Disruption of the Interaction of α-Synuclein with Microtubules Enhances Cell Surface Recruitment of the Dopamine Transporter†

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ABSTRACT: Mutations in α-synuclein have been implicated in the genesis of Parkinson's disease. A probable normative function of α-synuclein is the maintenance of dopamine homeostasis, partly through a negative modulation of dopamine transporter (DAT) activity, by reducing its level at the cell surface. To study the possible involvement of the microtubular network in the α-synuclein-dependent trafficking of DAT, we treated cotransfected cells and primary mesencephalic neurons with either colchicine, vinblastine, or nocodazole, each of which disrupts microtubules or affects microtubule dynamics. Treatment of both types of cells with vinblastine, colchicine, or nocodazole reversed α-synuclein-mediated inhibition of DAT activity, resulting in an increased rate of dopamine uptake and and increased level of extracellular dopamine-induced oxidative stress, with accelerated cell death. Treatment with these agents also reversed the α-synuclein-induced decrease in levels of cell surface-associated DAT. This effect of colchicine, vinblastine, or nocodazole was not linked to a disruption of formation of the α-synuclein−DAT complex but paradoxically caused an increased level of interaction between these proteins. Both α-synuclein and DAT co-immunoprecipitated with both α- and *β*-tubulins, in both transfected cells and rat primary mesencephalic dopaminergic neurons, suggesting heteromeric complex formation between these various proteins. Treatment with the microtubule depolymerizing agents disrupted the heteromeric protein complex between either α-synuclein or the DAT, and α- or *β*-tubulins. These results indicate a previously unappreciated role of microtubules in the modulation of DAT trafficking, and provide insight into a novel mechanism by which α-synuclein regulates DAT activity, by tethering the transporter to the microtubular network.

α-Synuclein is a major component of Lewy bodies (LBs)[1] in Parkinson's disease (PD) (*1*, *2*), and its mutations are associated with some familial forms of PD (*3*, *4*). Although the normal function(s) of α-synuclein is unknown, recent data suggested that a primary function for α-synuclein in dopaminergic neurons may be the regulation and maintenance of DA homeostasis (*5*, *6*); α-synuclein attenuates by 35% the functional activity of the dopamine transporter (DAT), with formation of a heteromeric complex with DAT (*7*−*9*). Since DAT is the primary determinant of dopamine re-uptake (*10*, *11*), its regulation is central in understanding the preferential degeneration of dopaminergic neurons in PD. In this regard, very little about the mechanisms by which DAT activity is regulated is known, although the transporter has been shown to be rapidly trafficked to and away from the plasma membrane through processes involving protein kinases (*12*−*15*). Some evidence from our laboratories suggested that the cytoskeleton may be crucial in the modulation of DAT trafficking by α-synuclein, since the amount of cell surface-associated DAT was increased when cell adhesion was impaired (*8*, *9*), a condition which is accompanied by significant modifications of the cytoskeleton (*16*). Thus, α-synuclein may be a probable intervening component between DAT and the cytoskeleton in the trafficking of DAT.

Cytoskeletal proteins, including tau, tubulin, actin, microtubule-associated protein 1B (MAP1B), MAP2, neurofilament H, and torsin A (*17*−*20*), colocalize with α-synuclein aggregates in LBs. Moreover, α-synuclein interacts with some cytoskeletal proteins, such as tubulin (*19*, *21*), tau (*22*), MAP1B (*23*), MAP2 (*18*), and torsin A (*24*). α-Synuclein was shown to colocalize with microtubules in cultured cells and to induce polymerization of purified tubulin into microtubules (*25*). Although the physiological and/or pathological relevance of interactions of α-synuclein with the cytoskeletal components remains enigmatic, a role for α-synuclein in axonal transport has been hypothesized (*26*, *27*), as well as modulation of synaptic vesicle synthesis and recycling (*5*, *28*).

In this study, we examine the putative role of the microtubular network in the modulatory interactions between

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[1] Abbreviations: PD, Parkinson's disease; DAT, dopamine transporter; hDAT, human DAT; LBs, Lewy bodies; ROS, reactive oxygen species; MAP, microtubule-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SN, substantia nigra; DA, dopamine; ROS, reactive oxygen species; SMBS, sodium metabisulfite; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium ion; DCF, 2′,7′-dichlorodihydrofluorescein diacetate; INDT, indatraline; co-IP, co-immunoprecipitation; SEM, standard error of the mean.

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α-synuclein and DAT, particularly with regard to the trafficking of DAT. We show here that the ability of α-synuclein to attenuate DAT function and cell surface expression is directly dependent on its ability to tether DAT to microtubules. Destabilization of the microtubular network disrupts the ability of α-synuclein to modulate DAT function, resulting in an increased level of trafficking to the plasma membrane, which is accompanied by an increased rate of dopamine uptake, dopamine-induced oxidative stress, and accelerated cell death.

EXPERIMENTAL PROCEDURES

Materials. DMEM (Cellgro 10-013-CM) was from BioSource International (Camarillo, CA). Fetal bovine serum (FBS), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], trypan blue, bovine serum albumin (BSA), vinblastine, colchicine, nocodazole, sodium orthovanadate, indatraline hydrochloride, dopamine (DA), sodium metabisulfate (SMBS), and sodium nitrite were purchased from Sigma-Aldrich (St. Louis, MO). Aliquoted stock solutions of sodium orthovanadate were prepared and used as described by Goodno (*29*) and Ringel et al. (*30*). [^{3}H]-Dopamine ([^{3}H]DA, NET-131, 31.6 Ci/mmol) was from Perkin-Elmer. All other chemicals were analytical grade.

cDNAs and Transfection. Human α-synuclein, its A53T mutant, and human DAT (hDAT) cDNA constructs were subcloned into the mammalian expression vector pcDNA3-1 as described previously (*8*, *9*). Cells were transiently transfected [2−3 μg of DNA/(1.0×10^5 cells)] at 80% confluence (seeding density of 10^5 cells/12-well plate) by the DEAE-dextran method, as previously described (*8*), and grown for a further 48 h after transfection to allow expression of the transgenes.

Cell Culture and Treatment. L*tk*$^-$ fibroblasts, a mouse fibroblast cell line (ATCC CCL-1.3) derived from subcutaneous connective tissue from C3H/An male mice, were grown at 37 °C and 5% CO_2 in DMEM supplemented with 4 mM L-glutamine and 10% FBS. Forty-eight hours after transfection, cells were rinsed with PBS and pretreated for 4 h with either 10 μM colchicine, 10 μM vinblastine, or 10 μM nocodazole, or vehicle (0.2% DMSO). After pretreatment, cells were assessed for [^{3}H]DA uptake, or washed twice with "warm" PBS (37 °C-warmed PBS) and treated with 200 μM dopamine for 24 h to measure the level of oxidative stress or cell death. Dopamine-induced oxidative stress and cell death were assessed in the presence or absence of either the DAT blocker indatraline (INDT, 10 μM) or the antioxidant sodium metabisulfate (SMBS, 200 μM). Control cells were processed in a similar way and treated with an equal concentration of solvent. Under all experimental conditions, the final concentration of solvent (DMSO) was kept constant at 0.2%. The level of oxidative stress was determined either by assessing nitrite levels, a stable byproduct of NO, by the Griess method using sodium nitrite standards, as described previously (*31*), or by determining the level of reactive oxygen species (ROS) production by measuring the fluorescence emission, at 525 nm, of cells preloaded (45 min) with 2′,7′-dichlorodihydrofluorescein diacetate (DCF), using a fluorescent microplate reader (CytoFluor2350, Millipore; fluorescence excitation at 475 nm), as described previously (*8*). DCF is a fluorescent dye which is sensitive to ROS production, since the wavelength of its maximal emission fluorescence is shifted after reaction with ROS. The intensity of fluorescence emission at the new wavelength of emission is proportional to the quantity of ROS present. Cell death was assessed by the MTT cell viability assay, as described previously (*32*).

Culture and Treatment of Rat Primary Mesencephalic Cultures. Mesencephalons from 18-day-old rat embryos were isolated and dissociated as described previously (*8*), and neuronal cells were cultured for 8 days as described previously (*8*). Half of the culture media was renewed every 2 days. Effects of colchicine, vinblastine, or nocodazole pretreatment (10 μM for 4 h) on [^{3}H]DA uptake were assessed as described above for L*tk*$^-$ cells. To assess dopamine-induced oxidative stress and neuronal death, 8-day-old neurons were washed twice with warm PBS, pretreated for 4 h with 10 μM vinblastine, colchicine, or nocodazole, washed again twice with warm PBS, and treated with increasing concentrations of dopamine for 16 h. Dopamine-induced oxidative stress and cell death were assessed in the presence or absence of the DAT blocker INDT (100 nM) or the antioxidant SMBS (10 μM). Oxidative stress was assessed by measuring ROS production by DCF fluorescence emission as described above, whereas neuronal death was assessed by cell counting using trypan blue. Apoptosis was challenged at 1 μM dopamine (12 h treatment) and measured by the TUNEL method according to the manufacturer's protocol (Roche Molecular Biochemicals). Results are expressed as the mean number of TUNEL positive (apoptotic) cells ± the standard error of the mean, as a percentage of total cells, counted in representative fields of 1000 cells in quadruplicate.

[^{3}H]Dopamine Uptake. Dopamine uptake was assessed by incubation of cells with 20 nM [^{3}H]DA for 10 min, as described previously (*8*, *33*). For kinetic analysis, cells were preincubated with unlabeled dopamine (10^{-11}−10^{-4} M) for 5 min prior to addition of [^{3}H]DA; 10 μM INDT was used to define nonspecific uptake. An aliquot of cells was collected for cell counting using trypan blue; the remaining cells in each well were lysed by freezing and thawing in 0.1 N NaOH, and the radioactivity incorporated into cells was measured by scintillation counting.

