⌘ *Author's Choice*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 23, pp. 17648–17661, June 4, 2010

Serine 129 Phosphorylation Reduces the Ability of α-Synuclein to Regulate Tyrosine Hydroxylase and Protein Phosphatase 2A *in Vitro* and *in Vivo*[*] [S]

Received for publication, January 8, 2010, and in revised form, March 6, 2010 Published, JBC Papers in Press, March 31, 2010, DOI 10.1074/jbc.M110.100867

Haiyan Lou[‡§1,2], Susana E. Montoya[‡1], Tshianda N. M. Alerte[‡1], Jian Wang[‡3], Jianjun Wu[‡], Xiangmin Peng[‡4], Chang-Sook Hong[‡], Emily E. Friedrich[‡], Samantha A. Mader[‡], Courtney J. Pedersen[‡], Brian S. Marcus[‡], Alison L. McCormack[¶], Donato A. Di Monte[¶], S. Colette Daubner[‖], and Ruth G. Perez[‡‡‡5]**

*From the [‡]Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, the [§]Department of Pharmacology, Shandong University School of Medicine, Jinan, Shandong 250012, China, [¶]The Parkinson's Institute, Sunnyvale, California 94085, the [‖]Department of Biological Sciences, St. Mary's University, San Antonio, Texas 78229, and the Departments of **Neurology and [‡‡]Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261*

α-Synuclein (a-Syn), a protein implicated in Parkinson disease, contributes significantly to dopamine metabolism. a-Syn binding inhibits the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis. Phosphorylation of TH stimulates its activity, an effect that is reversed by protein phosphatase 2A (PP2A). In cells, a-Syn overexpression activates PP2A. Here we demonstrate that a-Syn significantly inhibited TH activity *in vitro* and *in vivo* and that phosphorylation of a-Syn serine 129 (Ser-129) modulated this effect. In MN9D cells, a-Syn overexpression reduced TH serine 19 phosphorylation (Ser(P)-19). In dopaminergic tissues from mice overexpressing human a-Syn in catecholamine neurons only, TH-Ser-19 and TH-Ser-40 phosphorylation and activity were also reduced, whereas PP2A was more active. Cerebellum, which lacks excess a-Syn, had PP2A activity identical to controls. Conversely, a-Syn knock-out mice had elevated TH-Ser-19 phosphorylation and activity and less active PP2A in dopaminergic tissues. Using an a-Syn Ser-129 dephosphorylation mimic, with serine mutated to alanine, TH was more inhibited, whereas PP2A was more active *in vitro* and *in vivo*. Phosphorylation of a-Syn Ser-129 by Polo-like-kinase 2 *in vitro* reduced the ability of a-Syn to inhibit TH or activate PP2A, identifying a novel regulatory role for Ser-129 on a-Syn. These findings extend our understanding of normal a-Syn biology and have implications for the dopamine dysfunction of Parkinson disease.

α-Synuclein (a-Syn)[6] is a presynaptic protein (1) that contributes to neuronal plasticity and neurodegeneration (2). a-Syn also modulates multiple aspects of dopamine metabolism (3–8), including its ability to inhibit the activity of the rate-limiting catecholamine biosynthetic enzyme tyrosine hydroxylase (TH; EC 1.14.16.2) (9).

TH activity is stimulated by phosphorylation, leading to catecholamine synthesis (10). Phosphorylation of TH serine 19 (Ser(P)-19) plays the unique role of recruiting 14-3-3 chaperone proteins to TH (11–14), an event that enhances TH activity (15, 16). Phosphorylation of TH serine 40 (Ser(P)-40) dissociates the binding of inhibitory catecholamines (17–20). TH-Ser-19 phosphorylation is evident in all dopaminergic brain regions (21), raising the possibility that this is a key site for TH regulation. The binding of TH by 14-3-3 (22, 23) induces a 3-fold rate increase in Ser-40 phosphorylation (11, 23, 24), revealing an enhancing effect for Ser-19 phosphorylation on Ser(P)-40 that may be counter-regulated by a-Syn and 14-3-3. a-Syn and 14-3-3 share homology, and a-Syn is thought to have chaperone-like activity (25–30), a function that may underlie its ability to interact with targets such as TH (9, 26, 27, 31).

a-Syn mutations (A30P, A53T, or E46K) (32–34) and multiplications (35, 36) induce rare forms of familial Parkinson disease (PD), and a-Syn is a major protein in Lewy bodies, the intraneuronal protein aggregates characteristic of PD (37). a-Syn that is phosphorylated at serine 129 (Ser(P)-129) is plentiful in Lewy bodies (38, 39), yet no one knows how a-Syn Ser-129 phosphorylation affects a-Syn function.

PP2A (EC 3.1.3.16) is the main phosphatase for TH dephosphorylation (24, 40–42). a-Syn binds to and stimulates PP2A activity, and furthermore, a-Syn overexpression in dopaminergic cells reduces TH-Ser(P)-40 levels (43). These findings raised the possibility that phosphorylation of other TH serines might be similarly affected by a-Syn overexpression. Therefore, this

* This work was supported, in whole or in part, by National Institutes of Health Grants NS42094 (to R. G. P. with a supplement provided for S. E. M. (NINDS)) and GM47291 (to S. C. D.). This work was also supported by the China Scholarship Council (to H. L.).

This work is dedicated to M. J. Fox, R. Byer, and J. Cordy and in memory of L. "Rusty" Lanelli.

⌘ *Author's Choice*—Final version full access.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

[1] These authors contributed equally to this work.

[2] Present address: Institute of Pharmacology, Shandong University School of Medicine, Jinan 250012, China.

[3] Present address: Dept. of Neurology, Fudan University Huashan Hospital, 12 Wulumuqi Zhong Rd., Shanghai 200040, China.

[4] Present address: Dept. of Neurology, University of Michigan School of Medicine, 109 Zina Pitcher Pl., Ann Arbor, MI 48109.

[5] To whom correspondence should be addressed: 3501 Fifth Ave., BST3 7021, Pittsburgh, PA 15260. Tel.: 412-383-9776; Fax: 412-648-8373; E-mail: perezrg@pitt.edu.

[6] The abbreviations used are: a-Syn, α-synuclein; TH, tyrosine hydroxylase; PD, Parkinson disease; PP2A, protein phosphatase 2A; DOPA, dihydroxyphenylalanine; ANOVA, analysis of variance; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; Non-Tg, non-transgenic; ASKO, a-Syn knock-out mice; PLK2, Polo-like kinase 2; SNc, substantia nigra pars compacta; AAV, adeno-associated virus.

study was undertaken to 1) further elucidate the molecular mechanisms underlying the effects of a-Syn on TH, especially Ser-19 phosphorylation, which represents a key step in 14-3-3-mediated-TH activation, 2) determine the *in vivo* significance of a-Syn in TH regulation in transgenic overexpressing and null mice, and 3) assess if a-Syn Ser-129 phosphorylation affects a-Syn function toward PP2A or TH. We found significant *in vivo* and *in vitro* effects of a-Syn on the regulation of TH and PP2A that are modulated by phosphorylation of a-Syn at Ser-129. Because a-Syn is strongly associated with nigrostriatal dopaminergic neuron loss in disease, clarifying its biological role in these neurons holds promise for elucidating novel PD pharmacotherapies.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, Transfection, Mutagenesis—MN9D cells were grown as previously described (9). Stably transfected MN9D cells were grown in media containing 200 μg/ml G418 (Invitrogen) and used at low passage to sustain a-Syn expression as confirmed using immunoblots (9, 43, 44). Transient transfections of MN9D cells used Lipofectamine 2000 (Invitrogen) to express green fluorescent protein (GFP), wild type (WT) human a-Syn, or serine to alanine at amino acid 129 (S129A) a-Syn in pcDNA3.1 or the human PP2A catalytic domain in pcDNA4c (a gift of J. Haendeler, University of Düsseldorf) on 60% confluent cultures in 6-well plates. Transiently transfected cells were harvested at 48–72 h. S129A mutant a-Syn was generated by PCR using established methods (45) in which we used human WT a-Syn and 5′ forward priming with CAAGAATGAAGAAGGAC and 3′ reverse priming to change serine to alanine and introduce an XbaI site in the 3′-non-coding region of a-Syn cDNA, accomplished using the TCTAGATTAGGCTTCAGGTTCGTAGTCTTGATACCCTTCCTCAG**C**AGGCATTTCA sequence to create a 125-bp fragment that was digested with EcoRI and XbaI. This formed a 98-bp fragment that was ligated into EcoRI- and XbaI-digested WT human a-Syn in pcDNA3.1. S129A mutagenesis was confirmed by sequencing.

Animals—The wild type (WT-Syn++) and A53T a-Syn (A53T-Syn++) transgenic mice overexpress human a-Syn under control of a 4.8-kb rat TH-promoter, which restricts expression to catecholaminergic neurons. These TH-promoter mice are healthy and lack movement disorders (46). It is noteworthy that humans get PD symptoms only after an ~80% loss of nigrostriatal dopamine (47). a-Syn overexpression is ~2–3-fold greater than base-line a-Syn levels in non-transgenic (Non-Tg) control mice in the same genetic background and filial generations (F43–47). The a-Syn knock-out mice (ASKO) (3) were compared with their own Non-Tg controls in the 129 × C57BL/6 genetic background (F12-F15). All Syn++ mice and their controls were 6 months old, whereas ASKO mice for non-lentiviral analyses and their controls were 12 months old at the time of tissue harvest. For lentiviral transduction (detailed below), we delivered lentivirus using established methods (48) into substantia nigra of 2-month-old ASKO mice, and tissues were collected 7 days later. Mice were transduced with lentiviruses for GFP, WT-Syn, or a dephosphorylation mutant S129A a-Syn expression. Microdissected tissues were cryoprotected for immunostaining or flash-frozen, weighed, coded for blinded evaluation, and stored at −80 °C until use. Animals were handled according to National Institutes of Health guidelines on approved protocols at the Parkinson's Institute and the University of Pittsburgh.

Immunoblotting—Cells were harvested in 1% Nonidet P-40, 140 mM NaCl, 3 mM KCl, 25 mM Tris containing protease and phosphatase inhibitors, sonicated, and centrifuged as described below. Mouse tissues were sonicated in 10 volumes (w/v) of 1% Triton X-100, 0.2% SDS buffer containing 50 mM Tris, 150 mM NaCl, 0.02% NaN_3, 10 μg/ml leupeptin, 15 μg/ml aprotinin, 100 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride, and phosphatase inhibitor mixture (Pierce) at 4 °C. Samples were cleared by 14,000 × *g* 10-min centrifugations at 4 °C. Protein was measured by bicinchoninic acid (Pierce) or Bradford assay (Bio-Rad). Equal amounts of protein were separated by Tris-glycine-SDS-PAGE. Membranes, blocked in 10% milk, Tris-buffered saline, were incubated overnight at 4 °C in primary antibody. Antibodies included: a-Syn (Syn-1, 610786, BD Biosciences; C-20, sc-7011R, Santa Cruz Biotechnology); human a-Syn (LB509, Invitrogen); total TH (MAB318 mouse or AB151 rabbit, Chemicon, Temecula, CA); TH-Ser(P)-19 (AB5425, Chemicon); TH-Ser(P)-40 (AB5935; Chemicon); actin (A5441, Sigma); PP2A (1D6, Millipore). Signals from peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were visualized by chemiluminescence, and data, within a linear range, were quantified by ImageQuant as previously described (GE Healthcare) (9). Phosphorylated TH was always normalized to total TH, with β-actin serving as a loading control.

Immunohistochemistry—Brains were post-fixed in 4% formaldehyde and cryoprotected in phosphate-buffered saline sucrose at 4 °C as previously described (48). Cryomicrotome sections (30 μm) were stored in cryoprotectant at −20 °C until use. Tissues were permeabilized and blocked in a solution of phosphate-buffered saline, 5% bovine serum albumin, 10% goat serum, 0.1% glycine, and 0.05% Triton X-100 then incubated free-floating in primary antibody at 4 °C overnight. Antibodies included: chicken TH (TH1205, 1:100, Aves Labs, Tigard, OR), rabbit TH-Ser(P)-19 (AB5425, 1:1000, Chemicon), rabbit TH-Ser(P)-40 (AB5935, 1:1000, Chemicon), and sheep a-Syn (AB5334P, Chemicon, 1:500) as primary antibodies followed by Cy3 (Jackson ImmunoResearch), Cy5 (Jackson ImmunoResearch), or Alexafluor 488-, 546-, or 647-tagged secondary antibodies (Invitrogen). Tissues mounted in Vectashield (Vector Laboratories, Burlingame, CA) were analyzed by confocal microscopy (Olympus IX81) and digital image capture using FluoView™ 10 software (Olympus, Center Valley, PA). Settings were identical between conditions.

