striatum ipsilateral (*L*) or contralateral (*I*) to the 6-OHDA lesion collected 3–7 months following surgery were immunoprecipitated using antibodies to spinophilin or PP1γ_1 or control antibodies. Aliquots of the extract (*Input*) and the immunoprecipitates were immunoblotted for tyrosine hydroxylase (*TH*), spinophilin, and PP1γ_1, as indicated. A >90% depletion of tyrosine hydroxylase in the dopamine-depleted hemisphere of this animal confirmed substantial degeneration of nigrostriatal dopaminergic neurons. Inputs from the two hemispheres contained comparable amounts of spinophilin and PP1γ_1, and similar amounts of the cognate antigen were immunoprecipitated by each antibody from the two hemispheres. However, more spinophilin was detected in PP1γ_1 immunoprecipitates from the lesioned hemisphere (*L*) than from the control (*I*) hemisphere. Similarly, more PP1γ_1 was detected in the spinophilin immunoprecipitates from the lesioned hemisphere (*L*) than from the control (*I*) hemisphere. Quantitative analyses (*right*, plotted as mean ± S.E.) revealed statistically significant increases in the amount of spinophilin in PP1γ_1 immune complexes ($p = 0.029$, $n = 7$ animals) and the amount of PP1γ_1 in spinophilin immune complexes ($p = 0.031$, $n = 7$).

ment of neurabin (Nb-(146–493)) that inhibits the purified PP1γ_1 catalytic subunit more potently than it inhibits PP1β (12). We extended these findings by showing that Nb-(146–493) (1 μM) inhibits ≈90% of the activity of PP1γ_1 holoenzymes immunoprecipitated from striatal extracts but does not inhibit immunoprecipitated PP1β holoenzymes (Fig. 5*A*). Moreover, Nb-(146–493) only partially inhibits the total PP1 activity in whole striatal extracts (Fig. 5*B*). The overall reduced inhibitory potency and enhanced selectivity of Nb-(146–493) in whole tissue extracts relative to our previous assays of purified catalytic subunits presumably reflects the fact that the added Nb-(146–493) fragment has to competitively displace endogenous regulatory subunits from the catalytic subunits in the extracts. Development of this tool allowed us to show that dopamine depletion has no effect on the phosphatase activity that is insensitive to Nb-(146–493) (Fig. 6), demonstrating that dopamine depletion selectively reduces the activity of the PP1 that is sensitive to Nb-(146–493), presumably the PP1γ_1 isoform. Similar reductions in PP1γ_1 activity were detected 3–4 weeks and 11 months after 6-OHDA lesion surgery.

We cannot formally exclude a contribution of PP1α to Nb-(146–493)-sensitive activity. Spinophilin and neurabin associate preferentially with PP1γ_1 rather than PP1α (11), and PP1α is expressed in the striatum at ≈50% lower total levels than PP1γ_1 (42, 43). In addition, PP1α and PP1γ_1 are differentially distributed within dendritic spines, whereas PP1β is localized to dendritic shafts and the soma (14). Thus, it seems most likely that Nb-(146–493) preferentially inhibits PP1γ_1. In combination, our data demonstrate that dopamine depletion differentially affects the PP1 isoforms, apparently selectively reducing PP1γ_1 activity. These observations represent the first direct evidence that activities of PP1 isoforms are differentially modulated *in situ*.

Mechanisms for Selective Regulation of Striatal PP1 Isoforms—PP1γ_1 is selectively enriched in dendritic spines (associated with the postsynaptic density), as well as inconsistently (and to a lesser extent) in presynaptic terminals (14, 41). We considered the possibility that reduced activity of PP1γ_1 following

dopamine depletion directly resulted from the partial loss of spines containing PP1γ_1 and other proteins from indirect pathway MSNs (18), or from the loss of dopaminergic terminals. However, it is likely that such an underlying mechanism would decrease total protein levels, in contrast to data showing no significant changes in levels of PP1γ_1 (in whole extracts or in isolated PSD-enriched cytoskeletal fractions) or in total levels of several other postsynaptic or presynaptic proteins (Fig. 4) (28). Nevertheless, the reduced PP1γ_1 activity may be associated with changes in its subcellular localization in specific subpopulations of striatal MSNs. Careful immunofluorescence and/or immunoelectron microscopic studies will be required to address this possibility. However, such studies would not address the activity in different compartments, and PP1γ_1 activity may be altered without detectable changes in localization or vice versa.