Co-Immunoprecipitations and Western Blotting. Rat substantia nigra tissue (Zymed Labs Inc.), transfected L*tk*$^-$ cells ($1-2 \times 10^7$ cells), or rat primary mesencephalic cultures (2×10^6 cells) were solubilized at room temperature for 15 min with 0.5% (v/v) Nonidet P-40 (NP-40) in a microtubule-stabilizing buffer [20 mM Tris buffer (pH 6.9) containing 0.5% (v/v) NP-40, 2 M glycerol, 10% (v/v) DMSO, 1 mM $MgCl_2$, 2 mM EGTA, 200 μM sodium orthovanadate, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 500 μM PMSF, and protease inhibitor cocktail (Sigma) at 1 mL/100 mL of lysate]. NP-40 at this concentration and in a similar microtubule-stabilizing buffer permits solubilizations while maintaining the integrity of microtubules and is widely used for purifications of microtubules by several authors which are leaders in the field of microtubules (*34*−*37*). The composition of the microtubule-stabilizing buffer used in this study was chosen to conciliate all the protocols used by these authors (*34*−*37*). Solubilized proteins were harvested by centrifugation at 15000*g* for 20 min at room temperature. To the soluble extracts (400 μL/assay tube, 0.5 mg/mL

protein) were added the following antisera: anti-DAT rabbit polyclonal (4 μg; Chemicon AB5802 for transfected cells, Chemicon AB1591P for rat tissue), anti-α-synuclein goat polyclonal [4 μg, sc-7011 (C-20), Santa Cruz Biotechnology], anti-α-tubulin rabbit polyclonal (4 μg, Santa Cruz sc-5546), and anti-β-tubulin rabbit polyclonal (4 μg, Santa Cruz sc-9104) antibodies, or nonimmune sera (4 μg of protein; normal rabbit serum was the control for anti-DAT, anti-α-tubulin, and anti-β-tubulin polyclonal antibodies, and normal goat serum was the control for the anti-α-synuclein polyclonal antibodies). After the samples had been rocked for 8 h at room temperature (RT), immune complexes were precipitated with protein A−Sepharose beads (CL-4B, Pharmacia), and pellets were washed five times and subjected to SDS−PAGE and Western blotting, as previously described (*8*, *32*). Blots were probed with antibodies against either DAT (1:1000, Chemicon MAB 369 monoclonal), α-synuclein (1:500, BD Biosciences monoclonal 610787), α-tubulin (1:1000, Santa Cruz monoclonal sc-5274), β-tubulin (1:1000, Santa Cruz monoclonal sc-8035), or β-actin (1:500, goat polyclonal sc-1616, Santa Cruz Biotechnology). Proteins were visualized using peroxidase-conjugated secondary antibodies (1:7500, Santa Cruz Biotechnology) and enhanced chemiluminescence (Amersham, Arlington Heights, IL). Immunoblots were scanned with a pdi model DNA 35 scanner with QualityOne version 2.0 (pdi, Huntington Station, NY) software, and the level of protein associated with the specific bands was calculated through computer analysis of the scans. Protein standards were initially run to ensure that the protein in the blots was in the linear range. Moreover, multiple exposures, to obtain reduced band intensity, were also routinely performed.

Biotinylation of Plasma Membrane Proteins. Biotinylation of cell surface-associated hDAT was performed 2 days after transfection, using a freshly prepared 0.5 mg/mL solution of the water-soluble, cell-impermeable biotin analogue, EZ-link NHS-biotin (Pierce), as previously described (*8*, *9*). Cells were collected in ice-cold lysis buffer [50 mM Tris buffer (pH 7.6) containing 150 mM NaCl, 250 mM sucrose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 200 μM sodium orthovanadate, leupeptin and pepstatin (5 μg/mL each), and 500 μM PMSF] containing 0.5% (w/v) Triton X-100 and 0.5% (w/v) Igepal CA 630. The lysates (1−2 mg/mL protein) were rocked for 1 h at 4 °C in Eppendorf tubes and cleared by centrifugation (18500*g* for 20 min at 4 °C), and the supernatants containing solubilized proteins were diluted to 0.5 mg/mL with ice-cold lysis buffer. Immunoprecipitation of hDAT was performed as described above, with anti-DAT polyclonal antibody (4 μg, Chemicon AB5802) or rabbit normal serum (4 μg of protein) used as a control. Blots were blocked with 5% (w/v) BSA and probed for 1 h with HRP-conjugated avidin (1:2000, Pierce) to quantify biotin-bound hDAT, or with anti-DAT monoclonal antibody (1:1000, Chemicon MAB 369) to quantify total hDAT (biotinylated and non-biotinylated), as described previously (*8*, *9*). In parallel, solubilized proteins (~0.5 mg/mL protein) were affinity purified with Sepharose beads conjugated to NeutrAvidin (Pierce), and blots were probed with antibodies for DAT (1:1000, Chemicon MAB 369) to quantify biotin-bound hDAT, as described previously (*8*, *9*). We ensured that equal expression levels of either hDAT or α-synuclein protein were present in each sample by directly analyzing the whole cell lysates on Western blots, as described above.

Subcellular Fractionation. Pellets of rat primary mesencephalic neurons (2×10^6 cells) were resuspended in 5 volumes of low-salt buffer [100 mM Tris (pH 7.4) containing 50 mM KCl, 0.5% (v/v) NP-40, 2 M glycerol, 10% (v/v) DMSO, 1 mM $MgCl_2$, 2 mM EGTA, 200 μM sodium orthovanadate, leupeptin and pepstatin (5 μg/mL each), 500 μM PMSF, and protease inhibitor cocktail (Sigma) at 1 mL/100 mL of lysate] and homogenized at room temperature (RT) by repetitive pipetting ("total fraction"). After centrifugation of the lysate at 10000*g* for 15 min at RT, the supernatant was carefully pipetted off and stored in Eppendorf tubes ("soluble fraction"). The pellet was resuspended in 5 volumes of high-salt buffer [100 mM Tris (pH 7.4) containing 600 mM KCl, 2 M glycerol, 10% (v/v) DMSO, 1 mM $MgCl_2$, 2 mM EGTA, 200 μM sodium orthovanadate, leupeptin and pepstatin (5 μg/mL each), 500 μM PMSF, and protease inhibitor cocktail (Sigma) at 1 mL/100 mL of lysate], homogenized by repetitive pipetting at RT, and centrifuged at 10000*g* for 15 min at RT. The resulting supernatant was carefully pipetted off and discarded. The pellets were resuspended in 3 volumes of solubilization buffer [50 mM cacodylate, 150 mM NaCl, 1 mM DTT, and 1% (v/v) SDS (pH 6.0)] and disrupted by vortexing and homogeneization at RT ("cytoskeletal fraction"). After determination of the protein content in each fraction by the method of Lowry, samples were diluted in 2× Laemmli buffer and proteins were resolved by SDS−PAGE and subjected to Western blotting.

Data Analysis. Each experimental measurement was performed in at least triplicate and is the mean ± the standard error of the mean of at least three experiments. Kinetic parameters of [^{3}H]DA uptake were calculated by linear regressions of the Eadie−Hoffstee plots and confirmed by a nonlinear regression program on Kaleidagraph (version 3.0.8 D, Abelbeck Software). The statistical significance of the experimental results was obtained by Variance Analysis with a Fisher's test, using Instat Statistical Software (Graphpad, Sorrento Valley, CA). A p of <0.05 was accepted to denote statistical significance.

RESULTS

Microtubule-Depolymerizing Agents Relieve the Inhibitory Effect of α-Synuclein on DAT Activity in both Cotransfected Ltk⁻ Cells and Rat Primary Mesencephalic Neurons. L*tk*⁻ cells were cotransfected with 1 μg of DNA each/10^5 cells of hDAT and *wt* α-synuclein DNAs, since at these concentrations and ratios, the expression levels of these proteins were similar to the physiological levels found in the endogenously expressing rat substantia nigra (*38*). To investigate the participation of cytoskeletal proteins in the α-synuclein-mediated negative modulation of DAT activity, cotransfected L*tk*⁻ cells were pretreated for 4 h with the microtubule-destabilizing agents (at 10 μM) colchicine (Figure 1A), vinblastine (Figure 1A), and nocodazole (data not shown) or vehicle [control, 0.2% DMSO (Figure 1A)], and hDAT activity was measured by [^{3}H]DA uptake studies. In vehicle-treated cotransfected cells, α-synuclein caused a significant ($p < 0.05$) attenuation of ~40% of the translocation velocity (V_{max}) of hDAT-mediated [^{3}H]DA uptake,