Lentiviral Transduction—Mice were anesthetized with Ketaset (80 mg/kg; NADA 45–290, Fort Dodge, IA) for bilateral delivery of a-Syn or GFP lentivirus to substantia nigra pars compacta. Mice, immobilized in a stereotaxic frame, were treated with lubricant eye drops (Refresh, Allergan, Irvine, CA), after which 0.5 μl of lentiviral solution was infused at a rate of 0.2 μl/min (Stoelting, Wood Dale, IL). Coordinates were anterior-posterior −3.08, medial-lateral ± 0.8 and 1.2, dorsal-ventral −4.2 and −4.0 relative to Bregma. Wounds, sutured with

surgical silk, were topically treated with 2.5% lidocaine/prilocaine (Fougera, Mellville, NY). Post-surgical pain was alleviated with ketoprofen (3.0 mg/kg; Spectrum Chemical MFG, Gardena, CA). Mice were sacrificed by CO_2 inhalation on University of Pittsburgh Institutional Biosafety Committee and Environmental Health and Safety approved protocols ($n = 24$; eight WT a-Syn, eight S129A a-Syn, eight GFP). All evaluations were made by experimenters blinded to the conditions.

In Vitro Phosphorylation of Recombinant a-Syn—Two μg of recombinant human a-Syn (kind gifts of Drs. A. Grigorescu and J. Rosenberg, University of Pittsburgh) were used for each reaction. a-Syn was phosphorylated by Polo-like kinase 2 (PLK2; Calbiochem) in pH 7.5 buffer containing 50 mM HEPES, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij® 35 detergent, 200 μM adenosine triphosphate (ATP), and 1 mg/ml soybean L-α-phosphatidylcholine (Sigma) at 30 °C for 1 h. For "unphosphorylated" samples, a-Syn was incubated in the same buffer but without added PLK2. Aliquots of the initial sample and samples after incubations were separated using 15% Tris-glycine SDS-PAGE and transferred to nitrocellulose for confirmation of a-Syn Ser-129 phosphorylation by probing with an antibody specific for phosphorylated serine 129 (11A5, 1:250, gift of Dr. J. Anderson, Elan Pharmaceuticals). From the remaining sample, a-Syn was immunoprecipitated with Syn-1 antibody (610786, BD-Transduction Laboratories) and 25 μl of protein G-Sepharose beads (Zymed Laboratories Inc., South San Francisco, CA) to eliminate PLK2 and ATP. Immunoprecipitation was confirmed using rabbit a-Syn antibody (C20, sc7011-R, 1:500) for immunoblots. The effect of phosphorylated and unphosphorylated a-Syn on recombinant human PP2A (Cayman Chemical, Ann Arbor, MI) was then measured as detailed below.

PP2A Assay in Tissues and Cells—Samples were sonicated in 20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA, pH 7.0, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride and cleared by centrifugation. Free phosphates were removed using Microspin™ G-25 columns (GE Healthcare), after which samples were diluted 1:8 in assay buffer and incubated 30 min at 4 °C followed by 10-min incubations at 30 °C with KRpTIRR threonine phosphopeptide (pT) substrate. Triplicate samples were assayed spectrophotometrically at 650 nm relative to known standards using the malachite green assay (17–127; Millipore/Upstate, Billerica, MA). Protein concentrations for each condition were confirmed by Bradford assay, and data were calculated as pmol/min/μg of protein. Specificity controls included 50 nM protein phosphatase inhibitor 2 (a PP1 inhibitor), 10 nM fostriecin (PP2A-specific inhibitor), or 50 nM okadaic acid.

PP2A Assay with Recombinant Proteins—Recombinant PP2A activity was measured in response to phosphorylated and unphosphorylated recombinant a-Syn. The a-Syn was immunoprecipitated from kinase buffers to eliminate ATP from the reactions. Samples were incubated for 90 min at 4 °C with shaking. Activity toward phosphopeptide was performed in duplicate as described above.

TH Activity Assay—Dihydroxyphenylalanine (DOPA) production, catalyzed by tissue TH, was measured by colorimetric assay (49) with minor modifications. Briefly, tissues were sonicated in 4–8 volumes of 50 mM Hepes buffer, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride at 4 °C. After centrifugation, 2 μl of supernatant was added to 0.5 ml assays containing 40 mM Hepes, 100 μM tyrosine, 100 μg/ml catalase, 10 μM ferrous ammonium sulfate, and 1 mM dithiothreitol, pH 7.0. The reaction was initiated by the addition of 400 μM tetrahydrobiopterin, BH4, and allowed to continue for 2 min at 30 °C. Reactions were halted by the addition of 20 μl of 4.8 M HCl and 200 μl of 12.5% sodium nitrite and 12.5% sodium molybdate in Milli-Q water at room temperature for 10 min. Samples were vortexed after the addition of 100 μl of 2.1 M NaOH and assayed at 490 and 800 nm by diode array spectrophotometry (Agilent Technologies, Santa Clara, CA). DOPA levels were determined relative to freshly prepared standards. For lentiviral studies, tissues were prepared exactly as above, but instead of 2 μl, we used 5 μl aliquots of supernatant/assay for all conditions. Protein concentrations, determined by Bradford assay, allowed the calculation of DOPA levels in nmol/min/μg protein from duplicate or triplicate samples for each condition.

Recombinant Proteins—Purification of recombinant wild type rat TH and the TH assay have been previously described (50). Otherwise for these assays we utilized the method described above for tissues and cells but with 0.3 μM recombinant TH for each reaction.

Reagents—Okadaic acid and fostriecin were from Calbiochem. All reagents not otherwise indicated were from Sigma or Fisher.

Statistics—ANOVA and Student's *t* tests were performed using Instat or Prism (GraphPad Software, San Diego, CA) with significance set at $p < 0.05$. Post-hoc analyses (Tukey-Kramer multiple comparisons) were performed after significance was confirmed. EC_{50} and efficacy values were calculated using SigmaPlot software (Systat, Chicago, IL). Data represent the means ± S.E. of the mean for experiments repeated 2–6 times on independent occasions.

RESULTS

Overexpression of WT or A53T Mutant a-Syn in MN9D Cells Reduces TH-Ser-19 Phosphorylation—The first aim of this study was to elucidate the effects of a-Syn on TH-Ser(P)-19, a site that contributes to 14-3-3 binding to TH. We measured the impact of a-Syn on TH-Ser(P)-19 in MN9D cells by comparing untransfected MN9D cells to cells stably overexpressing GFP, WT a-Syn, or A53T a-Syn. We find that a-Syn levels in our stably transfected MN9D cells are maintained at ~13-fold higher levels than endogenous a-Syn for cells utilized at low passage number (9) (see also Fig. 5*A*). Total TH levels remain similar; however, on occasion we noted less total TH in a-Syn-overexpressing MN9D cells. To control for such potential differences in TH, we normalized TH-Ser(P)-19 to total TH in all experiments. We saw diminished TH-Ser(P)-19 levels on immunoblots from WT and A53T a-Syn cells compared with control MN9D cells (Fig. 1*A*). Data from four independent experiments confirmed significantly less TH-Ser(P)-19 in WT-Syn and A53T-Syn cells compared with control untransfected MN9D or GFP-transfected control cells (Fig. 1*B*) ($p < 0.0001$, one-way ANOVA). We noted similar effects of transiently overexpressed a-Syn on TH-Ser(P)-19 in MN9D cells as well as

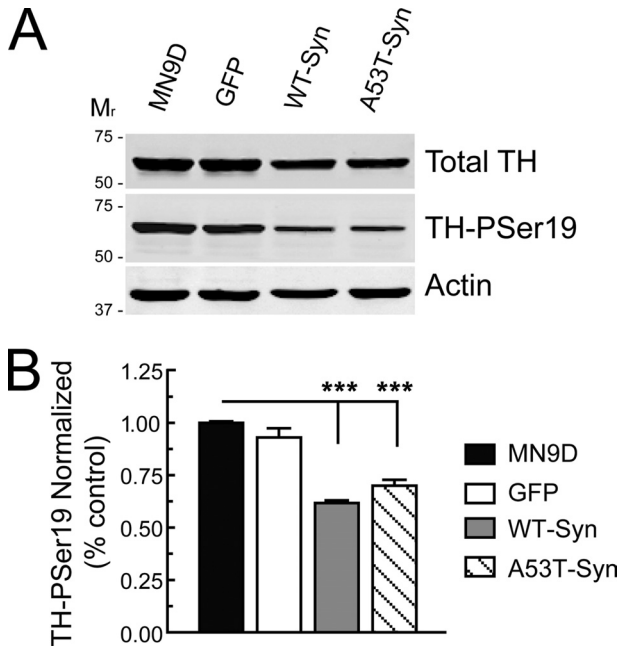

FIGURE 1. **Wild type and A53T mutant a-Syn-overexpressing MN9D cells have lower levels of TH phosphorylated at serine 19.** *A*, representative parallel immunoblots from untransfected MN9D cells, MN9D cells stably overexpressing GFP, wild type a-Syn (*WT-Syn*), or A53T mutant a-Syn (*A53T-Syn*) were evaluated for total-TH, TH-Ser(P)-19, and β-actin (*Actin*). Apparent molecular weights (M_r) as determined from prestained standards are shown on the *left*. *B*, quantitative analysis of Ser(P)-19 TH levels normalized to total TH reveal significantly less phosphorylation of Ser-19 on TH in WT-Syn and A53T-Syn MN9D cells compared with controls. β-Actin is a loading control. The histogram represents the mean ± S.E. of data from four independent experiments. **, $p < 0.01$; ***, $p < 0.001$, one-way ANOVA.

in PC12 cells in which a-Syn overexpression is inducible (not shown), confirming an association between a-Syn and TH that reduces TH-Ser(P)-19 levels. We previously measured TH activity in these cells and note that WT and A53T a-Syn cells, which have low TH-Ser(P)-19 levels in this study, also have lower TH activity, whereas control cells have more TH activity (9), identifying a contribution of TH-Ser(P)-19 to TH activity in MN9D cells.

PP2A Dephosphorylates TH-Ser(P)-19 in MN9D Cells—As previously mentioned, PP2A is the main TH phosphatase. To assess if PP2A dephosphorylates TH-Ser(P)-19 in our cells, we first treated untransfected MN9D cells with okadaic acid and measured Ser(P)-19 levels by immunoblot. We saw a dose-dependent increase in TH-Ser(P)-19 levels in MN9D cells after okadaic acid (1.0–1000 nM) with a significant increase noted at 10 nM okadaic acid as compared with vehicle-treated control cells (Fig. 2*A*; $p < 0.05$, one-way ANOVA). Because maximal effects were apparent at 1000 nM, a concentration known to affect other phosphatases, we also used fostriecin (0.1–10 nM), a PP2A-specific inhibitor (51). Fostriecin produced a similar increase in Ser(P)-19 levels on TH, confirming the role of PP2A in TH-Ser(P)-19 dephosphorylation in MN9D cells (Fig. 2*B*; $p < 0.05$, one-way ANOVA).

To verify this by non-pharmacological means, we transiently overexpressed the wild type PP2A catalytic domain (PP2A-WT) (52) in MN9D cells and then measured TH-Ser(P)-19 in comparison to mock-transfected MN9D cells. Overexpression of PP2A-WT did not alter total TH levels; however, TH-Ser(P)-19 levels were measurably reduced (Fig. 2*C*). Data from multiple experiments confirmed significantly lower TH-Ser(P)-19 levels in PP2A-WT cells compared with mock-transfected controls, directly demonstrating a role for PP2A in TH-Ser(P)-19 dephosphorylation in dopaminergic MN9D cells (Fig. 2*C*; $p < 0.01$, Student's *t* test).

We next explored the impact of PP2A inhibition in WT a-Syn-overexpressing MN9D cells and saw a pronounced increase in TH-Ser(P)-19 levels. This increase was apparent at 1.0 μM okadaic acid (Fig. 2*D*, TH-Ser(P)-19 blots) and also in cells treated with the PP2A-specific inhibitor fostriecin (not shown). Data from repeated okadaic acid experiments confirmed significantly higher TH-Ser(P)-19 levels in WT-Syn cells compared with control MN9D cells (Fig. 2*D*; $p < 0.001$, Student's *t* test), findings that firmly support the role of PP2A in TH-Ser-19 dephosphorylation in MN9D cells.

