Trypsin Disrupts the Trafficking of the Human Dopamine Transporter by α-Synuclein and Its A30P Mutant†

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ABSTRACT: α-Synuclein modulates dopamine homeostasis in dopamine-producing neurons of substantia nigra, partly through regulation of human dopamine transporter (hDAT) activity. To identify the underlying mechanisms, we disrupted the modulation of hDAT activity by wild-type (wt) α-synuclein, and its familial Parkinson's disease linked mutants A30P and A53T, by mild trypsinization (0.1%, 30 s) of L*tk*⁻ cotransfected cells. Trypsin completely reversed the attenuation of hDAT function mediated by wt and the A30P mutant. In A53T coexpressing cells, where DAT activity is not downregulated, trypsinization did not induce any changes. These effects of trypsin were mimicked by collagenase I and Dispase (0.1%, 1 min each) but not by chymotrypsin, Pronase, or papain (0.1%, up to 2 min each). Trypsin increased dopamine uptake in rat primary mesencephalic neurons, suggesting that DAT activity is also subjected to modulation by α-synuclein in these neurons that endogenously coexpress both proteins. In trypsinized cells, dopamine accelerated both production of reactive oxygen species and cell death in hDAT and wt or A30P, but not A53T, coexpressing cells, compared to nontrypsinized cells. Paradoxically, trypsin increased the protein−protein interactions between the synuclein variants and hDAT, without any noticeable proteolysis of these proteins. hDAT−α-synuclein protein−protein interactions occurred through residues 58−107 (NAC domain) of the α-synuclein variants and residues 598−620 of the carboxy-terminal tail of hDAT, in both trypsinized and nontrypsinized cells. Confocal microscopy and biotinylation studies show that, in cells expressing the wt or A30P variants, but not the A53T mutant, hDAT is sequestered away from the plasma membrane into the cytoplasm, an effect that is reversed by trypsin. These results show that α-synuclein modulates hDAT function through trafficking of the transporter in a process that can be disrupted by trypsin.

α-Synuclein has a fundamental role in the genesis of neurodegeneration since it is a major component of Lewy bodies (LBs)[1] characteristic of idiopathic Parkinson's disease (PD) (*1*−*6*) and of neuronal and glial cytoplasmic inclusions found in multiple system atrophy (*7*−*9*). α-Synuclein-immunoreactive LBs have also been observed in variants of Alzheimer's disease (*6*) and Downs syndrome with Alzheimer's disease (*10*) and in other neurodegenerative diseases, collectively known as synucleopathies (*11*, *12*), which include, among others, dementia with LBs or diffuse Lewy body disease (*3*−*7*). In addition, rare autosomal-dominant familial forms of PD are associated with the missense alanine^{30} → proline (A30P) and alanine^{53} → threonine (A53T) mutations in α-synuclein (*13*, *14*), which are not found in idiopathic PD (*2*, *15*).

Recent studies have suggested various cellular roles for α-synuclein at the presynaptic level in vesicle formation (*16*, *17*) and in the inhibition of phospholipase D2 activity with decreased production of phosphatidic acid (*18*) and subsequent modulation of synaptic vesicle synthesis/recycling (*16*, *18*). Additional evidence suggests the involvement of α-synuclein as a chaperone protein, closely related to the function of 14-3-3 chaperone molecules (*19*, *20*), in the protein ubiquitinylation process (*21*, *22*), and as a possible substrate and/or inhibitor of protein kinase-dependent pathways (*23*, *24*). In both dopaminergic and nondopaminergic neurons, α-synuclein may have a prominent role in neuronal development and plasticity (*25*−*29*) and in neuroprotection (*30*). α-Synuclein is also known to interact with several proteins that either bind to, interact with, or are part of the cytoskeleton, such as tubulin (*31*, *32*), *tau* (*24*), MAP1B (*33*), MAP2 (*34*), synphilin-1 (*35*, *36*), and torsin A (*37*). Although the physiological consequences of α-synuclein interactions with the cytoskeletal components are unclear, a role for this protein in axonal transport has been implicated (*27*).