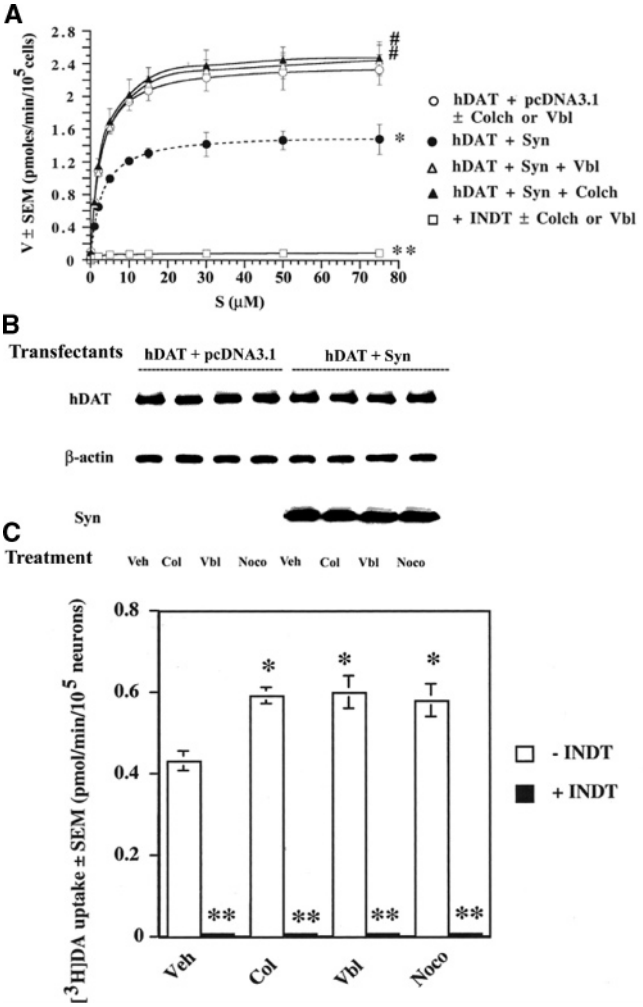

FIGURE 1: Reversal of α-synuclein-mediated attenuation of hDAT functional activity upon pretreatment with microtubule-depolymerizing agents. (A) Effect of a pretreatment with either colchicine or vinblastine on the saturation curves of [^{3}H]DA uptake in cotransfected L*tk*$^-$ cells. L*tk*$^-$ cells were cotransfected with hDAT and either pcDNA3.1 or α-synuclein (Syn) DNA [1 μg of DNA each/(1×10^5 cells)]. Forty-eight hours after transfection, cells were subjected to vehicle (0.2% DMSO) or 10 μM colchicine (+Colch) or vinblastine (+Vbl) for 4 h, and [^{3}H]DA uptake was assessed as described in Experimental Procedures. The specificity of the uptake was measured with the DAT blocker INDT at 10 μM. The asterisk ($p < 0.05$) indicates a value significantly different from that of cells expressing only hDAT; the number sign ($p < 0.05$) indicates a value significantly different from that of vehicle-treated hDAT and Syn coexpressing cells. (B) Western blots of whole cell lysates of cotransfected L*tk*$^-$ cells (30 μg of protein/lane) show that the expression levels of neither hDAT nor α-synuclein (Syn) proteins are affected by the 4 h pretreatment with 10 μM vinblastine (Vbl), colchicine (Col), or nocodazole (Noco), compared to vehicle (DMSO)-treated cells (control, Veh). β-Actin was used as a control to verify equal protein loading between samples. Data are representative of four independent studies. (C) Rat primary mesencephalic neurons were pretreated for 4 h with either vehicle (Veh, 0.2% DMSO) or 10 μM colchicine (Col), vinblastine (Vbl), or nocodazole (Noco), and the rate of [^{3}H]DA uptake (20 nM) was measured, in the presence or absence of the DAT blocker INDT at 100 nM, as described in Experimental Procedures. Two asterisks ($p < 0.01$) and one asterisk ($p < 0.05$) indicate values significantly different from those of vehicle-treated, control, neurons. In panels A and C, data shown are the mean ± SEM of four experiments, performed at least in triplicate.

relative to cells singly expressing hDAT [$V_{max} = 1.55 \pm 0.07$ and 2.32 ± 0.04 pmol min^{-1} (10^5 cells)$^{-1}$, respectively; $p < 0.05$ (Table 1)], which is similar to our previous observations (*7*, *8*). In pretreated cells, however, colchicine, vinblastine, and nocodazole fully reversed the α-synuclein-mediated inhibition of hDAT activity [$V_{max} = 2.57 \pm 0.12$, 2.50 ± 0.09, and 2.52 ± 0.11 pmol min^{-1} (10^5 cells)$^{-1}$ for colchicine, vinblastine, and nocodazole, respectively; $p < 0.05$, $n = 4$ (Table 1)], restoring the the level of uptake of [^{3}H]DA to levels seen in cells expressing hDAT alone. Neither colchicine, vinblastine, nor nocodazole treatments alone affected [^{3}H]DA uptake in cells expressing only hDAT [$V_{max} = 2.45 \pm 0.11$, 2.41 ± 0.09, and 2.42 ± 0.12 pmol min^{-1} (10^5 cells)$^{-1}$ for colchicine, vinblastine, and nocodazole, respectively; $p > 0.05$, $n = 4$ (Table 1)], indicating that the modulation of hDAT activity does not proceed directly through the microtubular network itself. This reversal of hDAT function was not due to changes in K_m, a measure of the affinity of the transporter for its preferred ligand (Table 1). That the increase in the level of [^{3}H]DA uptake in coexpressing cells was entirely due to hDAT activity, and not passive diffusion or increased permeability of the plasma membrane, by compromising the integrity of the plasma membrane, is demonstrated by the ability of the transporter blocker, indatraline (INDT, 10 μM), to totally abolish [^{3}H]-DA uptake in both vehicle-treated cells and cells treated with vinblastine or colchicine (Figure 1A), or nocodazole (data not shown), indicating that the uptake was hDAT-dependent. Moreover, trypan blue or neutral red staining of cells treated with either vehicle or drugs showed that the plasma membrane permeability was not significantly modified by such treatments (data not shown). In all instances, [^{3}H]DA basal efflux was negligible under all the experimental conditions used in this study, and unaffected by the co-expression of hDAT with *wt* α-synuclein (data not shown). These effects of colchicine, vinblastine, or nocodazole were also unrelated to differences in expression levels of hDAT or α-synuclein protein, since Western blots of whole cell lysates showed similar expression levels of these proteins in vehicle-treated or colchicine-treated (Col), vinblastine-treated (Vbl), or nocodazole-treated (Noco) cells (Figure 1B).

Table 1: Kinetic Parameters of [^{3}H]DA Uptake in Transfected L*tk*$^-$ Cells Pretreated for 4 h with Vehicle (0.2% DMSO) or 10 μM Colchicine, Vinblastine, or Nocodazole

	hDAT and pcDNA3.1		hDAT and α-synuclein	
pretreatment	K_m (μM)	V_{max} [pmol min^{-1} (10^5 cells)$^{-1}$]	K_m (μM)	V_{max} [pmol min^{-1} (10^5 cells)$^{-1}$]
vehicle	2.57 ± 0.10	2.32 ± 0.07	2.72 ± 0.10	1.55 ± 0.07^a
colchicine	2.49 ± 0.09	2.45 ± 0.11	2.80 ± 0.18	2.57 ± 0.12^b
vinblastine	2.42 ± 0.13	2.41 ± 0.09	2.75 ± 0.15	2.50 ± 0.09^b
nocodazole	2.47 ± 0.12	2.42 ± 0.12	2.70 ± 0.15	2.52 ± 0.11^b

[a] $p < 0.05$; value significantly different from values of cells expressing only hDAT. [b] $p < 0.05$; value significantly different from values of vehicle-treated cells coexpressing hDAT and α-synuclein.

To ascertain whether these effects of colchicine, vinblastine, or nocodazole could be detected in endogenously expressing cells and not merely an artifact of transfected cells, concurrent studies were conducted in 8-day-old primary rat mesencephalic neurons, which endogenously coexpress both α-synuclein and DAT (Figure 1C). In these neurons, pretreatment with either colchicine, vinblastine, or nocodazole (10 μM, 4 h) caused a 40% increase in the rate of [^{3}H]-

DA uptake, relative to vehicle (0.2% DMSO)-treated neurons ($p < 0.05$, $n = 4$). This increase is similar in magnitude to the level of relief of the negative modulation of hDAT activity seen after colchicine, vinblastine, or nocodazole treatment in cotransfected L*tk*$^-$ cells expressing hDAT and α-synuclein (Table 1), suggesting that the expression levels of DAT and α-synuclein are similar in both our mesencephalic neurons and our cotransfected cells. That this increase in the rate of [^{3}H]DA uptake by neurons was due to DAT activity and not due to changes in membrane permeability or efflux of the radioligand was confirmed through the use of 10 μM INDT, which almost totally blocks [^{3}H]DA uptake in both vehicle-treated and cytoskeletal drug-treated neurons (Figure 1C). Moreover, trypan blue or neutral red staining of neuronal cultures treated with either vehicle or drugs showed that the plasma membrane permeability was not significantly modified by such treatments (data not shown). In all instances, [^{3}H]DA basal efflux was negligible, regardless of whether the neurons were treated with vinblastine, colchicine, nocodazole, or vehicle.