As an additional control, we also evaluated phosphorylation of the extracellular signal-regulated kinase (ERK) in a-Syn MN9D cells, because ERK is a PP2A substrate (53, 54) that also regulates TH (55, 56) and because others have shown that ERK activity is inhibited by a-Syn overexpression (57). ERK phosphorylation was diminished in a-Syn cells compared with GFP-transfected MN9D controls, and furthermore, inhibition of PP2A increased ERK phosphorylation in our cells (data not shown), suggesting that only a-Syn interacting proteins were affected by a-Syn-mediated PP2A activation. This regulation further appears to be selective, as another major PP2A target, the protein kinase B/Akt (58, 59), that does not bind a-Syn retains normal levels of phosphorylation in our a-Syn-overexpressing cells (44), suggesting the existence of a microdomain(s) where a-Syn localizes with PP2A and dopamine regulatory proteins (9, 31).

a-Syn Overexpression in TH-promoter Mice Reduces TH Phosphorylation in Dopaminergic Tissues—The second goal of the study was to determine the *in vivo* relevance of a-Syn-mediated TH modulation using a-Syn-overexpressing and null transgenic mice. The TH in striatum is localized in the axon termini of nigrostriatal dopaminergic neurons (60), where it co-localizes extensively with a-Syn (61). To evaluate if *in vivo* overexpression of a-Syn altered TH phosphorylation, we compared striatal immunoblots from homozygous WT a-Syn-overexpressing TH-promoter mice (WT-Syn++) to non-transgenic control mice (Non-Tg) in the same genetic background in which total a-Syn overexpression is ~2.5–5 times above levels noted in Non-Tg controls (46). Interestingly, aging primates have an ~2–3-fold increase in a-Syn levels in nigral neurons (62). In mouse striatum probed with a human-specific a-Syn antibody LB509, human a-Syn protein was measurable only in WT-Syn++ mice (Fig. 3*A*; $p < 0.001$, Student's *t* test). Total TH levels were comparable in all mice; however, TH-Ser(P)-19 levels were significantly lower in WT-Syn++ mice compared with Non-Tg controls (Fig. 3*A*; $p < 0.01$, Student's *t* test). It has been demonstrated that TH activation requires TH-Ser-40 phosphorylation; thus, we also measured Ser(P)-40 levels in mouse striatum. We saw significantly less TH-Ser(P)-40 in WT-Syn++ mice compared with Non-Tg controls (Fig. 3*A*; $p < 0.01$, Student's *t* test). When we assessed if higher levels of a-Syn expression correlated strongly with lower TH phosphor-

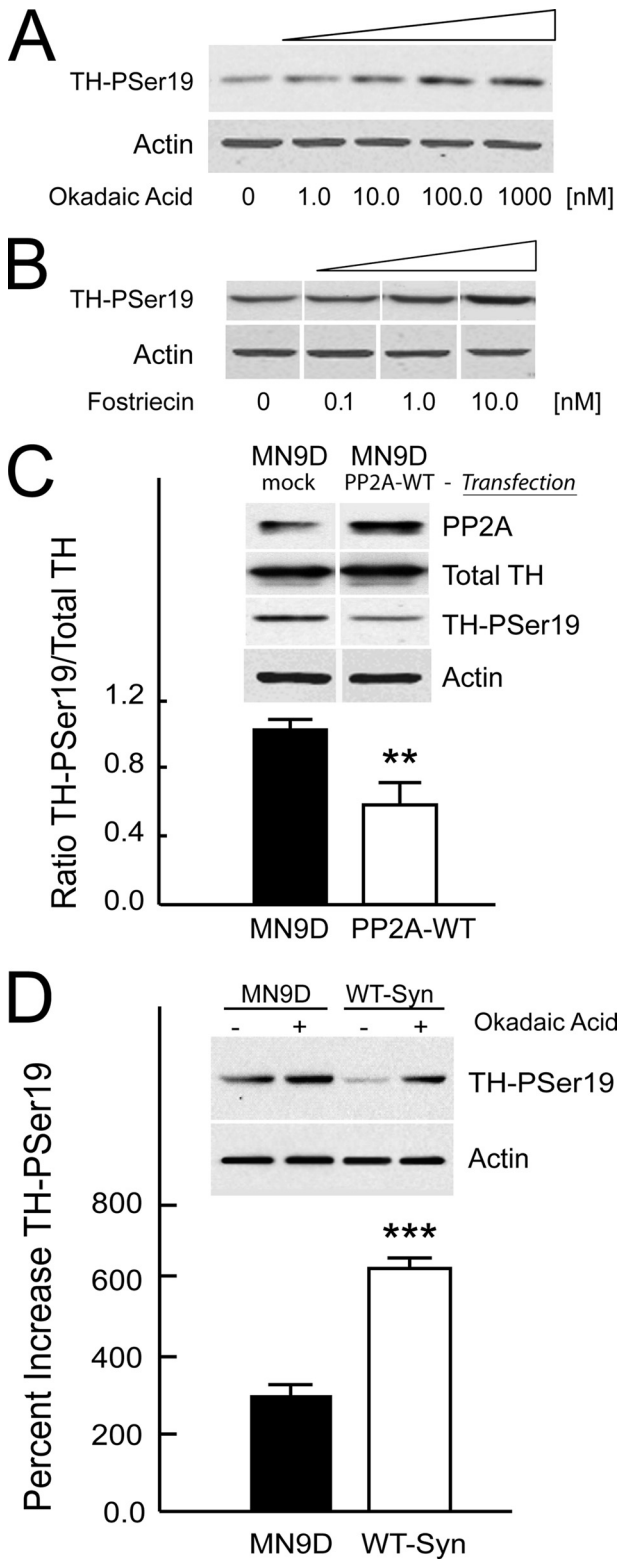

FIGURE 2. **PP2A dephosphorylates TH-Ser(P)-19 in MN9D cells.** Representative immunoblots show TH-Ser(P)-19 levels in response to pharmacological or molecular manipulations to assess PP2A contribution to Ser(P)-19 dephosphorylation. *A*, there was a dose-dependent increase in TH-Ser(P)-19 levels in untransfected MN9D cells treated for 1 h with different concentrations of okadaic acid. *B*, fostriecin, a PP2A-specific inhibitor, produced a similar dose-dependent increase in TH-Ser(P)-19 levels. *C*, transient overexpression of the wild type PP2A (*PP2A-WT*) catalytic subunit in MN9D cells increased PP2A levels and significantly decreased TH-Ser(P)-19 without affecting total TH levels. The histogram represents the mean ± S.E. of data from three independent experiments. *D*, MN9D cells and a-Syn-overexpressing MN9D cells (WT-Syn) were treated with 1.0 μM okadaic acid for 1 h, which produced a significantly larger increase in TH-Ser(P)-19 in WT-Syn cells, which have lower base-line levels of TH-Ser(P)-19 than do controls. β-Actin is a loading control. The histogram represents the mean ± S.E. from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$, Student's *t* tests.

ylation in individual WT-Syn++ mice, we found a significant negative correlation. Whenever a-Syn levels were higher, TH-Ser(P)-19 levels were concomitantly lower ($R^2 = 0.81$, $n = 5$, $p < 0.01$) as were TH-Ser(P)-40 levels ($R^2 = 0.78$, $n = 5$, $p < 0.01$). These data identify a close relationship between a-Syn levels and TH phosphorylation *in vivo*, which prompted us to measure for changes in TH activity.

a-Syn Overexpression in TH-promoter Mice Reduces TH Activity in Dopaminergic Tissues—To determine whether reduced TH phosphorylation reflected changes in TH enzymatic activity in striatum, we evaluated 15 mice per condition using a well characterized TH activity assay with minor modifications. This assay measures TH activity, whereby increased DOPA levels indicate more active TH (17, 63, 64). Consistent with the finding of less TH phosphorylation in WT-Syn++ striatum, there was significantly lower TH activity in striatum from WT-Syn++ mice compared with Non-Tg controls (Fig. 3*B*, $p < 0.001$, Student's *t* test). Together these findings strongly suggest that overexpression of a-Syn in TH-promoter mouse dopaminergic tissues reduced TH phosphorylation at TH-Ser(P)-19 and TH-Ser(P)-40, resulting in lower TH activity. Because we previously established that a-Syn overexpression reduces TH-Ser(P)-40 in MN9D cells (43) and reconfirmed this same effect here for wild type a-Syn-overexpressing mice, the data for the remainder of the manuscript will focus entirely on our novel findings related to TH-Ser(P)-19.

a-Syn Overexpression in TH-promoter Mice Reduces TH-Ser-19 Phosphorylation in Substantia Nigra Neuronal Cell Bodies—Although it is well established that a-Syn localizes presynaptically (1, 65), it has never been evaluated whether somal a-Syn in substantia nigra neuronal cell bodies may similarly impact TH phosphorylation. We assessed this by confocal microscopy using WT-Syn++ and Non-Tg substantia nigra immunostained with antibodies specific for human a-Syn, total TH, and TH-Ser(P)-19. As expected, human a-Syn was present only in WT-Syn++ substantia nigra somata and absent in Non-Tg controls (Fig. 3*C*, *left*). Total TH immunoreactivity was quite similar in WT-Syn++ mice and Non-Tg control nigral neurons (Fig. 3*C*, *second panel from the left*); however, TH-Ser(P)-19 was much lower in those neurons in WT-Syn++ mice compared with Non-Tg controls (Fig. 3*C*, *third panel from the left*). To further assess TH-Ser(P)-19 levels, we generated signal intensity plots from microscopic fields containing equivalent numbers of TH-positive neurons, which demonstrated lower TH-Ser(P)-19 signal in WT-Syn++ nigral neuronal somata compared with controls (Fig. 3*C*, *right*). Evaluation of eight microscopic fields per mouse from three mice per condition confirmed comparable total TH in substantia nigra of all mice (Non-Tg, 246 ± 4; WT-Syn++, 247 ± 4; $t = 0.126$, $p = 0.89$, Student's *t* test) but significantly lower TH-Ser(P)-19 in WT-Syn++ compared with Non-Tg substantia nigra cell bod-