Emerging studies describing the modulatory effects of α-synuclein on dopamine transporter (DAT) function (*38*−*41*, *47*), on tyrosine hydroxylase activity, the rate-limiting

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[1] Abbreviations: hDAT, human dopamine transporter; LBs, Lewy bodies; PD, Parkinson's disease; DA, dopamine; HBSS, Hank's balanced salt solution; co-IP, coimmunoprecipitation; CT, carboxy terminal; NAC, nonamyloid β component; INDT, indatraline; SMBS, sodium metabisulfite; wt, wild type.

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enzyme in DA biosynthesis (*42*, *43*), and on vesicular storage of DA (*16*, *17*) suggest that a primary normative function of α-synuclein in dopamine-producing neurons may be the regulation and maintenance of DA homeostasis. In particular, α-synuclein interactions with the DAT, which mediates the reuptake of dopamine released into the synaptic cleft by dopamine-producing neurons, regulate dopaminergic content, neurotransmission, and synaptic strength. Excessive intracellular dopamine levels have been linked to the genesis of PD. Since DAT remains the primary determinant in the reuptake of synaptic dopamine, as well as in the uptake of known neurotoxins causing Parkinsonian syndromes, its own regulation is central to the understanding of the neurodegenerative processes in PD. In this regard, very little is known of the mechanisms by which DAT activity is regulated, although the transporter has been shown to be rapidly trafficked to and from the plasma membrane through processes probably involving kinases and the cytoskeleton (*44*−*46*). We have recently shown that wt α-synuclein and its A30P mutant attenuate, by 35−45%, the functional activity of DAT, an effect not seen with the A53T variant (*39*, *41*, *47*). Critical to the modulatory effects of wt α-synuclein and A30P is the formation of stable complexes with DAT through protein−protein interactions, which are lacking with the A53T mutant (*39, 41, 47*).

More recently, we have shown that when L*tk*$^-$ cells are transfected by the batch transfection method, whereby transfection is conducted in a large dish and cells are detached by trypsinization and then replated into individual dishes, we did not observe any attenuation of human DAT (hDAT) activity by either wt α-synuclein or its A30P mutant (*47*). This effect was also seen in cells that were subjected to mild trypsinization (0.1% for 30 s) without cell detachment and replating, giving rise to a speculation that we may have inadvertently reversed, with trypsinization, the modulatory effects of α-synuclein on DAT and thereby disrupted either α-synuclein function or α-synuclein−hDAT protein−protein complex formation (*47*). We therefore investigated this phenomenon further, using mild trypsinization as a tool, to glean further insight into α-synuclein functions and the mechanisms implicated in its modulation of hDAT function. Here we show that mild trypsinization disrupts and reverses the inhibition of hDAT function obtained with α-synuclein and its A30P mutant. By contrast, consistent with the inability of A53T α-synuclein to attenuate hDAT function, there was no effect of trypsin on A53T-dependent events. These effects of trypsin did not disrupt the protein−protein interactions between hDAT and the α-synuclein variants, as trypsinization increased the levels of hDAT−α-synuclein variant complexes. Moreover, the protein−protein interaction between hDAT and α-synuclein variants seen after cell trypsinization occurred through the same protein domains as in nontrypsinized cells. The effects of α-synuclein and the A30P mutant on hDAT occur through modifications of the plasma membrane trafficking of the transporter, which are disrupted by trypsin, as shown by biotinylation studies and confocal microscopy analysis, suggesting that wt α-synuclein and its A30P mutant may be implicated in the hDAT targeting, both to and away from the plasma membrane. These findings provide unique insight into the processes by which α-synuclein modulates hDAT-mediated dopaminergic neurotransmission and dopamine content.

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], Hank's balanced salt solution (HBSS), trypsin (0.1% in HBSS), collagenase (type I; EC 3.4.24.3), Dispase, Pronase E (actinase E; EC 3.4.24.4), papain (papainase; EC 3.4.22.2), chymotrypsin (type II; EC 3.4.31.1), trypan blue, bovine serum albumin (BSA), indatraline hydrochloride, dopamine, sodium nitrite, and sodium metabisulfite (SMBS) were purchased from Sigma. [^{3}H]Dopamine (NET-131; 31.6 Ci/mmol) was from New England Nuclear. All other chemicals were of analytical grade.