Relief of α*-Synuclein-Mediated Inhibition of DAT Activity Accelerates Dopamine-Induced Neurotoxicity in both Cotransfected Ltk*$^-$ *Cells and Rat Primary Mesencephalic Neurons.* A consequence of the attenuation of hDAT activity by α-synuclein is the reduction of dopamine-mediated cytotoxicity, indexed by both reactive oxygen species (ROS) production and cell death, consistent with a reduced rate of uptake of dopamine and its subsequent autoxidation to cytotoxic species (*8*). Conversely, an increased level of uptake of dopamine would imply increased intracellular dopamine-mediated cytotoxicity. We, therefore, assessed this by exposing both vehicle (0.2% DMSO)-treated and either vinblastine-, colchicine-, or nocodazole-treated (10 μM, 4 h) L*tk*$^-$ cells to 200 μM dopamine for 24 h, followed by measurements of ROS production (Figure 2A) and cell death (Figure 2B). In vehicle-treated L*tk*$^-$ cells expressing hDAT alone, dopamine caused increased production of ROS (by 65%, $n = 4$, $p < 0.01$), associated with reduced cell viability (by 60%, $n = 4$, $p < 0.01$) compared to vehicle-treated cells that were not exposed to dopamine. That this increase was due to hDAT-mediated uptake of dopamine was demonstrated with INDT, which abolished dopamine-mediated ROS production (Figure 2A) and prevented cell death (Figure 2B). In vehicle-treated cells coexpressing hDAT with α-synuclein, there was a decrease in dopamine-elicited ROS production (by 59%, $n = 4$, $p < 0.05$) and cell death (by 40%, $n = 4$, $p < 0.05$) relative to cells expressing only hDAT, consistent with a reduced rate of uptake of dopamine, which is similar to our previous findings (*8*). Compared to that of vehicle-treated cotransfected cells, treatment of cells coexpressing hDAT and α-synuclein with cytoskeletal depolymerizing agents increased the levels of both dopamine-induced ROS production (by 75, 71, and 73% with colchicine, vinblastine, and nocodazole, respectively; $n = 4$, $p < 0.05$) and cell death (by 51, 49, and 50% with colchicine, vinblastine, and nocodazole, respectively; $n = 4$, $p < 0.05$) to levels not significantly different from those seen in vehicle-treated cells expressing only hDAT. In all instances, INDT ablated the cytotoxicity of dopamine in vinblastine-, nocodazole-, or colchicine-treated cells, demonstrating that the increased level of ROS production and cell death seen in treated cells is solely due to the increased rate of uptake of DA upon relief of the negative modulation of hDAT activity by α-synuclein. Similar results were observed when dopamine-induced oxidative stress was assessed by measuring the production of reactive nitrogen species, by assessing nitrite levels, a stable byproduct of nitric oxide (data not shown). In all instances, the levels of cell death and oxidative stress induced by a treatment with either colchicine, vinblastine, or nocodazole alone, without treatment with dopamine, were not significantly different from the levels of cell death and oxidative stress elicited by dopamine in the presence of the hDAT blocker INDT (10 μM) shown in panels A (mean DCF fluorescence emission ± SEM of 2.1 ± 1.1 after treatment of cells coexpressing hDAT and α-synuclein with only 10 μM vinblastine for 4 h) and B (ΔA_{564} ± SEM of 0.060 ± 0.019 after treatment of cells coexpressing hDAT and α-synuclein with only 10 μM vinblastine for 4 h, compared to vehicle-treated cells coexpressing hDAT and α-synuclein) of Figure 2, both for cells expressing hDAT alone and in cells coexpressing hDAT and α-synuclein (data not shown).

Parallel studies were conducted in rat primary mesencephalic neurons, which were pretreated with either colchicine or vinblastine (10 μM, 4 h) and exposed to increasing concentrations of dopamine for 16 h (Figure 2C,D). In vehicle-treated neurons, dopamine induced a dose-dependent increase in ROS production [by 69% with 10 μM dopamine; $n = 4$, $p < 0.05$ (Figure 2C)] and neuronal death [by 65.9% with 10 μM dopamine; $n = 4$, $p < 0.05$ (Figure 2D)], compared to vehicle-treated neurons not exposed to dopamine, that was blocked by INDT (100 nM). Pretreatment of neurons with either colchicine or vinblastine (10 μM, 4 h) increased the dopamine-induced, dose-dependent, increase in ROS production and neuronal death, which was totally blocked by INDT (100 nM), showing that this increase in dopamine-induced oxidative stress and neuronal death was due to an increased rate of DAT-mediated uptake of dopamine (Figure 2C,D). Indeed, pretreatment of neurons with either colchicine or vinblastine [10 μM, 4 h) followed by an exposure to 10 μM dopamine resulted in an ∼85% increase (for both agents) in ROS production ($n = 4$, $p < 0.05$) and an ∼82% increase (for both agents) in neuronal death ($n = 4$, $p < 0.05$), compared to vehicle-pretreated neurons not exposed to dopamine. In all instances, the levels of neuronal death and oxidative stress induced by a treatment with either colchicine, vinblastine, or nocodazole alone, without dopamine, were not significantly different from the levels of cell death and oxidative stress elicited by dopamine in the presence of the DAT blocker INDT (100 nM) shown in panels C (mean DCF fluorescence emission ± SEM of 16.00 ± 2.15 after treatment of neurons with only 10 μM vinblastine for 4 h) and D (mean number of neurons ± SEM of 505 300 ± 7659 after treatment of neurons with only 10 μM vinblastine for 4 h, compared to 508 000 ± 5490 neurons after a parallel treatment with vehicle) of Figure 2 (data not shown). Similar results were observed after pretreatment for 4 h with 10 μM nocodazole (data not shown). Both dopamine-induced ROS production and neuronal death were blocked by the antioxidant SMBS at 10 μM, showing that these effects of dopamine were linked to the genesis of an oxidative stress.

Apoptosis induced by dopamine (1 μM, 12 h) was assessed by the TUNEL method (Figure 2E). In vehicle (0.2% DMSO)-treated neurons, exposure to dopamine increased the