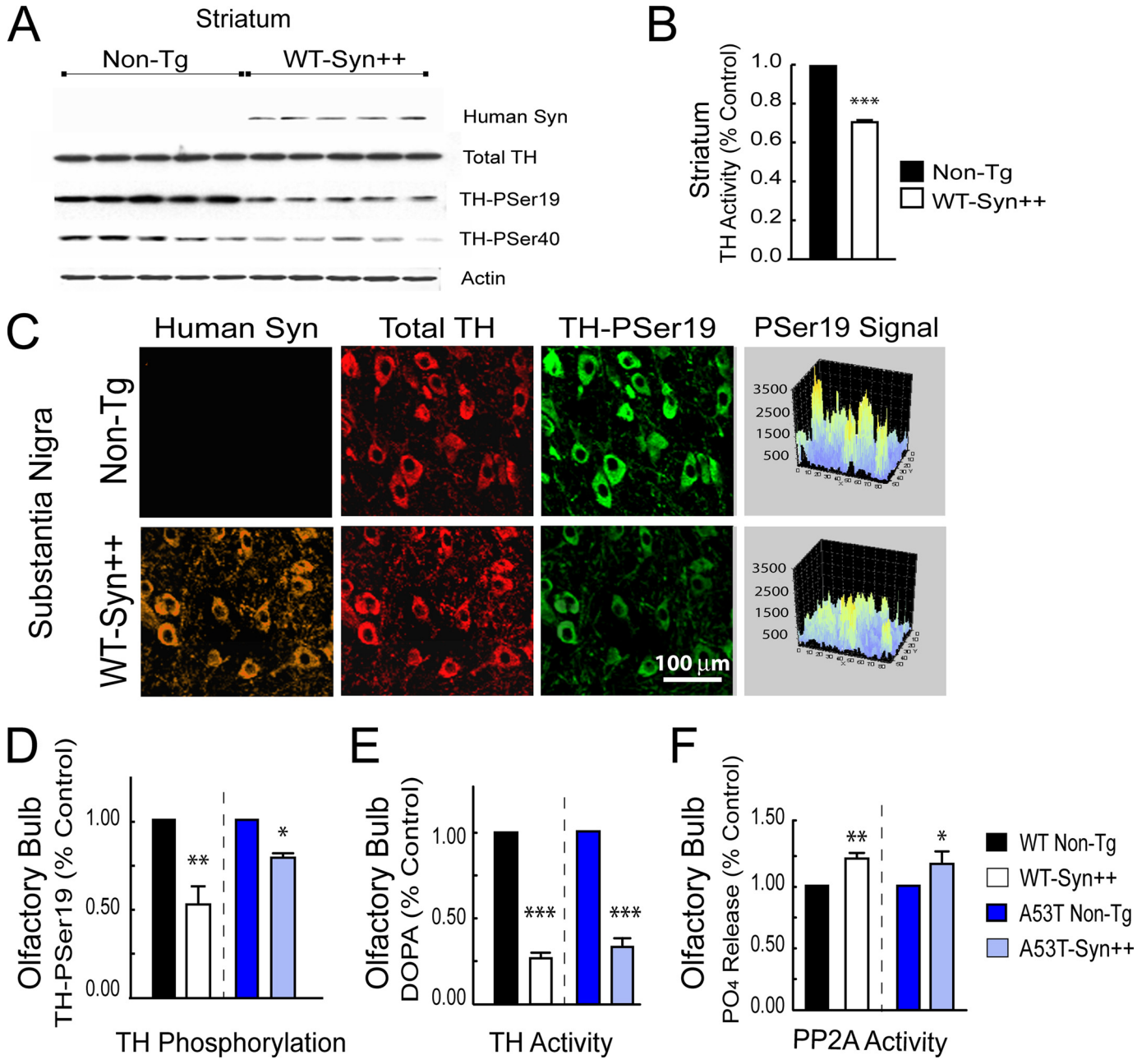

FIGURE 3. **a-Syn overexpression in TH-promoter transgenic mice reduces TH phosphorylation and activity but stimulates PP2A activity in PD relevant dopaminergic tissues.** *A*, representative immunoblots from Non-Tg controls and WT-Syn++ mouse striatal extracts reacted for human a-Syn, total TH, TH-Ser(P)-19, TH-Ser(P)-40, and β-actin from 10 μg of total protein per lane. Total TH and actin levels are similar, but TH-Ser(P)-19 and TH-Ser(P)-40 levels are reduced in WT-Syn++ mouse. *B*, TH activity, measured by the conversion of tyrosine to DOPA from striatal tissues, is significantly lower in WT-Syn++ mice compared with Non-Tg controls. *C*, immunostaining of substantia nigra pars compacta dopaminergic neurons revealed significantly less TH-Ser(P)-19 signal in WT-Syn++ mice compared with Non-Tg controls, as demonstrated in signal intensity plots from microscopic fields having equal numbers of TH+ neurons. *D*, histograms of quantitative immunoblot data from olfactory bulbs from age-matched Non-Tg control mice, WT-Syn++ mice, and A53T-Syn++ mice reveals significantly reduced levels of TH-Ser(P)-19 in a-Syn-overexpressing mice compared with controls. *E*, both WT-Syn++ and A53T-Syn++ mice had reduced TH activity in olfactory bulbs compared with their own age-matched controls. *F*, histogram of PP2A activity data from olfactory bulbs of WT-Syn++ mice and A53T-Syn++ mice reveals significantly higher PP2A activity in a-Syn-overexpressing mice compared with their age-matched Non-Tg controls. In *B*, *D*, *E*, and *F*, data represent the mean ± S.E. of 15 mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, all comparisons are by Student's *t* test. *Size bar*, 100 μm.

ies (Non-Tg, 2547 ± 4; WT-Syn++, 2194 ± 5; $t = 52$, $p < 0.0001$, Student's *t* test). Importantly, all samples had equivalent low background levels of immunoreactivity (Non-Tg, 192 ± 11.4; WT-Syn++, 200 ± 6; $t = 0.6$, $p = 0.57$, Student's *t* test). These findings parallel our data from MN9D cells (Fig. 1) and mouse striatum (Fig. 3*A*), identifying a previously undescribed effect of a-Syn on TH in nigral neuronal cell bodies *in vivo*.

Wild Type and A53T Mutant a-Syn Inhibit TH and Stimulate PP2A in Mouse Dopaminergic Tissues, a Finding Reversed in a-Syn Null Mice—To determine whether a-Syn affects TH in other dopaminergic tissues, we evaluated olfactory bulbs from the same 30 mice described above. An important reason to assess the olfactory bulb is that early-onset olfactory deficits occur in most clinical cases of PD, rendering the studies highly relevant (66, 67). Furthermore, we previously showed that peri-

glomerular neurons in the olfactory bulb contain abundant TH that is subject to a-Syn-mediated regulation (48). We confirmed equivalent total TH in olfactory bulbs from WT-Syn++ and Non-Tg mice by immunoblot using actin as a loading control (not shown) and found that TH-Ser(P)-19 levels were reduced in WT-Syn++ mouse olfactory bulbs compared with Non-Tg controls (Fig. 3*D*, *left side*; $p < 0.01$, Student's *t* test). As expected, for TH that is more highly phosphorylated, Non-Tg TH was also more active than WT-Syn++ TH from olfactory bulb (Fig. 3*E*, *left side*; $p < 0.01$, Student's *t* test). PP2A is widely expressed in all cells of olfactory bulb, whereas TH is expressed only in periglomerular dopaminergic neurons, which make up less than 10% of all olfactory cells. Yet despite a-Syn being overexpressed only in this small group of cells, we could measure a significant increase in PP2A activity in WT-Syn++ mice compared with Non-Tg control olfactory bulbs (Fig. 3*F*, *left side*; $p < 0.01$, Student's *t* test). The size of the effect was likely diluted by the presence of many more non-TH cells in the olfactory bulb than a-Syn-overexpressing cells. In control experiments using the PP2A-specific inhibitor fostriecin (10 nM), low dose okadaic acid (50 nM), or the protein phosphatase 1 (PP1)-specific inhibitor protein phosphatase inhibitor-2 (PPI2, 50 nM), we verified that fostriecin and low dose okadaic acid could both inhibit phosphatase activity in our assay, whereas the PP1 inhibitor did not (not shown). These data identify PP2A as the major TH phosphatase in mouse olfactory bulb. As a negative control, we evaluated cerebellum from WT-Syn++ and Non-Tg mice in which immunoblots showed that TH and PP2A levels were identical, and human a-Syn was undetected (not shown). TH activity was not measurable in cerebellar homogenates, and PP2A activity in WT-Syn++ and Non-Tg cerebellum was not significantly different (WT-Syn++, 0.92 ± 0.07 pmol of PO_4/min/μg of protein; Non-Tg, 0.83 ± 0.09 pmol of PO_4/min/μg of protein; Student's *t* test, $p = 0.67$).

Our earlier work using stably transfected MN9D cells and inducible PC12 cells demonstrated that overexpression of WT a-Syn activates PP2A (43); however, it is not known if A53T mutant a-Syn might have a similar effect on PP2A activity or, if so, whether it also occurs *in vivo*. To test for this we assessed the impact of human A53T a-Syn on TH and PP2A in olfactory bulbs from A53T mutant TH-promoter mice (A53T-Syn++) and their age matched Non-Tg controls (46). Immunoblots with equal protein loading had equivalent levels of TH and PP2A in A53T and control mice (not shown). Much like we found in WT-Syn++ mice above, the A53T-Syn++ mice also had lower TH-Ser(P)-19 levels compared with Non-Tg controls (Fig. 3*D*, *right side*; $p < 0.01$, Student's *t* test) as well as significantly lower TH enzymatic activity (Fig. 3*E*, *right side*; $p < 0.001$, Student's *t* test) and significantly higher PP2A activity (Fig. 3*F*, *right side*; $p < 0.05$, Student's *t* test). As noted for WT-Syn++ TH-promoter mice, the effect of a-Syn on PP2A in A53T-Syn++ olfactory bulb appeared to be diluted by a-Syn being overexpressed in such a small pool of neurons in that brain region. These data provide the first evidence that both WT a-Syn and A53T mutant a-Syn regulate TH and PP2A similarly *in vitro* and *in vivo*.

Based on the above findings, we surmised that if a-Syn inhibits TH and stimulates PP2A in dopaminergic neurons *in vivo*, then the absence of a-Syn in ASKO mice would produce effects opposite those of a-Syn overexpression. This possibility is also supported by findings by others in which RNAi-mediated silencing of endogenous a-Syn led to increased levels of TH phosphorylation, TH activity, and dopamine in MN9D cells (8). To assess the impact of a-Syn loss *in vivo*, we used striatum from 12-month-old ASKO mice and age-matched Non-Tg controls in which we measured TH phosphorylation, TH activity, and PP2A activity. We confirmed the absence of a-Syn in ASKO mice and noted equivalent total TH and PP2A levels in ASKO and Non-Tg controls on immunoblots with equal protein loading (not shown). When we assessed TH-Ser(P)-19 by immunoblot, ASKO mice had significantly higher TH-Ser(P)-19 levels than Non-Tg controls (Fig. 4*A*; $p < 0.01$, Student's *t* test). TH activity in tissue homogenates from ASKO mice was also significantly elevated (Fig. 4*B*, *left side*; $p < 0.05$, Student's *t* test), whereas PP2A activity was significantly lower compared with Non-Tg controls (Fig. 4*B*, *right side*; $p < 0.01$, Student's *t* test). Taken together, the mouse data provide compelling support for a physiological contribution of a-Syn to the regulation of TH and PP2A *in vivo*. To assess the potential contribution of a-Syn phosphorylation on TH and PP2A activity in a physiological model, we next evaluated MN9D cells after transient transfection with wild type or Ser-129 mutant a-Syn.

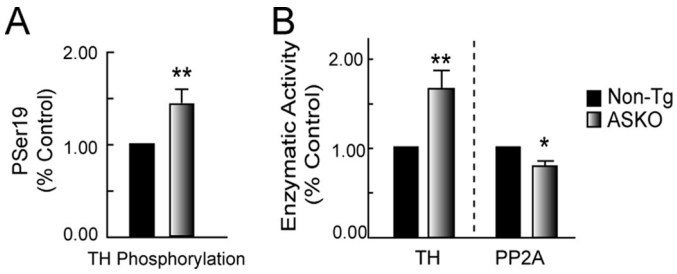

FIGURE 4. **a-Syn knock-out mouse striatum has significantly higher levels of TH-Ser(P)-19 and TH activity and reduced PP2A activity.** *A*, a histogram of immunoblot data from ASKO and Non-Tg control mice reveals significantly higher levels of TH-Ser(P)-19 in ASKO striatum compared with Non-Tg background strain control mice. *B*, TH activity was also significantly higher, and PP2A activity was significantly lower in ASKO striatum compared with striatum from Non-Tg controls. $n = 28$, 12 months of age; 14 mice/condition. *, $p < 0.05$; **, $p < 0.01$, Student's *t* test.

Overexpression of WT or Dephosphorylation Mutant a-Syn, S129A, Stimulates PP2A Activity and Reduces TH Phosphorylation in MN9D Cells, with a Bigger Effect of S129A—Although Ser(P)-129 a-Syn is strongly implicated in PD pathogenesis, no one has evaluated if Ser-129 phosphorylation on a-Syn affects a-Syn function. Therefore, the third aim of this study was to assess if phosphorylation of a-Syn, in particular of Ser-129, affected PP2A or TH activity *in vitro* or *in vivo*. In this first series of experiments we demonstrated Ser(P)-129 levels in untransfected, GFP-transfected, and MN9D cells stably transfected with A53T a-Syn or WT a-Syn. Immunoblots show Ser(P)-129 in all cells with higher levels noted for cells that overexpressed a-Syn, confirming Ser-129 phosphorylation in MN9D cells at base line (Fig. 5*A*). To measure the impact of Ser(P)-129 a-Syn on PP2A and TH, we transiently overexpressed WT-Syn or S129A-Syn mutant a-Syn, with alanine substituted for serine at amino acid 129. This mutation has been widely used to evaluate the impact of dephosphorylated a-Syn