We also considered the role of PP1γ_1 interactions with other proteins that are known to play critical roles in mediating acute effects of dopamine signaling in the striatum. Most prominently, PP1 is inhibited by DARPP-32 that has been phosphorylated by cAMP-dependent protein kinase at Thr[34] in response to D$_1$ dopamine receptor activation. Phosphorylation of DARPP-32 at Thr[75] in response to activation of D$_2$ receptors interferes with Thr[34] phosphorylation (reviewed in Ref. 33). However, there is no evidence that DARPP-32 is an isoform-selective modulator, and dopamine depletion has no significant effect on the total levels or Thr[34] phosphorylation of DARPP-32, although Thr[75] phosphorylation is substantially increased (25, 28, 44). Thus, DARPP-32 does not appear to be involved in selective suppression of PP1γ_1 activity following dopamine depletion.

Most PP1 regulatory proteins are not known to exhibit isoform selectivity, and their roles in the striatum are poorly understood. Prominent exceptions are spinophilin and neurabin, related F-actin-binding proteins that are also localized to dendritic spines (reviewed in Ref. 45). Spinophilin and neurabin selectively interact with PP1γ_1 (8, 11, 12) and are essential for dopaminergic modulation of corticostriatal synaptic plasticity (9, 15, 46). Chronic dopamine depletion does not change the total levels of spinophilin/neurabin or alter the amount of these proteins present in PSD-enriched cytoskeletal fractions (28). However, we show here that dopamine depletion increases the association of PP1γ_1 with spinophilin (Fig. 7). Because spinophilin and neurabin selectively inhibit the activity of PP1γ_1 catalytic subunits toward model substrates such as phosphorylase a, this increased interaction of PP1γ_1 with spinophilin is the likely mechanism accounting for the decreased activity of PP1 detected in striatal lysates following dopamine depletion.

Implications of Altered Spinophilin-PP1γ₁ Interaction Following Dopamine Depletion—Spinophilin and neurabin appear to function as classical scaffolding proteins with no intrinsic enzyme activity. Both proteins interact with a variety of signaling proteins in addition to PP1γ₁. For example, spinophilin interacts with D_2 dopamine receptors, p70 S6 kinase, and several small GTPase exchange factors that appear to pay key roles in regulating neuronal morphology (reviewed in Ref. 47). Moreover, alterations in spinophilin expression levels or overexpression of isolated domains from spinophilin results in altered neuronal morphology, including changes in dendritic spines. Interactions of spinophilin with F-actin are modulated by multiple protein kinases (46, 48), and some spinophilin-binding proteins are regulated by protein phosphorylation, including p70 S6 kinase and the rac GTPase exchange factor Tiam1 (49, 50). Moreover, PP1γ₁ and p70 S6 kinase appear to bind neurabins in a competitive, mutually exclusive manner to modulate cell morphology (10). Although most previous studies focused on changes in neuronal morphology during relatively early postnatal development and/or using neuronal cultures, we suggest that the increased binding of PP1γ₁ to spinophilin following dopamine depletion in the mature striatum reflects a re-organization of spinophilin signaling complexes, leading to altered protein phosphorylation/dephosphorylation. This reorganization might favor increased phosphorylation of PP1γ₁ substrates, giving rise to diverse effects on striatal signaling and cell morphology. Indeed, dopamine depletion increases the phosphorylation of several known synaptic PP1 substrates, such as subunits of N-methyl-D-aspartic acid- and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate-type glutamate receptors and CaMKIIα (28–30, 51), which may be important in the disruptions of corticostriatal synaptic plasticity and behavior following dopamine depletion (23–26, 29, 30). However, it is possible that additional signaling proteins are either directly or indirectly affected by dopamine depletion, including kinases and other phosphatases.

Our observations in this animal model of parkinsonism are similar to emerging evidence for disruptions in the balance between kinase and phosphatase activities in other neurodegenerative diseases or disease models, such as amyotrophic lateral sclerosis (52), multiple sclerosis (53), Charcot-Marie-Tooth disease (54), and Alzheimer disease (55). Perhaps most similarly, decreased protein phosphatase activity in a mouse model of Angelman mental retardation syndrome correlates with increased phosphorylation of hippocampal CaMKII and disruptions of synaptic plasticity, learning, and memory (56). Thus, we suggest that the development of strategies to increase the activity of critical protein phosphatases, including PP1γ₁, might be a fruitful strategy to normalize protein phosphorylation and treat some of the symptoms of Parkinson disease, Angelman syndrome, and possibly other neurodegenerative diseases.

Final Summary—The present data demonstrate the specificity of cellular signaling via highly homologous PP1 isoforms in the striatum. Dopamine depletion selectively decreases the activity of the PP1γ1 isoform. Selective inhibition of a specific PP1 isoform that is precisely targeted in dendritic spines provides new insights into the long term changes in striatal signaling associated with Parkinson disease.

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