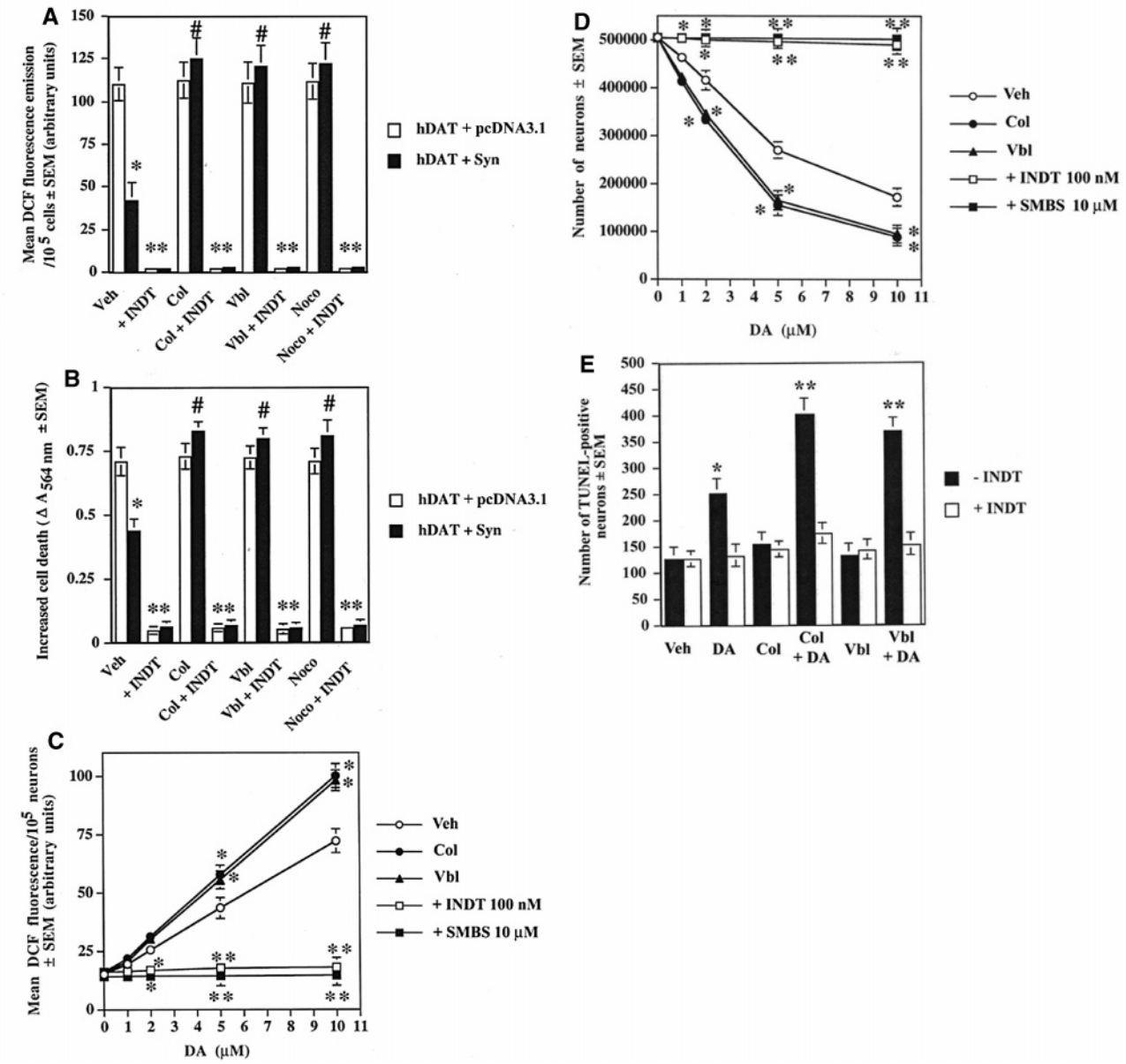

FIGURE 2: Relief of α-synuclein-mediated inhibition of hDAT activity by pretreatment with either colchicine, vinblastine, or nocodazole accelerates dopamine-induced ROS production and cell death in both cotransfected L*tk*$^-$ cells (A and B) and rat primary mesencephalic cultures (C−E). In panels A and B, L*tk*$^-$ cells were cotransfected with hDAT and either pcDNA3.1 or α-synuclein (Syn) DNA [1 μg of DNA each/(1 × 10^5 cells)]. Forty-eight hours after transfection, cells were pretreated for 4 h with vehicle (Veh, 0.2% DMSO) or 10 μM vinblastine (Vbl), nocodazole (Noco), or colchicine (Col), and then treated with 200 μM dopamine (DA) for 24 h, in the presence or absence of INDT (10 μM), followed by measurement of the level of ROS production (A) by DCF fluorescence emission, or cell death (B) by the MTT cell viability assay, as described in Experimental Procedures. Two asterisks ($p < 0.01$) and one asterisk ($p < 0.05$) indicate values significantly different from those of cells expressing only hDAT; the number sign ($p < 0.05$) indicates a value significantly different from those of vehicle-treated cells coexpressing hDAT and Syn. In panels C−E, 8-day-old rat primary mesencephalic cultures were pretreated with vehicle (Veh, 0.2% DMSO) or 10 μM vinblastine (Vbl) or colchicine (Col) for 4 h and treated with increasing concentrations of dopamine (DA) for 16 h, in the presence or absence of INDT (100 nM) or the antioxidant SMBS (10 μM), followed by measurement of ROS production (C) by DCF fluorescence emission or neuronal death (D) by counting the number of viable neuronal cells with Trypan Blue, as described in Experimental Procedures. Asterisks ($p < 0.05$) indicate values significantly different from those of vehicle-treated neurons. In panels C and D, identical results were obtained in the presence of the antioxidant SMBS or the DAT blocker INDT (see also panel E) for all treatment conditions. Therefore, for the sake of simplification, a single representative curve for all experimental conditions is shown for the blockade by INDT or SMBS. In panel E, dopamine (DA, 1 μM)-induced apoptosis, in the presence or absence of the DAT blocker INDT (100 nM), was checked by the TUNEL method, as described in Experimental Procedures, after pretreatment for 4 h of mesencephalic cultures with vehicle (Veh, 0.2% DMSO) or 10 μM vinblastine (Vbl) or colchicine (Col). Two asterisks ($p < 0.01$) and one asterisk ($p < 0.05$) indicate values significantly different from those of vehicle-treated neurons. In panels A−D, data shown are the mean ± SEM of four experiments, performed in quadruplicate. In panel E, data are the means ± SEM of the number of TUNEL-positive neurons, counted in representative fields of 1000 cells in quadruplicate.

degree of apoptosis (by 50%, $n = 4$, $p < 0.05$) compared to that of neurons not exposed to dopamine. After treatment with colchicine or vinblastine (10 μM, 4 h), dopamine-induced apoptotic death was further enhanced (59 and 46% apoptotic death after colchicine and vinblastine pretreatment followed by dopamine treatment, respectively; $n = 4$, $p < 0.05$), compared to neurons not exposed to dopamine. In all instances, dopamine-induced apoptosis of neurons was

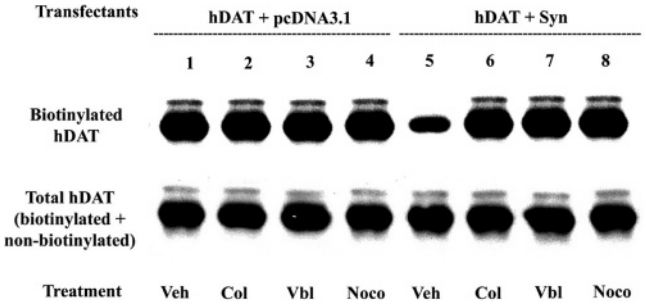

FIGURE 3: Biotinylation of hDAT in L*tk*$^-$ cells expressing hDAT in the presence or absence of α-synuclein, and pretreated or not pretreated with vinblastine or colchicine. L*tk*$^-$ cells were transfected with hDAT and either pcDNA3.1 control vector (columns 1−4) or α-synuclein (Syn, columns 5−8) DNA [1 μg of DNA each/(1.0×10^5 cells)], and cell surface proteins present in cells pretreated (10 μM, 4 h) with either vinblastine (Vbl, columns 3 and 7), colchicine (Col, columns 2 and 6), nocodazole (Noco, columns 4 and 8), or vehicle (control, Veh, columns 1 and 5) were labeled with EZ-link NHS-biotin, as described in Experimental Procedures. Biotinylated hDAT levels were assessed by immunoprecipitation of total hDAT and examination of the fraction of total hDAT that was biotinylated with avidin-bound HRP (top gel). Similar results were observed in parallel experiments, where total biotinylated proteins were immunoprecipitated with NeutrAvidin-conjugated beads and immunoprecipitates probed for hDAT (data not shown). The total hDAT present in each fraction was measured after IP with anti-hDAT polyclonal antibodies followed by Western blots using anti-hDAT monoclonal antibodies (bottom gel). Data are representative of three experiments.

blocked by INDT (100 nM), and treatment of neurons with either colchicine or vinblastine alone, in the absence of dopamine, did not cause neurotoxicity, confirming that these agents do not induce cell death or oxidative stress by themselves. These results clearly show that depolymerization of the microtubular network elicits the same changes in primary mesencephalic neurons, as in transfected L*tk*$^-$ cells, and that such depolymerization relieves the ability of α-synuclein to modulate DAT function.

Biotinylation of hDAT in Transfected Ltk$^-$ *Cells.* We have previously shown that α-synuclein-mediated inhibition of hDAT activity caused the transporter to be transported away from the plasma membrane, and disruption of the effect of α-synuclein on the transporter reversed such trafficking, causing increased levels of hDAT to be localized at the cell surface (*8*, *9*). To ascertain whether similar alterations in trafficking of hDAT occurred upon treatment of cells with the cytoskeletal depolymerizing agents, quantitative studies were conducted to assess the levels of plasma membrane-bound hDAT in transfected L*tk*$^-$ cells, whereby surface proteins were labeled with biotin, as described in Experimental Procedures. Biotinylated hDAT levels were assessed by probing with avidin-bound HRP, after immunoprecipitation of total hDAT (Figure 3). In vehicle-treated cells coexpressing hDAT and α-synuclein (column 5), there was a significant reduction (by 40%, $n = 3$, $p < 0.05$) in the levels of biotinylated hDAT, compared to vehicle-treated cells expressing only hDAT (column 1), indicating a diminished presence (compare columns 1 and 5) of hDAT at the cell surface (Figure 3, top gel), which is similar to our previous findings (*8*, *9*). In cells coexpressing hDAT and α-synuclein, treatment with either vinblastine (column 7), colchicine (column 6), or nocodazole (column 8) (10 μM, 4 h) significantly increased (by 45% after treatment with either colchicine, vinblastine, or nocodazole; $n = 3$, $p < 0.05$) the levels of biotinylated hDAT, compared to those of vehicle-treated, cotransfected cells (column 5), consistent with increased localization of the protein at the plasma membrane (compare column 5 with columns 6−8). Moreover, the levels of biotinylated hDAT, in treated cells coexpressing hDAT and α-synuclein (columns 6−8), were identical to the levels seen in vehicle-treated cells expressing only hDAT (column 1) (Figure 3, top gel). In cells coexpressing hDAT and α-synuclein, the increased level of hDAT at the plasma membrane elicited by colchicine (column 6), vinblastine (column 7), or nocodazole (column 8), compared to that of vehicle-treated cells (column 5), was also unrelated to changes in protein expression levels, as assessed by anti-hDAT monoclonal antibodies after immunoprecipitations of total hDAT protein levels (biotinylated and nonbiotinylated) with anti-hDAT polyclonal antibodies (Figure 3, bottom gel). In cells expressing only hDAT (columns 1−4), the levels of biotinylated hDAT were not significantly affected by a treatment of the cells with either colchicine (column 2), vinblastine (column 3), or nocodazole (column 4) (10 μM, 4 h) (compare column 1 with columns 2−4). Similar results were observed in parallel experiments, where total biotinylated proteins were immunoprecipitated with NeutrAvidin-conjugated beads and immunoprecipitates probed for hDAT immunoreactivity (data not shown).

Cytoskeletal Depolymerizing Agents Enhance the Physical Protein−Protein Interactions between DAT and α-Synuclein in both Cotransfected Cells and Rat Primary Mesencephalic Neurons. We have previously shown that α-synuclein and hDAT form heterodimeric complexes, through protein−protein interactions, and that such complexes are essential for attenuation of DAT activity in cotransfected L*tk*$^-$ cells (*8*). We, therefore, speculated that the reversal of α-synuclein-induced inhibition of hDAT activity by microtubule-destabilizing agents may be due to disruption of such complexes. To test for this, co-immunoprecipitation (co-IP) studies were conducted using detergent-solubilized lysates from hDAT and α-synuclein cotransfected L*tk*$^-$ cells, as described in Experimental Procedures. Surprisingly, colchicine, vinblastine, or nocodazole did not interfere with the formation of protein−protein complexes between hDAT and α-synuclein, but rather appeared to promote and/or stabilize such interactions (Figure 4). Thus, when using anti-DAT polyclonal antibodies, a 2-fold increase in the amount of α-synuclein protein (compare columns 1−4) was found to be associated with DAT immunopellets after treatment with either colchicine (column 2), nocodazole (column 4), or vinblastine (column 3), when compared to vehicle (0.2% DMSO; column 1)-treated control cells (Figure 4A). The increased level of association of α-synuclein with hDAT in the co-immunoprecipitates was not due to any increase in the level of expression of α-synuclein protein per se since the amount of α-synuclein detected by IP with its own antibody (columns 9−12) was identical in vehicle-treated cells (column 9) and in cells treated with the cytoskeletal depolymerizing (columns 10−12) agents (Figure 4A). Similarly, when anti-α-synuclein polyclonal antibodies were used in the reciprocal co-IPs, there was an ∼2-fold increase in the amount of hDAT co-immunoprecipitated by these antibodies (compare columns 9−12) from lysates of either vinblastine-treated (column 11), nocodazole-treated (column