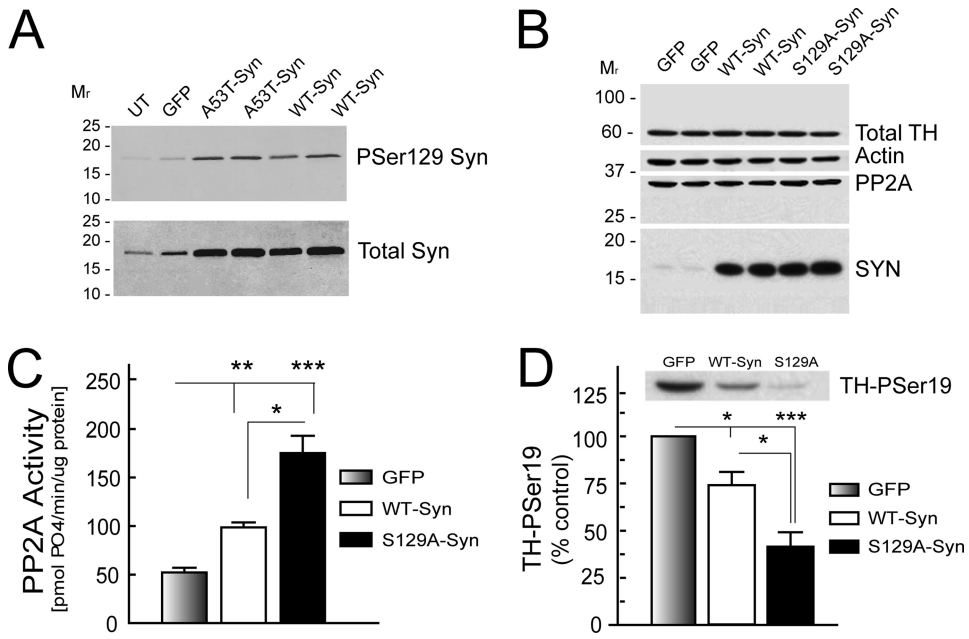

FIGURE 5. **a-Syn is phosphorylated on Ser-129 in MN9D cells, and cells overexpressing the S129A a-Syn dephosphorylation mimic have more potent PP2A activation and TH dephosphorylation than WT a-Syn or GFP control cells.** *A*, representative immunoblots demonstrating the relative amount of a-Syn Ser(P)-129 (*upper*, probed with 11A5 monoclonal antibody) and total a-Syn (*lower*, probed with sc-7011R polyclonal antibody) in untransfected MN9D cells (*UT*), MN9D cells stably transfected with GFP, and clonal lines overexpressing A53T mutant a-Syn (*A53T-Syn*) and wild type a-Syn (*WT-Syn*); 80 μg of total protein per lane. *B*, representative immunoblots from transiently transfected MN9D cells overexpressing GFP, WT a-Syn, or S129A a-Syn are shown. Total TH, PP2A, and β-actin levels are similar, and a-Syn levels are increased only in WT-Syn- and S129A-transfected cells compared with GFP-transfected control MN9D cells. Molecular weights (M_r) determined from prestained standards are shown on the *left*. *C*, MN9D cells transiently transfected with GFP (*gray*), WT a-Syn (WT-Syn, *white bar*), or S129A a-Syn (S129A-Syn, *black bar*) revealed that both WT a-Syn and S129A a-Syn cells had higher levels of PP2A activity compared with GFP-transfected MN9D cells. *D*, phosphorylated TH-Ser(P)-19 levels are significantly lower in WT-Syn and S129A-Syn-transfected cells compared with GFP-transfected control MN9D cells. A histogram of data from three independent experiments represents the mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, one-way ANOVA.

Ser-129 in both cellular and animal models (68–71). In our cells transfected with GFP, WT-Syn, or S129A-Syn for 48–72 h we saw equivalent transfection efficiency and cellular morphology (not shown). Immunoblots using actin as a loading control confirmed equivalent PP2A and total TH in all cells, with high a-Syn levels noted only in a-Syn-transfected cells, as expected (Fig. 5*B*). When we measured PP2A activity in MN9D cells, we found significantly elevated PP2A activity in both WT-Syn and S129A-Syn cells compared with GFP-transfected controls, with the largest activation of PP2A noted for S129A-Syn dephosphorylation mimic cells (Fig. 5*C*; $p < 0.0001$, one-way ANOVA). Regarding TH, we noted that TH-Ser(P)-19 levels were significantly lower in both WT-Syn and S129A-Syn cells compared with GFP-transfected cells, with a greater reduction noted in S129A-Syn cells (Fig. 5*D*; $p < 0.001$, one-way ANOVA). Together the data strongly suggest that Ser-129 phosphorylation modulates a-Syn ability to stimulate PP2A activity in MN9D cells. This interpretation is also supported by the finding of the much more diminished TH-Ser(P)-19 levels in S129A-Syn MN9D cells compared with GFP or WT-Syn MN9D cells, findings that prompted us to evaluate the impact of S129A-Syn and WT-Syn *in vivo* as detailed below.

S129A a-Syn Dephosphorylation Mutant Inhibits TH and Potentiates PP2A in Substantia Nigra of ASKO Mice Transduced with a-Syn Lentivirus—Using WT-Syn lentivirus in ASKO mice, we previously showed that TH phosphorylation is reduced when soluble a-Syn levels increase in dopaminergic neurons (48). Here we assessed the impact of a-Syn phosphorylation on TH and PP2A *in vivo*, by transducing substantia nigra of ASKO mice with GFP, WT-Syn, or S129A-Syn lentivirus. Brains were collected 7 days after stereotaxic delivery of the virus and evaluated by immunohistochemistry and biochemistry. Low magnification images near the site of the needle tract of lentivirally transduced ASKO mice revealed widespread expression of GFP or a-Syn around and within substantia nigra pars compacta (SNc) of mice transduced with GFP, WT-Syn, or S129A-Syn lentivirus (Fig. 6, *A* and *B*, *left panels*). FluoView™ assessment of the green signal in GFP-, WT-Syn-, or S129A-Syn-transduced mice demonstrated similar signal intensities in all mice (Fig. 6*B*, *second panels*). TH immunoreactivity, as denoted by red fluorescence, was also similar in all mice (Fig. 6*A*, *middle panels*, *B*, *third panels*). As expected for lentiviral transduction, non-TH neurons of ventral midbrain were also transduced by GFP and a-Syn virus, as can be appreciated in the merged images where non-TH cells still appear green (Figs. 6, *A* and *B*, *right panels*), whereas TH neurons that were transduced with GFP or a-Syn appear yellow due to red/green double fluorescent signal. To assess the impact of transduction on TH-Ser(P)-19, we also compared Ser(P)-19 immunoreactivity in GFP, WT-Syn, and S129A-Syn mice. Ventral midbrain from GFP-transduced mice had strong TH-Ser(P)-19 immunoreactivity, but WT-Syn or S129A-Syn mice had much lower TH-Ser(P)-19 in SNc dopaminergic neurons (Fig. 6*B*, *fourth panels*). FluoView™ assessment of the pseudocolored Ser(P)-19 Cy5 signal (*orange*) confirmed significantly greater TH-Ser(P)-19 in GFP-transduced TH neurons (788 ± 34 pixels) compared with WT-Syn (404 ± 29 pixels)- or S129A-Syn-transduced SNc TH neurons (371 ± 18.5 pixels) ($p < 0.0001$, one-way ANOVA) (Fig. 6*B*, *fifth panel*). This reduction in TH-Ser(P)-19 in WT-Syn- and S129A-Syn-transduced SNc suggested that TH activity might also be reduced in a-Syn lentivirally transduced mice.

To assess for this we collected ventral midbrain at 7 days post-transduction and prepared tissue homogenates for immunoblots and enzymatic assays. Equivalent PP2A and TH protein levels were seen on immunoblots from all mice (not shown); however, significantly less TH activity was noted for mice transduced with WT-Syn or S129A compared with GFP lentiviral control mice, with a larger decrease in TH activity noted for S129A-Syn mice (Fig. 6*C*; $p < 0.001$, one-way ANOVA). Con-

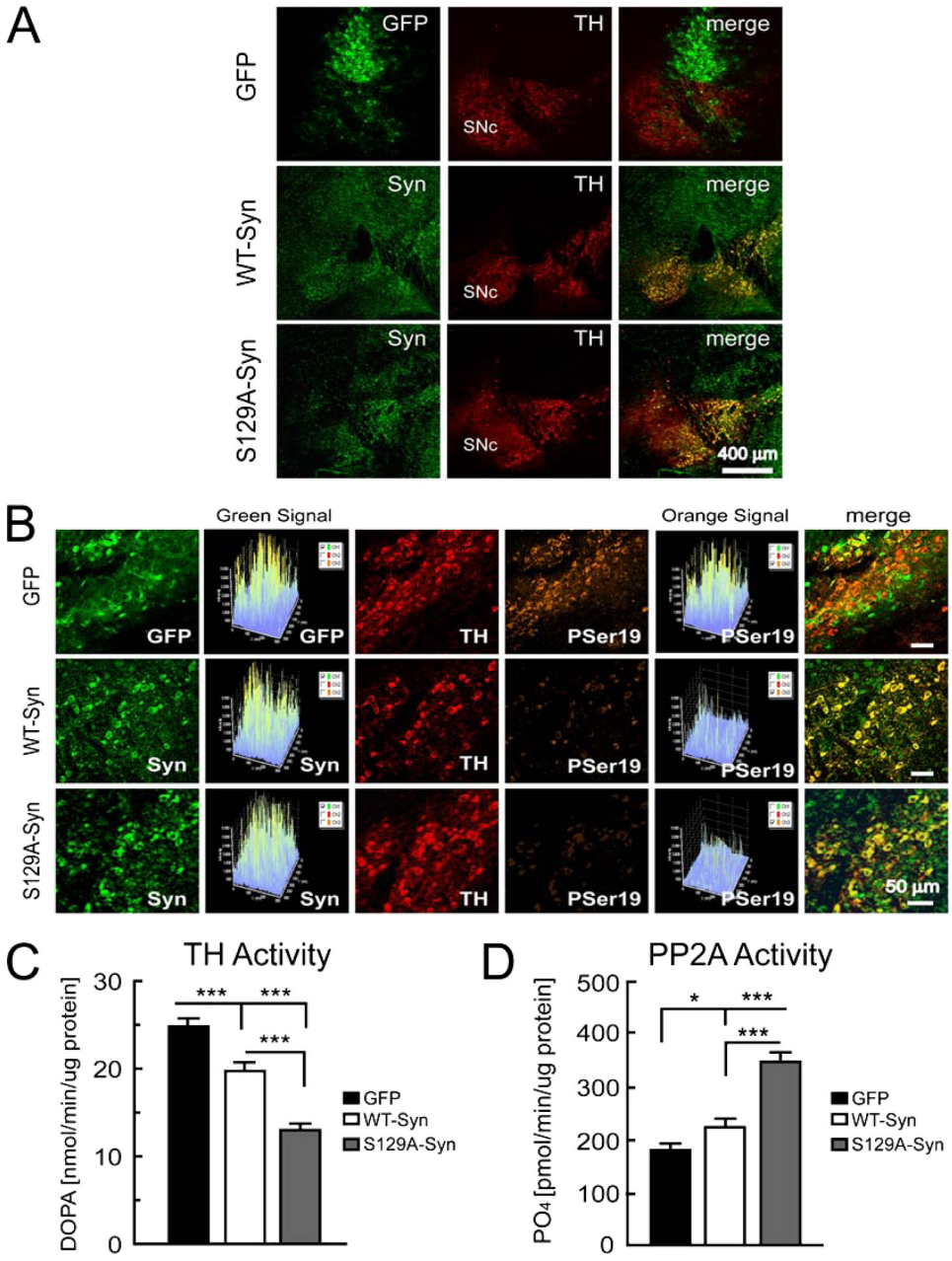

FIGURE 6. **Lentiviral transduction of ventral midbrain neurons reveals a-Syn effects on TH and PP2A *in vivo*, with the S129A-Syn mutant, which mimics dephosphorylated a-Syn, producing a larger effect.** Tissues were collected 7 days post-stereotaxic delivery of GFP, WT-a-Syn (WT-Syn), or S129A a-Syn (S129A-Syn) lentivirus into ASKO ventral midbrain substantia nigra. *A*, representative para sagittal sections of ventral midbrain of lentivirally transduced ASKO mice were immunostained for a-Syn (*green*) and TH (*red*). Low magnification images near the site of the needle tract confirm widespread GFP or a-Syn expression around and within *SNc* of mice transduced with GFP, WT-Syn, or S129A-Syn lentivirus. *B*, higher magnification images of SNc immunostained for a-Syn, total TH, and TH-Ser(P)-19 in ASKO mice transduced with GFP, WT-Syn, or S129A-Syn lentivirus are shown. No a-Syn immunoreactivity is apparent in SNc of GFP mice, whereas a-Syn signals are similar in WT-Syn and S129A-Syn-transduced mice, as can be appreciated from signal intensity plots of microscopic fields having equal numbers of TH+ neurons. Total TH immunoreactivity is similar in all mice; however, TH-Ser(P)-19 signal is reduced in WT-Syn and S129A-Syn-transduced mice SNc as also demonstrated in signal intensity plots from microscopic fields with equal numbers of TH+ neurons. *C*, TH activity was significantly inhibited in WT-Syn and S129A-Syn-transduced mice compared with GFP controls, with S129A-Syn producing a more pronounced effect. *D*, PP2A activity was significantly elevated in WT-Syn-transduced mice but even more so in S129A-Syn a-Syn dephosphorylation mimic-transduced mice compared with GFP controls. Histograms represent the mean ± S.E. of data from duplicate or triplicate samples from 3- 6 independent experiments. * $p < 0.05$; *** $p < 0.001$, one way ANOVA. *Scale bar*: *A*, 400 μm; *B*, 50 μm.

versely, activity of PP2A was significantly elevated in ventral midbrain of WT-Syn- and S129A-Syn transduced mice compared with GFP lentivirus controls (Fig. 6*D*; $p < 0.001$, one-way ANOVA), with S129A again producing a bigger effect, much like our data from MN9D cells (Fig. 5). Together these data imply that when a-Syn is dephosphorylated on Ser-129, the molecule becomes a more potent regulator of TH and PP2A than when Ser-129 on a-Syn is phosphorylated. To directly measure the impact of Ser-129 phosphorylation of a-Syn on PP2A and TH, we used recombinant proteins and cell-free assays.