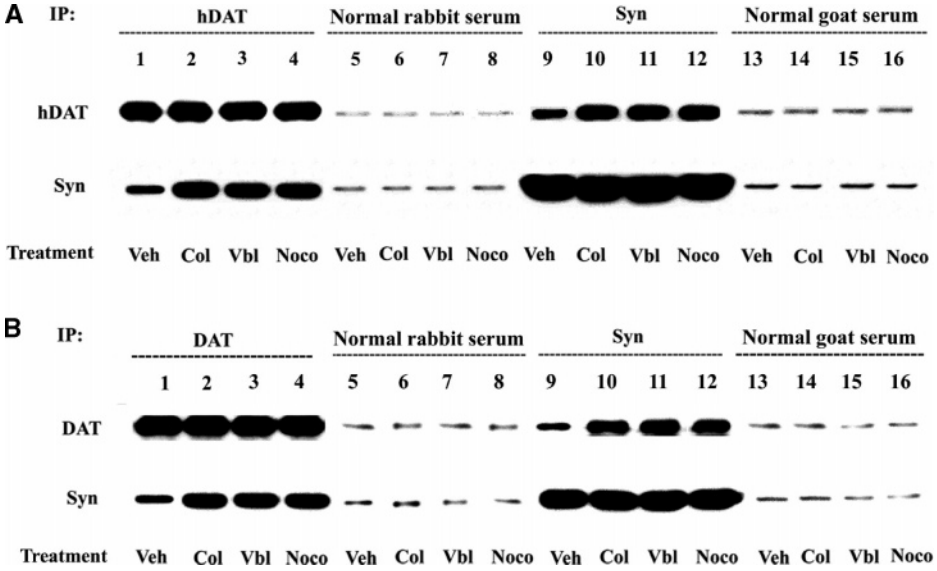

FIGURE 4: Treatment of cotransfected cells (A) or rat primary mesencephalic cultures (B) with microtubule-depolymerizing agents increases the co-immunoprecipitation of α-synuclein with DAT. hDAT and α-synuclein (Syn) cotransfected [1 μg of DNA each/(1×10^5 cells)] L*tk*$^-$ cells (A) or rat primary mesencephalic cultures (B) were pretreated (10 μM, 4 h) with either colchicine (Col), vinblastine (Vbl), nocodazole (Noco), or vehicle (0.2% DMSO, Veh), as described in Experimental Procedures. Solubilized lysates were prepared, and IP studies were conducted using either anti-DAT (IP DAT, columns 1−4) or anti-α-synuclein (IP Syn, columns 9−12) antibodies, or nonimmune sera [normal rabbit serum used as a control for the anti-DAT antibodies (columns 5−8); normal goat serum used as a control for the anti-Syn antibodies (columns 13−16)], as described in Experimental Procedures. Proteins present in the immunopellets were probed by Western blots, using monoclonal antibodies against α-synuclein (Syn, bottom gel) or DAT (top gel). Blots were scanned to measure relative levels of immunoprecipitated proteins present in immunopellets. Data shown in panels A and B are representative of three experiments.

12), or colchicine-treated (column 10) cotransfected cells, as compared to vehicle-treated (column 9) control cells (Figure 4A). This increase in the amount of hDAT protein in the α-synuclein immunopellets was also not due to any changes in the expression of hDAT protein per se, since the total amount of hDAT protein immunoprecipitated by its own antibody (compare columns 10−12 to column 9) was identical in these lysates irrespective of whether the cells were treated with vinblastine, colchicine, or nocodazole (Figure 4A).

Similar co-IP results were observed by using detergent-solubilized lysates of rat primary mesencephalic cultures (Figure 4B). Thus, colchicine, nocodazole, or vinblastine pretreatment of neurons (10 μM each, 4 h) increased by ~2-fold the amount of DAT present in the α-synuclein immunopellet (compare column 1 with columns 2−4) and the amount of α-synuclein found in the DAT immunopellet (compare column 9 to columns 10−12), when compared to vehicle-treated control neurons. In all such studies with neurons or L*tk*$^-$ cells, no co-IP between DAT or α-synuclein was detected when specific antibodies were replaced with either nonimmune sera [normal goat serum and normal rabbit serum were used as controls for the anti-α-synuclein polyclonal and the anti-DAT polyclonal antisera, respectively (Figure 4A,B); compare columns 5−8 and columns 13−16] or heat-inactivated (boiled) specific antisera (data not shown). These combined data suggest that microtubule depolymerization somehow increases the availability of these proteins to physically interact with one another such that there is now an increased number of protein−protein interactions between DAT and α-synuclein. The relief of α-synuclein-induced negative modulation of DAT activity by depolymerization of the microtubular network is therefore not due to disruption of DAT−α-synuclein protein−protein complexes.

Both DAT and α-Synuclein Form Protein−Protein Heteromeric Complexes with α- and β-Tubulin in Transfected Ltk$^-$ *Cells, in Rat Substantia Nigra Tissue, and in Rat Primary Mesencephalic Cultures.* To better understand the role of cytoskeletal proteins in our system, we conducted co-IP studies between either α-synuclein or DAT and α-tubulin, as well as β-tubulin, using lysates of hDAT and α-synuclein cotransfected cells, or from rat substantia nigra, as described in Experimental Procedures. Solubilization of the proteins and the co-immunoprecipitations were performed on fresh tissue or cells at room temperature, and in microtubule-stabilizing buffers, since microtubules spontaneously depolymerize into α-tubulin−β-tubulin dimers in the cold. Anti-α-synuclein antibodies were able to co-immunoprecipitate both α-tubulin and β-tubulin from detergent-solubilized lysates of rat substantia nigra (SN) tissue (Figure 5A). Similarly, anti-DAT antibodies were able to co-immunoprecipitate both α-tubulin and β-tubulin from rat SN lysates. Reciprocal co-IP studies showed that anti-α-tubulin and anti-β-tubulin antibodies were also able to co-immunoprecipitate α-synuclein and DAT (Figure 5A), showing that α-synuclein forms protein−protein complexes with both α-tubulin and β-tubulin, and that DAT also forms protein−protein complexes with α-tubulin and β-tubulin, in rat SN tissue (Figure 5A).

To ascertain that the effects of the cytoskeletal depolymerizing agents on [^{3}H]DA uptake in cotransfected cells were linked to some putative effects of these drugs on the interactions between the four proteins, cotransfected L*tk*$^-$ cells were treated with either colchicine, vinblastine, or nocodazole (10 μM, 4 h) prior to co-IPs being conducted in the microtubule-stabilizing buffers. In vehicle-treated cells, co-IP studies demonstrated clear protein−protein interactions between α-synuclein and both the tubulin variants (column

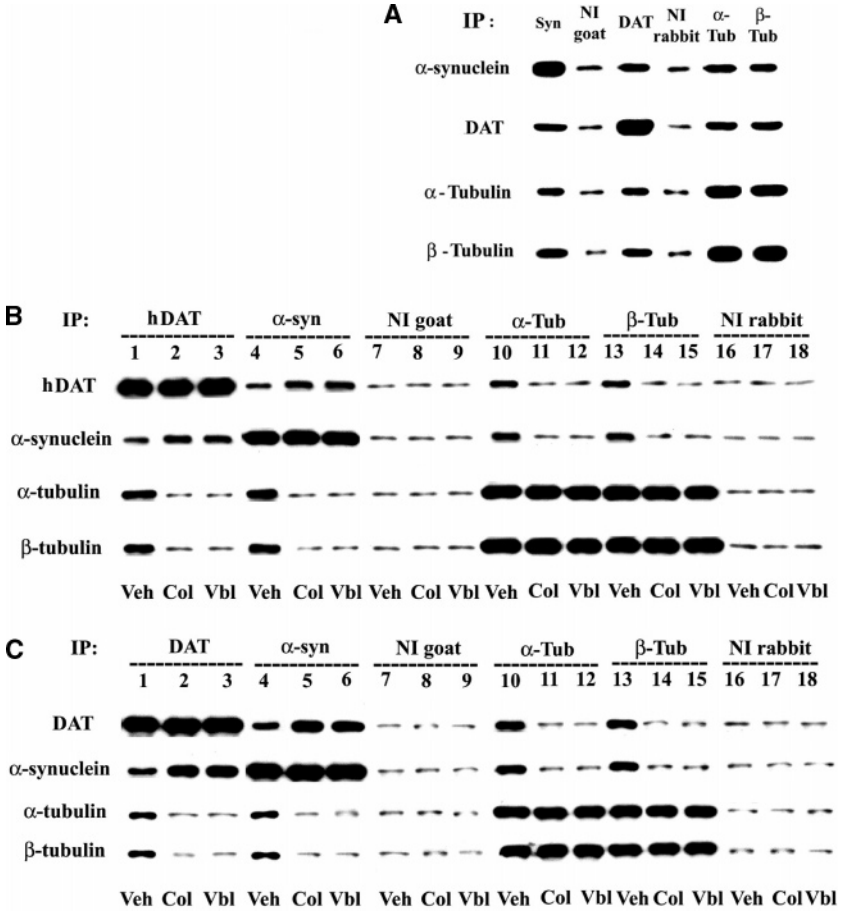

FIGURE 5: Co-immunoprecipitations between DAT and α- and *β*-tubulins, and between α-synuclein and α- and *β*-tubulins, are blocked by a pretreatment with microtubule-depolymerizing agents. (A) Co-immunoprecipitation between α-synuclein (Syn) and either α- or *β*-tubulin, and between DAT and either α- or *β*-tubulin, in rat substantia nigra (SN) tissue. In panels B and C, treatment of cotransfected cells (B) or rat primary mesencephalic cultures (C) with microtubule-depolymerizing agents blocks the co-IPs between DAT and the tubulins, and between α-synuclein and the tubulins. hDAT and α-synuclein cotransfected [1 μg of DNA each/(1 $\times$ 10^5 cells)] L*tk*$^-$ cells (B) or rat primary mesencephalic cultures (C) were pretreated (10 μM, 4 h) with either colchicine (Col), vinblastine (Vbl), or vehicle (0.2% DMSO, Veh), as described in Experimental Procedures. In panels A−C, IP studies were conducted on either solubilized rat SN (A), solubilized cotransfected L*tk*$^-$ cells (B), or solubilized rat mesencephalic cultures (C), as described in Experimental Procedures, using either anti-DAT (IP DAT, columns 1−3), anti-α-synuclein (IP Syn, columns 4−6), anti-α-tubulin (IP α-Tub, columns 10−12), or anti-*β*-tubulin (IP *β*-Tub, columns 13−15) antibodies, or nonimmune sera [normal rabbit serum (NI rabbit) used as a control for the anti-DAT, anti-α-tubulin, and anti-*β*-tubulin antibodies (columns 16−18); normal goat serum (NI goat) used as a control for the anti-Syn antibodies (columns 7−9)]. Proteins present in the immunopellets were probed by Western blots, using monoclonal antibodies against α-synuclein, DAT (hDAT in cotransfected cells), α-tubulin, or *β*-tubulin, as described in Experimental Procedures. Blots were scanned to measure relative levels of immunoprecipitated proteins present in immunopellets. Data are representative of three independent experiments.

4), or between DAT and the tubulins (column 1). In cells treated with colchicine (columns 2 and 5) or vinblastine (columns 3 and 6), neither DAT nor α-synuclein was found to be associated with either α-tubulin or *β*-tubulin (compare column 1 to columns 2 and 3, and compare column 4 to columns 3 and 5) (Figure 5B). This was not due to changes in expression levels of the tubulins that could have been induced by the microtubule-depolymerizing agents, since pretreatment of the cells with these agents did not alter the levels of tubulins that can be immunoprecipitated by their specific antisera (compare columns 10−15). Similar co-IP results were observed after pretreatment for 4 h with 10 μM nocodazole (data not shown). When co-IP studies were performed on L*tk*$^-$ cells expressing only DAT (DAT and *pcDNA3.1* cotransfected cells), no co-IP was noticed between DAT and either α-tubulin or *β*-tubulin (data not shown), in agreement with the lack of an effect of the microtubular drugs on DAT-mediated [^{3}H]DA uptake in cells expressing only DAT (Figure 1A).

The physiological relevance of these findings was further confirmed using detergent-solubilized lysates of rat primary mesencephalic cultures (Figure 5C). Indeed, treatment of neurons with colchicine or vinblastine (10 μM, 4 h) almost totally blocked the interaction between α-synuclein and both the α- and *β*-tubulins (compare column 4 with columns 5 and 6), as well as the association between DAT and the α- and *β*-tubulins (compare column 1 with columns 2 and 3), without affecting the overall expression levels of the four proteins since similar levels of DAT (columns 1−3), α-synuclein (columns 4−6), α-tubulin (columns 10−12), and *β*-tubulin (columns 13−15) could be immunoprecipitated by their own specific antisera (Figure 5C). Similar co-IP results were observed after pretreatment for 4 h with 10 μM nocodazole (data not shown).

In all the co-IP studies of panels B and C of Figure 5, no co-immunoprecipitation between either DAT or α-synuclein and the tubulins could be noticed when specific antibodies were replaced either with nonimmune sera (normal goat

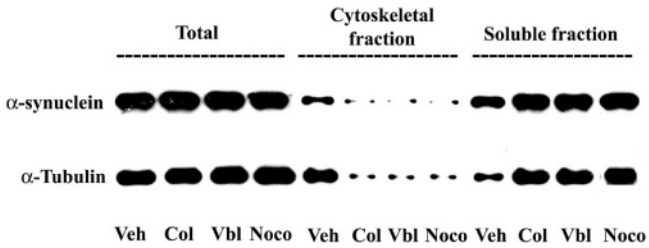

FIGURE 6: Effect of microtubule-depolymerizing agents on the subcellular fractionation of α-synuclein and α-tubulin in rat primary mesencephalic cultures. Neurons were pretreated for 4 h with vehicle (Veh, 0.2% DMSO) or 10 μM colchicine (Col), vinblastine (Vbl), or nocodazole (Noco), as described in Experimental Procedures, and cell lysates (Total) were fractionated into a cytoskeletal fraction and a soluble fraction, as described in Experimental Procedures. Samples were immunoblotted for either α-synuclein (top gel) or α-tubulin (bottom gel) as described in Experimental Procedures. Data are representative of three experiments.

serum, columns 7−9, was used as a control for the anti-α-synuclein polyclonal, whereas normal rabbit serum, columns 16−18, was used as a control for the anti-DAT, anti-α-tubulin, and anti-β-tubulin polyclonal antisera) (Figure 5B,C) or heat-inactivated (boiled) specific antisera (data not shown). These combined data clearly show that depolymerization of the microtubules disrupts the protein−protein interactions between α-synuclein and α- and β-tubulins, and between DAT and α- and β-tubulins, suggesting that blockade of such interactions causes increased trafficking of the DAT at the plasma membrane.

Subcellular Fractionation of Cytoskeleton-Associated α*-Synuclein in Rat Primary Mesencephalic Neurons.* To ascertain whether some α-synuclein was associated with the microtubules of rat primary mesencephalic neurons, and whether treatment of neurons with microtubule-depolymerizing agents could induce a redistribution of α-synuclein away from microtubules, thereby explaining the reversal of the inhibitory effect of α-synuclein on DAT activity induced by the microtubule-depolymerizing agents, we performed subcellular fractionation studies by isolating a cytoskeleton fraction (containing microtubules) and a soluble fraction (containing the free α-tubulin−β-tubulin dimers), as described in Experimental Procedures. In vehicle-treated cells, α-synuclein immunoreactivity was mostly associated with the soluble fraction, but significant immunoreactivity was also found in the cytoskeletal fraction. Pretreatment of mesencephalic neurons with microtubule-depolymerizing agents significantly decreased the amount of α-synuclein associated with the cytoskeletal fraction, with a corresponding increase in the amount of α-synuclein found in the soluble fraction (Figure 6). The microtubule-depolymerizing agents also affected the subcellular distribution of α-tubulin (cytoskeleton-associated vs soluble form), with a significant decrease in α-tubulin immunoreactivity found in the cytoskeletal fraction, and a corresponding increase in α-tubulin immunoreactivity found in the soluble fraction.

Lack of Modulation of DAT Trafficking by the A53T Mutant of α*-Synuclein with Microtubule-Depolymerizing Agents.* In our earlier studies, we have shown that the A53T mutant of α-synuclein binds only weakly to DAT and is unable to regulate its trafficking and function (*8*). To test whether there were any effects of the microtubule-depolymerizing agents in cells coexpressing hDAT with A53T,

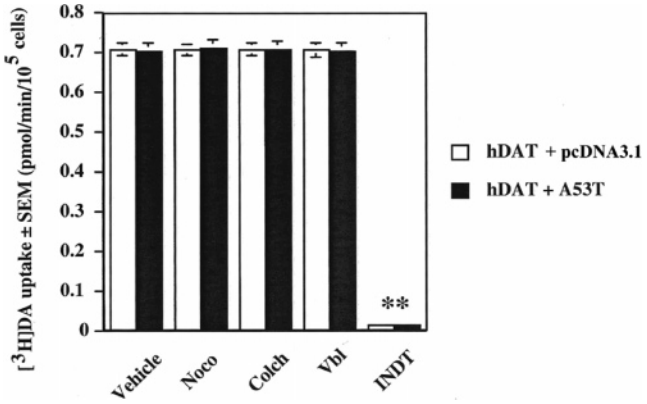

FIGURE 7: Microtubule-depolymerizing agents do not affect the lack of modulation of DAT activity by the A53T mutant of α-synuclein. L*tk*$^-$ cells were cotransfected with hDAT and either pcDNA3.1 or the A53T mutant of α-synuclein DNA [1 μg of DNA each/(1×10^5 cells)]. Forty-eight hours after transfection, cells were subjected to treatment with vehicle (0.2% DMSO) or 10 μM nocodazole (Noco), colchicine (Colch), or vinblastine (Vbl) for 4 h, and [^{3}H]DA uptake (20 nM) was assessed as described in Experimental Procedures. The specificity of the uptake was measured with the DAT blocker INDT at 10 μM. Identical results were obtained in the presence of INDT for all treatment conditions. Therefore, for the sake of simplification, a single representative column for all experimental conditions is shown for the blockade by INDT. Two asterisks ($p < 0.01$) indicate values significantly different from those of cells expressing hDAT. Data are the mean ± SEM of four experiments, performed in triplicate.

cotransfected L*tk*$^-$ cells were treated with either colchicine, vinblastine, or nocodazole (10 μM, 4 h), as described in Experimental Procedures, and DAT activity was assayed by conducting [^{3}H]DA uptake. As seen in Figure 7, none of the microtubule-destabilizing agents was able to alter DAT function in the presence of the A53T mutant, confirming that this mutant is completely unable to regulate DAT function, and that it cannot tether the transporter to the micotubular network.