Phosphorylation of a-Syn Ser-129 in a Cell-free System Attenuates a-Syn Modulatory Effects on PP2A and TH—Because the above experiments were performed using S129A dephosphorylation mutant, this series of experiments was performed to directly compare unphosphorylated a-Syn to a-Syn phosphorylated on Ser-129 for potential differences in PP2A activity. Samples were treated identically except for the presence or absence of PLK2 in reaction buffers. We confirmed equivalent a-Syn in each reaction and verified Ser-129 phosphorylation by immunoblots using an antibody specific for a-Syn Ser(P)-129 (Fig. 7*A*). We next compared Ser(P)-129 a-Syn *versus* unphosphorylated a-Syn on PP2A activity in a cell-free assay (detailed under "Experimental Procedures").

PP2A does not dephosphorylate a-Syn Ser-129 (72), and we confirmed this in our experiments where background signal-only was noted for both PLK2+- and PLK2- a-Syn-treated samples (Fig. 7*B*, *left*). Immunoblots also revealed a persistent Ser(P)-129 signal on PLK2+ a-Syn after PP2A incubation (not shown, but similar to Ser(P)-129 blot in Fig. 7*A*). In experiments utilizing a phosphopeptide substrate (pT), PP2A efficiently cleaved phosphate from pT (362 ± 108 pmol/min/μg of protein), and both phosphorylated and unphosphorylated a-Syn enhanced PP2A activity; however, unphosphorylated a-Syn activated PP2A more (1421 ± 73 pmol/min/μg of protein) than Ser(P)-129 a-Syn (839 ± 61 pmol/min/μg of protein) (Fig. 7*B*, *right side*; $p < 0.0001$, one-way ANOVA). When we performed a dose response, the EC_{50} was

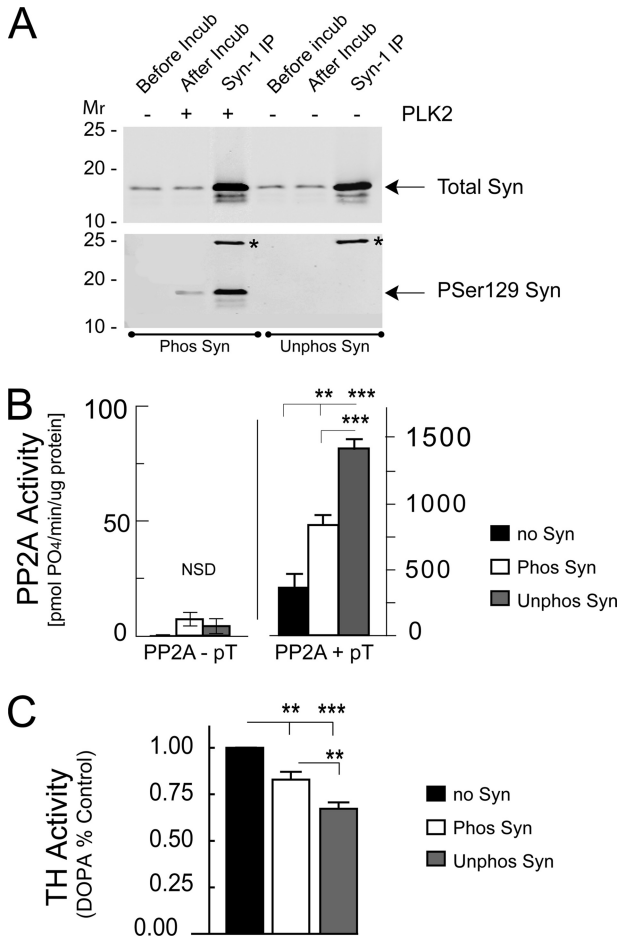

FIGURE 7. **Ser-129 phosphorylation reduces the impact of a-Syn on the regulation of PP2A and TH in cell-free assays.** *A*, shown are representative immunoblots of recombinant a-Syn treated with PLK2 (*Phos Syn*) or without PLK2 (*Unphos Syn*). Blots show 0.025 μg of the initial a-Syn sample (*Before Incub*), 0.025 μg of a-Syn after incubation with or without PLK2 in the reaction buffer (*After Incub*), and a-Syn immunoprecipitated from the buffers (*Syn-1 IP*). The *upper blot*, reacted with a rabbit a-Syn antibody (C20, sc7011-R, 1:500), demonstrates total a-Syn in each sample. The *lower blot*, reacted with a Ser(P)-129-specific a-Syn monoclonal antibody (11A5, 1:250), confirms Ser(P)-129 phosphorylation of PLK2 samples. IgG bands, at the *asterisks*, appear due to the use of mouse antibodies for immunoprecipitation and immunoblot. Molecular weights (M_r) determined from prestained standards are shown on the *left*. *B*, PP2A assay without any phosphopeptide substrate (*PP2A-pT*) demonstrates that PP2A does not dephosphorylate a-Syn that was phosphorylated on Ser-129 by PLK2, as can be appreciated by comparing PP2A alone (*black bar*), PP2A in the presence of 5 μg of Phos Syn (*white bar*), and 5 μg of Unphos Syn (*gray bar*), which show background signal only. Note: the *left y axis* maximum is set to 100, whereas the right *y axis* is set to 1500 to allow visualizing the extremely low background values on the *left graph*. On the *right graph*, in the presence of phosphorylated pT substrate (*PP2A + pT*), PP2A activity was lower at base line (*black bar*) but increased in the presence of Phos Syn (*white bar*) and even more so in response to Unphos Syn (*gray bar*). *C*, activity of recombinant TH alone (*no Syn*, *black bar*) was reduced in the presence of Phos Syn (*white bar*) and even more so in the presence of Unphos Syn (*gray bar*). Data represent the mean ± S.E. from 2 to 4 independent experiments performed in duplicate or triplicate. *NSD*, not significantly different; **, $p < 0.01$; ***, $p < 0.001$, one-way ANOVA.

identical for phosphorylated and unphosphorylated a-Syn (EC_{50} = 6.574e-7, $R^2 = 0.99$), revealing equal potency; however, at saturation Ser(P)-129-phosphorylated a-Syn had significantly less effect on PP2A activity than unphosphorylated a-Syn (Ser(P)-129, 2707 pmol/min/μg of protein; unphosphorylated Syn, 4511 pmol/min/μg of protein) revealing reduced efficacy for Ser(P)-129 a-Syn (see supplemental Fig. 1). These data provide the first evidence that native phosphorylation of a-Syn Ser-129 attenuates a-Syn ability to stimulate PP2A activity.

We next measured the effect of a-Syn Ser-129 phosphorylation on TH activity using a cell-free *in vitro* TH assay (as detailed under "Experimental Procedures"). We did immunoblots to confirm a-Syn phosphorylation as above (Fig. 7*A*) with similar results (not shown). Recombinant TH alone had ample activity (9.8 ± 0.9 nmol/min/μg of protein), whereas TH incubated in the presence of Ser(P)-129 a-Syn (5.7 ± 0.5 nmol/min/μg of protein) or unphosphorylated a-Syn (3.8 ± 0.4 nmol/min/μg of protein) had significantly lower TH activity, with unphosphorylated a-Syn having a greater effect on TH inhibition (Fig. 7*C*; $p < 0.0001$, one-way ANOVA). These results provide the first evidence that native phosphorylation of a-Syn Ser-129 can attenuate a-Syn ability to inhibit TH.

DISCUSSION

Our studies using cell-free assays, dopaminergic neuronal cells, and four independent a-Syn mouse models have generated three major and novel findings regarding a-Syn-mediated regulation of TH and PP2A. First, we observed that both WT and A53T mutant a-Syn overexpression diminished TH-Ser(P)-19 levels and inhibited TH activity. Second, we found that both WT and A53T mutant a-Syn stimulated the activity of PP2A *in vitro* and *in vivo*. Third, we established that phosphorylation of a-Syn on Ser-129 lessened the a-Syn ability to stimulate PP2A or inhibit TH *in vitro* and *in vivo*.

Early suggestions that a-Syn might affect dopamine levels arose from studies of transgenic mice that overexpress human wild type a-Syn driven by a PDGF promoter (73) and from rats virally transduced to overexpress a-Syn in the nigrostriatal system (74). Animals in both of the aforementioned studies had less striatal dopamine and reduced TH activity. Although those findings were intriguing, some argued that the data resulted from a loss of dopaminergic terminals rather than reflecting a functional inhibition by a-Syn on dopamine metabolism. Our early *in vitro* findings identified an inhibitory role for a-Syn on TH (9) occurring by the effects on TH- Ser-40 (43). Those studies provided the basis for the present work in which we evaluated a-Syn effects on TH-Ser(P)-19 *in vitro*. *In vivo* evidence, showing that a-Syn reduced Ser(P)-19 on TH, was obtained in transgenic mice expressing human a-Syn under the control of the TH-promoter (Syn++). These mice are healthy with no overt nigrostriatal pathology (46), thus allowing for systematically analyzing TH phosphorylation and TH activity in healthy dopaminergic tissues that are relevant to PD.

a-Syn overexpression in WT-Syn++ and A53T-Syn++ mice reduced TH-Ser-19 phosphorylation *in vivo*, suggesting that, as in dopaminergic cell lines, both forms of a-Syn regulate TH similarly in the brain. Several laboratories have shown that the A53T mutation does not affect a-Syn activity toward various proteins (9, 27, 44, 75, 76), suggesting the possibility that the toxicity associated with the A53T mutation occurs by its earlier aggregation. Because humans remain unaffected at up to an ~80% loss of nigrostriatal dopamine input (47), transgenic mice may compensate in order to sustain functional dopamine levels. Evidence for regulation of TH by a-Syn *in vivo* was further corroborated by our data in mice lacking a-Syn, in which

we saw effects opposite to those observed in a-Syn-overexpressing mice; namely, the ASKO mice had higher TH-Ser(P)-19 levels and significantly more active TH. The results in the ASKO mice, which have β-synuclein and γ-synuclein at levels comparable to controls (3), suggest that a-Syn has non-redundant functions for dopamine regulation that cannot be compensated for entirely by β- or γ-synuclein.

With regard to TH-Ser(P)-19, this post-translational modification contributes to TH activation by recruiting the binding of 14-3-3 "activator proteins" to TH (15, 16), an event that also stimulates catecholamine synthesis (11, 14, 22, 77). TH-Ser-19 is phosphorylated chiefly by Ca^{2+}/calmodulin-dependent protein kinase II (78, 79), a kinase that integrates signals associated with depolarization with the synthesis of neurotransmitter (80). Ser-19 phosphorylation also helps stabilize TH in an active conformation (10) that then sustains TH- Ser-40 phosphorylation (81). Our findings raise the distinct possibility that reversible phosphorylation of TH-Ser-19 may actively regulate the recruitment of either a-Syn or 14-3-3 to TH to serve as regulators of dopamine homeostasis (9, 31, 82).

Our data further suggest that a-Syn exerts important regulatory effects on PP2A, because a-Syn overexpression stimulates PP2A activity *in vitro* (43), whereas its absence reduced PP2A activity in dopaminergic tissues from ASKO mice (Fig. 4*B*, *right side*). Although the exact mechanism underlying PP2A regulation by a-Syn is not yet defined, it likely involves a-Syn binding to the PP2A catalytic subunit based on our prior co-immunoprecipitation data (43) and our current findings using recombinant proteins, which require preincubation to affect PP2A activity (Fig. 7). *In vivo*, a-Syn may also bind particular PP2A B subunits that contribute to TH dephosphorylation (83). Or dephosphorylation of TH may be stimulated by PKCδ, a kinase that binds both PP2A (84) and a-Syn (26). Taken together, the data suggest the intriguing possibility that a-Syn is a member of a dopamine regulatory complex localized to mitochondria (31), a possibility that we are further investigating.

Effects of a-Syn on TH and PP2A, as modeled in this study, may also play a role in neuronal pathology, including that associated with familial PD. For instance, genetic variability in the a-Syn promoter is associated with increased PD risk (85–88) and occurs even with relatively modest increases in a-Syn expression. Aging is also a major risk factor for most neurodegenerative diseases, and a-Syn protein levels significantly increase in aged human nigral neurons (62). Aged rhesus monkeys have increased a-Syn levels and are more vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity, and those monkeys fail to up-regulate dopamine levels after toxin exposure (62, 89) perhaps due to TH inhibition, which may be associated with elevated a-Syn levels. Up-regulation of a-Syn also is a consistent feature of dopaminergic neurons in response to toxins (90–92), and animal models characterized by changes in dopamine metabolism after toxin exposure frequently exhibit pathology reminiscent of PD (93, 94). The data from our current study have demonstrated that alterations in a-Syn levels *in vivo* have the potential to alter dopamine synthesis in a manner to stress dopaminergic neurons by shifting dopamine levels as soluble a-Syn levels go up or down (82).

a-Syn Ser(P)-129 has been implicated in pathology (38, 95); however, whether Ser(P)-129 functionally affected PP2A activity or TH activity has never been evaluated. Importantly, Ser(P)-129 is not dephosphorylated by PP2A (72), a finding that we verified (Fig. 7*B*). a-Syn can be phosphorylated by at least three kinases, including casein kinase 2 (72, 96–99), G-protein-coupled receptor kinase 5 (100), and Polo-like kinases, especially PLK2, which exclusively phosphorylates Ser-129 (101). PLK2 levels are affected by oxidative stress (102), yet untreated mice have measurable brain levels of Ser(P)-129 a-Syn (103), suggesting that this particular post-translational modification contributes to normal a-Syn physiology. Curiously, flies that overexpress the S129A mutant form of a-Syn remain healthy, whereas those that overexpress an S129D to mimic phosphorylation develop pathology (69). In contrast, rats that overexpress WT or S129A a-Syn delivered by adeno-associated virus (AAV) develop pathology by 8 weeks, although rats transduced with S129D AAV do not (68, 70). In our study there was no evidence of pathology in any S129A-transduced ASKO mice (Fig. 7) as confirmed by the absence of pro-apoptotic caspase activation.[7] It is noteworthy that both flies and the ASKO mice lack endogenous a-Syn, and both were entirely unaffected by S129A overexpression, raising the possibility that levels of endogenous a-Syn in wild type rodents may have contributed to the pathology noted in the AAV S129A-transduced rats described above. High levels of a-Syn induce pathology in families with extra copies of the a-Syn gene, as recently reviewed (104). Another major difference between our studies and the AAV rats is the timing of S129A overexpression, implying that perhaps long term S129A overexpression induces excessive and, hence, detrimental PP2A hyperactivation as we observed a potentiation of PP2A activity by S129A both in cells (Fig. 5) and in mice (Fig. 6). It is known that prolonged activation of PP2A can induce apoptosis in part by enhancing pro-apoptotic Bax activity (105), which stimulates ceramide production, a factor also known to contribute to PD pathology (106). Importantly, Ser(P)-129 is not dephosphorylated by PP2A (72), a finding that we have also reconfirmed (Fig. 7*B*). Collectively our findings provide compelling support for physiological regulation of PP2A and TH by a-Syn, with a-Syn Ser-129 phosphorylation attenuating the effects.

Finally, it remains to be determined the exact mechanism(s) whereby a-Syn mediates TH inhibition and whether it occurs by a direct interaction between a-Syn and TH or indirectly by a-Syn-mediated PP2A activation. An important clue from our current studies is the discovery that as a-Syn levels increase in MN9D cells, Ser(P)-129 levels also increased (Fig. 5). Because a-Syn Ser-129 phosphorylation reduced the a-Syn effect on TH and PP2A, a shift in a-Syn levels along with changes in the levels of Ser(P)-129 could dysregulate a-Syn-modulated proteins, especially in aging brain (62). A recent report in transduced MN9D cells overexpressing the a-Syn S129D phosphomimic saw an increase in TH-Ser(P)-40 phosphorylation (107). Although that study did not measure the effect of native a-Syn

[7] H. Lou, S. E. Montoya, T. N. M. Alerte, J. Wang, J. Wu, X. Peng, C.-S. Hong, E. E. Friedrich, S. A. Mader, C. J. Pedersen, B. S. Marcus, A. L. McCormack, D. A. Di Monte, S. Colette Daubner, and R. G. Perez, unpublished information.

phosphorylation, their work clearly supports our findings that TH inhibition is attenuated by a-Syn Ser-129 phosphorylation (Fig. 7*C*), which likely led to the measurable increase in TH-Ser(P)-40 and dopamine noted in their S129D cells.

In summary, our work using *in vitro* models has demonstrated that PP2A dephosphorylates TH-Ser(P)-19 (Fig. 2), that a-Syn activates PP2A and inhibits TH, and further, that a-Syn Ser(P)-129 attenuates both effects (Figs. 5 and 7). These findings are supported by our *in vivo* studies (Figs. 3 and 6), suggesting the possibility that (i) TH inhibition may be caused by an up-regulation of PP2A activity that is triggered by a-Syn overexpression, (ii) PP2A activation and TH inhibition may be independently triggered by a-Syn overexpression leading to interactions that affect both enzymes, or (iii) that TH inhibition may be an additive consequence of a-Syn overexpression and its ability to activate PP2A. Our findings in ASKO mice demonstrated that a loss of a-Syn produced the opposite effect on TH and PP2A imply that enzymatic dysregulation can also occur by a loss of soluble a-Syn, as those mice express normal levels of β- and γ-synuclein. Studies to clarify the precise mechanisms underlying a-Syn effects on TH and PP2A are under way. Delineating those mechanisms underlying a-Syn-mediated modulation of PP2A and TH holds promise to aid in the design of novel therapies to optimize dopaminergic neurotransmission for the treatment of PD, without overproducing potentially toxic dopamine levels.

Acknowledgments—We thank Drs. A. Heller and L. Won for cells, J. Haendeler and S. Dimmeler for PP2A plasmids, J. Anderson for antibody, B. Freeman for instrumentation, and A. Grigorescu and J. Rosenberg for recombinant a-Syn. Key experimental support was also provided by K. Farrell, D. Holland, J. John, K. Mackett, S. Reid, and S. Slusher. We thank S. Bharathi, M. A. Collins, and R. Leak for critical reading of the manuscript.

REFERENCES

1. Maroteaux, L., Campanelli, J. T., and Scheller, R. H. (1988) *J. Neurosci.* **8,** 2804–2815
2. Clayton, D. F., and George, J. M. (1998) *Trends Neurosci.* **21,** 249–254
3. Abeliovich, A., Schmitz, Y., Fariñas, I., Choi-Lundberg, D., Ho, W. H., Castillo, P. E., Shinsky, N., Verdugo, J. M., Armanini, M., Ryan, A., Hynes, M., Phillips, H., Sulzer, D., and Rosenthal, A. (2000) *Neuron* **25,** 239–252
4. Baptista, M. J., O'Farrell, C., Daya, S., Ahmad, R., Miller, D. W., Hardy, J., Farrer, M. J., and Cookson, M. R. (2003) *J. Neurochem.* **85,** 957–968
5. Larsen, K. E., Schmitz, Y., Troyer, M. D., Mosharov, E., Dietrich, P., Quazi, A. Z., Savalle, M., Nemani, V., Chaudhry, F. A., Edwards, R. H., Stefanis, L., and Sulzer, D. (2006) *J. Neurosci.* **26,** 11915–11922
6. Lotharius, J., and Brundin, P. (2002) *Hum. Mol. Genet.* **11,** 2395–2407
7. Wersinger, C., and Sidhu, A. (2003) *Neurosci. Lett.* **340,** 189–192
8. Liu, D., Jin, L., Wang, H., Zhao, H., Zhao, C., Duan, C., Lu, L., Wu, B., Yu, S., Chan, P., Li, Y., and Yang, H. (2008) *Neurochem. Res.* **33,** 1401–1409
9. Perez, R. G., Waymire, J. C., Lin, E., Liu, J. J., Guo, F., and Zigmond, M. J. (2002) *J. Neurosci.* **22,** 3090–3099
10. Dunkley, P. R., Bobrovskaya, L., Graham, M. E., von Nagy-Felsobuki, E. I., and Dickson, P. W. (2004) *J. Neurochem.* **91,** 1025–1043
11. Bobrovskaya, L., Dunkley, P. R., and Dickson, P. W. (2004) *J. Neurochem.* **90,** 857–864
12. Haycock, J. W., Lew, J. Y., Garcia-Espana, A., Lee, K. Y., Harada, K., Meller, E., and Goldstein, M. (1998) *J. Neurochem.* **71,** 1670–1675
13. Haycock, J. W., and Wakade, A. R. (1992) *J. Neurochem.* **58,** 57–64
14. Toska, K., Kleppe, R., Cohen, P., and Haavik, J. (2002) *Ann. N.Y. Acad. Sci.* **971,** 66–68
15. Ichimura, T., Isobe, T., Okuyama, T., Takahashi, N., Araki, K., Kuwano, R., and Takahashi, Y. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85,** 7084–7088
16. Tanji, M., Horwitz, R., Rosenfeld, G., and Waymire, J. C. (1994) *J. Neurochem.* **63,** 1908–1916
17. Daubner, S. C., Lauriano, C., Haycock, J. W., and Fitzpatrick, P. F. (1992) *J. Biol. Chem.* **267,** 12639–12646
18. Haavik, J., Martínez, A., and Flatmark, T. (1990) *FEBS Lett.* **262,** 363–365
19. Ramsey, A. J., and Fitzpatrick, P. F. (1998) *Biochemistry* **37,** 8980–8986
20. Kumer, S. C., and Vrana, K. E. (1996) *J. Neurochem.* **67,** 443–462
21. Salvatore, M. F., Garcia-Espana, A., Goldstein, M., Deutch, A. Y., and Haycock, J. W. (2000) *J. Neurochem.* **75,** 225–232
22. Itagaki, C., Isobe, T., Taoka, M., Natsume, T., Nomura, N., Horigome, T., Omata, S., Ichinose, H., Nagatsu, T., Greene, L. A., and Ichimura, T. (1999) *Biochemistry* **38,** 15673–15680
23. Toska, K., Kleppe, R., Armstrong, C. G., Morrice, N. A., Cohen, P., and Haavik, J. (2002) *J. Neurochem.* **83,** 775–783
24. Bevilaqua, L. R., Cammarota, M., Dickson, P. W., Sim, A. T., and Dunkley, P. R. (2003) *J. Neurochem.* **85,** 1368–1373
25. Kim, T. D., Paik, S. R., Yang, C. H., and Kim, J. (2000) *Protein Sci.* **9,** 2489–2496
26. Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Choi, P., Hardy, J., and Wolozin, B. (1999) *J. Neurosci.* **19,** 5782–5791
27. Souza, J. M., Giasson, B. I., Lee, V. M., and Ischiropoulos, H. (2000) *FEBS Lett.* **474,** 116–119
28. Kim, T. D., Paik, S. R., and Yang, C. H. (2002) *Biochemistry* **41,** 13782–13790
29. Ahn, M., Kim, S., Kang, M., Ryu, Y., and Kim, T. D. (2006) *Biochem. Biophys. Res. Commun.* **346,** 1142–1149
30. Fujita, M., Wei, J., Nakai, M., Masliah, E., and Hashimoto, M. (2006) *Neuropathology* **26,** 383–392
31. Wang, J., Lou, H., Pedersen, C. J., Smith, A. D., and Perez, R. G. (2009) *J. Biol. Chem.* **284,** 14011–14019
32. Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J. T., Schöls, L., and Riess, O. (1998) *Nat. Genet.* **18,** 106–108
33. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) *Science* **276,** 2045–2047
34. Zarranz, J. J., Alegre, J., Gómez-Esteban, J. C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Muñoz, D. G., and de Yebenes, J. G. (2004) *Ann Neurol.* **55,** 164–173
35. Chartier-Harlin, M. C., Kachergus, J., Roumier, C., Mouroux, V., Douay, X., Lincoln, S., Levecque, C., Larvor, L., Andrieux, J., Hulihan, M., Waucquier, N., Defebvre, L., Amouyel, P., Farrer, M., and Destée, A (2004) *Lancet* **364,** 1167–1169
36. Singleton, A. B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M. R., Muenter, M., Baptista, M., Miller, D., Blancato, J., Hardy, J., and Gwinn-Hardy, K. (2003) *Science* **302,** 841
37. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and Goedert, M. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6469–6473
38. Anderson, J. P., Walker, D. E., Goldstein, J. M., de Laat, R., Banducci, K., Caccavello, R. J., Barbour, R., Huang, J., Kling, K., Lee, M., Diep, L., Keim, P. S., Shen, X., Chataway, T., Schlossmacher, M. G., Seubert, P., Schenk, D., Sinha, S., Gai, W. P., and Chilcote, T. J. (2006) *J. Biol. Chem.* **281,** 29739–29752
39. Saito, Y., Kawashima, A., Ruberu, N. N., Fujiwara, H., Koyama, S., Sawabe, M., Arai, T., Nagura, H., Yamanouchi, H., Hasegawa, M., Iwatsubo, T., and Murayama, S. (2003) *J. Neuropathol. Exp. Neurol.* **62,** 644–654
40. Berresheim, U., and Kuhn, D. M. (1994) *Brain Res.* **637,** 273–276
41. Haavik, J., Schelling, D. L., Campbell, D. G., Andersson, K. K., Flatmark, T., and Cohen, P. (1989) *FEBS Lett.* **251,** 36–42