DISCUSSION

Using agents that disrupt the microtubular network as a tool, we have examined the extent to which microtubules contribute to the regulation of DAT activity and trafficking by α-synuclein. Our results show that compounds which depolymerize microtubules, such as colchicine, vinblastine, and nocodazole, can reverse the negative modulation of DAT activity by α-synuclein. Our data show that these agents do not directly modify the DAT itself, since transporter activity, in the absence of any α-synuclein protein, was identical in both vehicle-treated cells and in cells treated with cytoskeleton-depolymerizing agents. Moreover, these agents did not affect the expression levels of either DAT or α-synuclein proteins. Instead, the biotinylation data clearly show that depolymerization of the microtubules causes increased levels of mobilization or targeting of the DAT protein to the cell plasma membrane, only in cells that have been cotransfected with DAT and α-synuclein. That the magnitude of the relief of inhibition was the same in neurons and in cotransfected cells indicates that this process also occurs physiologically.

Our previous findings (*5*, *8*, *9*) showed that a prerequisite for α-synuclein-mediated attenuation of DAT activity was the formation of protein−protein complexes between DAT and α-synuclein. The data presented in this paper, however,

show that the formation of a protein−protein complex between α-synuclein and DAT does not in itself ensure the manifestation of an inhibitory response. Thus, although vinblastine, colchicine, or nocodazole treatments reverse the inhibition mediated by α-synuclein on DAT activity, such treatments did not abrogate the protein−protein interactions between these proteins. Instead, there was a paradoxical increase in the number of co-associations between α-synuclein and DAT, as indexed by co-IP studies. Therefore, the mechanism by which vinblastine, colchicine, and nocodazole disrupt α-synuclein-mediated attenuation of DAT activity is not linked to a disruption of the ability of DAT and α-synuclein to physically interact. This is consistent with our overall hypothesis that rearrangements of the microtubular network induced by these drugs may release any α-synuclein and DAT which are bound to cytoarchitectural components, thereby increasing the size of the intracellular free pool of these proteins, resulting in an increased number of protein−protein interactions. Together, these results imply that in addition to protein−protein interactions, there is another microtubule-dependent component which modulates both the protein−protein interactions and the functional outcome of such interactions.

α-Synuclein binds to and interacts closely with the cytoskeleton and with a variety of proteins which act to stabilize or organize the cytoskeleton (*18*, *19*, *21*−*25*). A physiological role for α-synuclein in axonal transport has, therefore, been hypothesized (*26*, *27*), and is entirely consistent with our observations that α-synuclein is implicated in the regulation of DAT targeting both to (this study and refs *9* and *33*) and away from (*7*−*9*, *32*) the plasma membrane. In the study presented here, we observed interactions between α-synuclein and both α-tubulin and *β*-tubulin, confirming previous studies that α-synuclein binds to tubulin (*19*, *21*, *25*).

Our study also shows that DAT can co-immunoprecipitate with both α-tubulin and *β*-tubulin, suggesting the existence of protein−protein complexes between DAT and the tubulins, and to our knowledge, this is the first study to describe such interactions. The fact that DAT−α/*β*-tubulin interactions are lost under conditions in which the negative modulation of DAT activity by α-synuclein is disrupted, i.e., after treatment with colchicine, vinblastine, or nocodazole, suggests that protein−protein complexes between DAT and the tubulins are physiologically relevant. Thus, from our combined data, a role for α-synuclein as a functional link between microtubules and DAT can be envisaged. An attractive hypothesis would be that the role of synuclein is to maintain or stabilize the sequestration of DAT in a cytoplasmic vesicular compartment, which requires the presence of stable microtubules or microtubule polymerization to occur. As such then, α-synuclein may function as a tethering protein, tethering DAT to the microtubular network. Since there does appear to be a difference in the co-immunoprecipitation results after treatment of the cells with the microtubule-depolymerizing drugs, it appears that whatever linkage between the three components existed in the cell in the absence of colchicine or vinblastine was preserved during the immunoprecipitation but was destroyed under intracellular microtubule-depolymerizing conditions. Thus, the three-way interaction between α-synuclein bound to DAT, and the α- and *β*-tubulins, must depend on the conformation of the α- or *β*-tubulins when present in the microtubules or when polymerized as microtubules or must at least depend on some aspect of the three-dimensional arrangement that is promoted along the surface of microtubules.

Studies showing colocalization of hyperphosphorylated tau and α-synuclein in LBs (*20*) have led to the proposal that α-synuclein may cause the collapse of the intraneuronal organization of microtubules (*39*). Our studies suggest that the converse may also occur, whereby minor perturbations in the structural integrity of the cytoskeleton could trigger α-synuclein cytotoxicity, upon dysregulation of DAT function, causing an increased level of recruitment of the protein at the cell surface. Since DAT is the only means by which dopaminergic neurons can re-uptake synaptically released dopamine (*11*), the increased presence of DAT at the plasma membrane clearly results in an increased rate of re-uptake of dopamine, causing excessive accumulation of high intracellular levels of ROS and reactive nitrogen species, contributing to cytotoxic events which culminate in neuronal death (*5*). The increased degree of stabilization of α-synuclein soluble protofibrils, the most toxic molecular forms of α-synuclein, by oxidized dopamine (*40*) and the disaggregation of α-synuclein amyloid fibrils into soluble toxic protofibrills, induced by exposure of cells to dopamine or L-DOPA (*41*), are consistent with this hypothesis. In this regard, it is also important to note that in PD, the initial degeneration of SN dopamine-producing neurons is seen at distal striatal nerve terminals, which contain the highest levels of DAT, α-synuclein, and tubulins.

Numerous studies show that cytoskeletal proteins are themselves susceptible to modifications by etiological factors of PD: oxidative stress and free radicals (*42*, *43*), MPTP (*44*−*47*), and rotenone (*48*). In particular, MPTP induces abnormal aggregation of filamentous material of the cytoskeleton (*49*), disorganization of actin filaments (*50*), an increased rate of phosphorylation of neurofilament-H (*45*), an increased level of expression of tau (*44*) and α-tubulin (*51*), oxidative (nitration) post-translational modification of α-synuclein in mouse striatum and ventral midbrain (*52*), and a decrease in the levels of MAP2 and *β*-actin expression (*50*, *53*). MPTP is therefore able to induce rearrangements of the cytoskeletal network. Interestingly, our previous studies performed on cotransfected L*tk*$^-$ cells and on rat primary mesencephalic neurons showed that a short pretreatment with very low, pathologically relevant concentrations of MPP^+ (<50−100 nM) reversed the negative modulation of DAT activity by α-synuclein, with an increased level of shuttling of DAT to the cell surface, and increased oxidative stress and cell death induced upon exposure to extracellular dopamine (*8*). It is therefore tempting to speculate that this effect of MPP^+ occurred through a dysregulation of DAT mediated by a kind of destabilization of the microtubular network. Additionally, MPP^+ induces intracellular oxidative stress by generation of free radicals through inhibition of complex I of the mitochondrial respiratory chain. Therefore, in conjunction with destabilization of the cytoskeletal network and the ability of reactive oxygen and nitrogen species to accelerate the formation of α-synuclein protofibrils (*40*, *41*, *54*), MPTP-mediated neurotoxicity can be partly explained by our model. This suggestion is underscored by a recent report documenting the relative resistance of α-synuclein −/− mice to MPTP-

induced degeneration (*55*), indicating the central role of α-synuclein in mediating neurotoxicity. Although the findings in syn−/− mice may appear at first to contradict our findings, whereby a lack of α-synuclein would implicate excessive DAT at the cell surface, the possibility that other compensatory mechanisms exist which allow for normal function of nigrostriatal neurons (*56*), which also may affect DAT trafficking, has been raised. In addition, it must be borne in mind that the intracellular accumulation of α-synuclein, secondary to an increased level of expression of the gene or probably mostly secondary to a decreased level of degradation of the protein through either the proteasomal or lysosomal pathway or both, is essential for aggregation of this protein, a key criterion for neurodegeneration. In this regard, our model also accounts for the degeneration seen in animal models upon the overexpression of α-synuclein in the absence of toxins (reviewed in refs *2* and *57*), since elevated α-synuclein levels could lead to an increased number of interactions with cytoskeleton-binding proteins, preventing these proteins from performing their normal functions, with eventual cytoskeleton destabilization and disruption of axonal transport.

In addition to α-synuclein, there are two other members of the synuclein family, *β*-synuclein (phosphoneuroprotein-14) and *γ*-synuclein (persyn), although their role in neurodegeneration is poorly understood. Since the NAC domain of α-synuclein is essential for binding to DAT, and since the *β*-synuclein lacks a substantial portion of this component (*58*), it is likely that *β*-synuclein may not be able to appropriately regulate DAT. However, *γ*-synuclein, which has this domain, may be able to modulate DAT, but since this protein appears to be predominantly expressed in peripheral tissues and in the spinal cord (*59*), the relevance of such interactions may not be physiological.

In conclusion, our findings highlight a previously unknown physiological role of the microtubule network in facilitating α-synuclein-mediated functions. The data show that α-synuclein may act in a neuroprotective manner, by weakening the neurotoxic consequences of excessive cell surface expression of DAT. Ablation of this protective effect is reversed by microtubule-depolymerizing agents, resulting in conditions that are more neurotoxic. At least part of this neurotoxicity is associated with relief of the α-synuclein-mediated inhibition of DAT activity, promoting an increased level of recruitment of the transporter to the cell surface, with an increase in the dopamine-uptake capacity of the transporter.

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