42. Leal, R. B., Sim, A. T., Gonçalves, C. A., and Dunkley, P. R. (2002) *Neurochem Res.* **27,** 207–213
43. Peng, X., Tehranian, R., Dietrich, P., Stefanis, L., and Perez, R. G. (2005) *J. Cell Sci.* **118,** 3523–3530
44. Tehranian, R., Montoya, S. E., Van Laar, A. D., Hastings, T. G., and Perez, R. G. (2006) *J. Neurochem.* **99,** 1188–1196
45. Perez, R. G., Soriano, S., Hayes, J. D., Ostaszewski, B., Xia, W., Selkoe, D. J., Chen, X., Stokin, G. B., and Koo, E. H. (1999) *J. Biol. Chem.* **274,** 18851–18856
46. Matsuoka, Y., Vila, M., Lincoln, S., McCormack, A., Picciano, M., LaFrancois, J., Yu, X., Dickson, D., Langston, W. J., McGowan, E., Farrer, M., Hardy, J., Duff, K., Przedborski, S., and Di Monte, D. A. (2001) *Neurobiol. Dis.* **8,** 535–539
47. Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K., and Seitelberger, F. (1973) *J. Neurol. Sci.* **20,** 415–455
48. Alerte, T. N., Akinfolarin, A. A., Friedrich, E. E., Mader, S. A., Hong, C. S., and Perez, R. G. (2008) *Neurosci. Lett.* **435,** 24–29
49. Daubner, S. C., and Fitzpatrick, P. F. (1993) *Adv. Exp. Med. Biol.* **338,** 87–92
50. Royo, M., Daubner, S. C., and Fitzpatrick, P. F. (2005) *Proteins* **58,** 14–21
51. Walsh, A. H., Cheng, A., and Honkanen, R. E. (1997) *FEBS Lett.* **416,** 230–234
52. Haendeler, J., Hoffmann, J., Rahman, S., Zeiher, A. M., and Dimmeler, S. (2003) *FEBS Lett.* **536,** 180–186
53. Zhou, B., Wang, Z. X., Zhao, Y., Brautigan, D. L., and Zhang, Z. Y. (2002) *J. Biol. Chem.* **277,** 31818–31825
54. Adams, D. G., Coffee, R. L., Jr., Zhang, H., Pelech, S., Strack, S., and Wadzinski, B. E. (2005) *J. Biol. Chem.* **280,** 42644–42654
55. Salvatore, M. F., Waymire, J. C., and Haycock, J. W. (2001) *J. Neurochem.* **79,** 349–360
56. Haycock, J. W., Ahn, N. G., Cobb, M. H., and Krebs, E. G. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 2365–2369
57. Hashimoto, M., Takenouchi, T., Rockenstein, E., and Masliah, E. (2003) *J. Neurochem.* **85,** 1468–1479
58. Millward, T. A., Zolnierowicz, S., and Hemmings, B. A. (1999) *Trends Biochem. Sci* **24,** 186–191
59. Van Kanegan, M. J., Adams, D. G., Wadzinski, B. E., and Strack, S. (2005) *J. Biol. Chem.* **280,** 36029–36036
60. Fallon, J. H., and Moore, R. Y. (1978) *J. Comp. Neurol.* **180,** 545–580
61. Jakowec, M. W., Donaldson, D. M., Barba, J., and Petzinger, G. M. (2001) *Dev. Neurosci.* **23,** 91–99
62. Chu, Y., and Kordower, J. H. (2007) *Neurobiol. Dis.* **25,** 134–149
63. Royo, M., Fitzpatrick, P. F., and Daubner, S. C. (2005) *Arch. Biochem. Biophys.* **434,** 266–274
64. Daubner, S. C., Lohse, D. L., and Fitzpatrick, P. F. (1993) *Protein Sci.* **2,** 1452–1460
65. Hsu, L. J., Mallory, M., Xia, Y., Veinbergs, I., Hashimoto, M., Yoshimoto, M., Thal, L. J., Saitoh, T., and Masliah, E. (1998) *J. Neurochem.* **71,** 338–344
66. Bohnen, N. I., Gedela, S., Kuwabara, H., Constantine, G. M., Mathis, C. A., Studenski, S. A., and Moore, R. Y. (2007) *J. Neurol.* **254,** 84–90
67. Liberini, P., Parola, S., Spano, P. F., and Antonini, L. (2000) *J. Neurol.* **247,** 88–96
68. Azeredo da Silveira, S., Schneider, B. L., Cifuentes-Diaz, C., Sage, D., Abbas-Terki, T., Iwatsubo, T., Unser, M., and Aebischer, P. (2009) *Hum. Mol. Genet.* **18,** 872–887
69. Chen, L., and Feany, M. B. (2005) *Nat. Neurosci.* **8,** 657–663
70. Gorbatyuk, O. S., Li, S., Sullivan, L. F., Chen, W., Kondrikova, G., Manfredsson, F. P., Mandel, R. J., and Muzyczka, N. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 763–768
71. Smith, W. W., Margolis, R. L., Li, X., Troncoso, J. C., Lee, M. K., Dawson, V. L., Dawson, T. M., Iwatsubo, T., and Ross, C. A. (2005) *J. Neurosci.* **25,** 5544–5552
72. Waxman, E. A., and Giasson, B. I. (2008) *J. Neuropathol. Exp. Neurol.* **67,** 402–416
73. Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000) *Science* **287,** 1265–1269
74. Kirik, D., Rosenblad, C., Burger, C., Lundberg, C., Johansen, T. E., Muzyczka, N., Mandel, R. J., and Björklund, A. (2002) *J. Neurosci.* **22,** 2780–2791
75. Iwata, A., Miura, S., Kanazawa, I., Sawada, M., and Nukina, N. (2001) *J. Neurochem.* **77,** 239–252
76. Ahn, B. H., Rhim, H., Kim, S. Y., Sung, Y. M., Lee, M. Y., Choi, J. Y., Wolozin, B., Chang, J. S., Lee, Y. H., Kwon, T. K., Chung, K. C., Yoon, S. H., Hahn, S. J., Kim, M. S., Jo, Y. H., and Min, D. S. (2002) *J. Biol. Chem.* **277,** 12334–12342
77. Kleppe, R., Toska, K., and Haavik, J. (2001) *J. Neurochem.* **77,** 1097–1107
78. Sutoo, D., Akiyama, K., and Yabe, K. (2002) *Brain Res.* **933,** 1–11
79. Sumi, M., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T., and Hidaka, H. (1991) *Biochem. Biophys. Res. Commun.* **181,** 968–975
80. Colbran, R. J. (1992) *Neurochem Int.* **21,** 469–497
81. Bobrovskaya, L., Gilligan, C., Bolster, E. K., Flaherty, J. J., Dickson, P. W., and Dunkley, P. R. (2007) *J. Neurochem.* **100,** 479–489
82. Perez, R. G., and Hastings, T. G. (2004) *J. Neurochem.* **89,** 1318–1324
83. Saraf, A., Virshup, D. M., and Strack, S. (2007) *J. Biol. Chem.* **282,** 573–580
84. Zhang, D., Kanthasamy, A., Yang, Y., Anantharam, V., and Kanthasamy, A. (2007) *J. Neurosci.* **27,** 5349–5362
85. Farrer, M., Maraganore, D. M., Lockhart, P., Singleton, A., Lesnick, T. G., de Andrade, M., West, A., de Silva, R., Hardy, J., and Hernandez, D. (2001) *Hum. Mol. Genet.* **10,** 1847–1851
86. Maraganore, D. M., de Andrade, M., Elbaz, A., Farrer, M. J., Ioannidis, J. P., Krüger, R., Rocca, W. A., Schneider, N. K., Lesnick, T. G., Lincoln, S. J., Hulihan, M. M., Aasly, J. O., Ashizawa, T., Chartier-Harlin, M. C., Checkoway, H., Ferrarese, C., Hadjigeorgiou, G., Hattori, N., Kawakami, H., Lambert, J. C., Lynch, T., Mellick, G. D., Papapetropoulos, S., Parsian, A., Quattrone, A., Riess, O., Tan, E. K., and Van Broeckhoven, C. (2006) *JAMA* **296,** 661–670
87. Mellick, G. D., Maraganore, D. M., and Silburn, P. A. (2005) *Neurosci. Lett.* **375,** 112–116
88. Pals, P., Lincoln, S., Manning, J., Heckman, M., Skipper, L., Hulihan, M., Van den Broeck, M., De Pooter, T., Cras, P., Crook, J., Van Broeckhoven, C., and Farrer, M. J. (2004) *Ann Neurol.* **56,** 591–595
89. McCormack, A. L., Di Monte, D. A., Delfani, K., Irwin, I., DeLanney, L. E., Langston, W. J., and Janson, A. M. (2004) *J. Comp. Neurol.* **471,** 387–395
90. Manning-Bog, A. B., McCormack, A. L., Li, J., Uversky, V. N., Fink, A. L., and Di Monte, D. A. (2002) *J. Biol. Chem.* **277,** 1641–1644
91. Purisai, M. G., McCormack, A. L., Langston, W. J., Johnston, L. C., and Di Monte, D. A. (2005) *Neurobiol. Dis.* **20,** 898–906
92. Vila, M., Vukosavic, S., Jackson-Lewis, V., Neystat, M., Jakowec, M., and Przedborski, S. (2000) *J. Neurochem.* **74,** 721–729
93. Caudle, W. M., Richardson, J. R., Wang, M. Z., Taylor, T. N., Guillot, T. S., McCormack, A. L., Colebrooke, R. E., Di Monte, D. A., Emson, P. C., and Miller, G. W. (2007) *J. Neurosci.* **27,** 8138–8148
94. McCormack, A. L., Mak, S. K., Shenasa, M., Langston, W. J., Forno, L. S., and Di Monte, D. A. (2008) *J. Neuropathol. Exp. Neurol.* **67,** 793–802
95. Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M. S., Shen, J., Takio, K., and Iwatsubo, T. (2002) *Nat. Cell Biol.* **4,** 160–164
96. Ishii, A., Nonaka, T., Taniguchi, S., Saito, T., Arai, T., Mann, D., Iwatsubo, T., Hisanaga, S., Goedert, M., and Hasegawa, M. (2007) *FEBS Lett.* **581,** 4711–4717
97. Lee, G., Tanaka, M., Park, K., Lee, S. S., Kim, Y. M., Junn, E., Lee, S. H., and Mouradian, M. M. (2004) *J. Biol. Chem.* **279,** 6834–6839
98. Miller, D. W., Patel, N., Clarimon, J., and Cookson, M. R. (2006) *Soc. Neurosci. Abstr.*, 381.314
99. Okochi, M., Walter, J., Koyama, A., Nakajo, S., Baba, M., Iwatsubo, T., Meijer, L., Kahle, P. J., and Haass, C. (2000) *J. Biol. Chem.* **275,** 390–397
100. Pronin, A. N., Morris, A. J., Surguchov, A., and Benovic, J. L. (2000) *J. Biol. Chem.* **275,** 26515–26522
101. Inglis, K. J., Chereau, D., Brigham, E. F., Chiou, S. S., Schöbel, S., Frigon, N. L., Yu, M., Caccavello, R. J., Nelson, S., Motter, R., Wright, S., Chian, D., Santiago, P., Soriano, F., Ramos, C., Powell, K., Goldstein, J. M., Babcock, M., Yednock, T., Bard, F., Basi, G. S., Sham, H., Chilcote, T. J., McConlogue, L., Griswold-Prenner, I., and Anderson, J. P. (2009) *J. Biol.*

Chem. **284,** 2598–2602
102. Schweikl, H., Hiller, K. A., Eckhardt, A., Bolay, C., Spagnuolo, G., Stempfl, T., and Schmalz, G. (2008) *Biomaterials* **29,** 1377–1387
103. Hirai, Y., Fujita, S. C., Iwatsubo, T., and Hasegawa, M. (2004) *FEBS Lett.* **572,** 227–232
104. McCormack, A. L., and Di Monte, D. A. (2009) *Curr. Protein. Pept. Sci.* **10,** 476–482
105. Xin, M., and Deng, X. (2006) *J. Biol. Chem.* **281,** 18859–18867
106. Bras, J., Singleton, A., Cookson, M. R., and Hardy, J. (2008) *FEBS J.* **275,** 5767–5773
107. Wu, B., Liu, Q., Duan, C., Li, Y., Yu, S., Chan, P., Ueda, K., and Yang, H. (2010) *Acta Histochem.*, in press