cDNAs and Minigene Constructs. Human α-synuclein (a gift from T. Dawson) and hDAT (a gift from F. Liu and H. B. Niznik) cDNA constructs were subcloned into *pcDNA3.1* using the *Eco*RI restriction site. hDAT and α-synuclein DNA constructs, encoding specific structural domains of the proteins, were generated by PCR using oligonucleotides directed to specific areas of cDNA incorporating *Kpn*I and *Xho*I restriction sites and subcloned into *pcDNA3.1*. The constructs incorporated stop codons in the 3′ oligonucleotides and methionine initiation codons in the 5′ oligonucleotides. The subcloned DNAs were resequenced to detect any spurious PCR-generated errors. The α-synuclein constructs were as follows: α-*syn1*, aa1−57; α-*syn2*, aa58−107; α-*syn3*, aa108−140. The hDAT constructs were as follows: *hDAT1*, aa579−620; *hDAT2*, aa579−597; *hDAT3*, aa598−620.

Cell Culture, Transfection, and Trypsinization. L*tk*$^-$ mouse fibroblasts and HEK293 human embryonic kidney and human neuroblastoma-derived SK-N-MC cells were grown in 12-well dishes (seeding density 1×10^5 cells/well) in DMEM plus 10% (v/v) heat-inactivated and selected FBS, antibiotics, and 2 mM L-glutamine at 37 °C and 5% CO_2 and transiently transfected (2−3 μg of DNA/1×10^5 cells) using hDAT and α-synuclein variant DNAs by the DEAE-dextran/chloroquine method as described before (*39*, *41*, *47*). In all transfections, and in particular for cells singly transfected with hDAT alone, the total amount of DNA used in transfections was equalized by addition of an equal amount of *pcDNA3.1* control vector DNA. After transfection (24 h), cells were rinsed twice with PBS, either grown for a further 24 h (control cells) or submitted to 0.1% (w/v) trypsin for 30 s, without cell detachment, rinsed twice with DMEM plus 10% FBS, and grown for another 24 h in DMEM plus 10% FBS. When the effect of other proteases on [^{3}H]DA uptake was studied, cotransfected L*tk*$^-$ cells were rinsed twice 24 h after transfection with PBS, either grown for a further 24 h (control cells) or submitted to 0.1% (w/v in D-PBS) collagenase I or Dispase for 1 min or 0.1% (w/v) chymotrypsin (in PBS), Pronase (in D-PBS), or papain (in D-PBS) for 2 min, without cell detachment, rinsed twice with DMEM plus 10% FBS, and grown for another 24 h in DMEM plus 10% FBS. To show the dose-dependent reversal of wt α-synuclein or its A30P mutant, but not the A53T mutant, induced modulation of hDAT-mediated [^{3}H]DA uptake (20 nM) by various concentrations of trypsin, cotransfected cells were processed as described before, except that mild trypsinization (30 s) was performed with various concentrations of trypsin (in % v/v), obtained by diluting, in HBSS,

the trypsin solution (0.1% in HBSS) purchased from Sigma. When oxidative stress and cell death were assessed, cells were washed three times with D-PBS 36 h after transfection and incubated overnight in DMEM without serum, followed by a 24 h treatment with dopamine (200 μM) in DMEM without serum, in the presence or absence of either the DAT blocker indatraline (INDT) or the antioxidant sodium metabisulfite (SMBS). INDT (10 μM) or SMBS (200 μM) was added 30 min before beginning the dopamine (DA) treatment. Control cells were treated with an equal concentration of the solvent (0.2% H_2O) and processed in a similar way.

Neuronal Cultures and Trypsinization. Mesencephalon from 18-day-old rat embryos were isolated; cells were dissociated by mechanical disruption, counted (600−800 cells seeded/mm^2), and grown in neurobasal medium supplemented with 2% (v/v) B-27 supplement and 50 μM β-mercaptoethanol in 35 mm Petri dishes precoated with poly(L-ornithine) (2 μg/mL in borate buffer, pH 8.4; Sigma) and laminin (3 μg/mL in PBS; Sigma) for 6 days. Neurons were carefully washed twice with PBS, subjected to mild trypsinization (0.1% trypsin for 30 s), and carefully washed twice with PBS, and neurons were allowed to recover for 24 h after the addition of fresh medium.

Nitrite Production and Cell Death Measurements. Oxidative stress was measured by assessing nitrite levels, a stable byproduct of NO, as described previously (*40*, *41*). Briefly, 0.3 mL of Griess reagent (1 part of 0.1% naphthylethylenediamine dihydrochloride in H_2O and 1 part of 1% sulfanilamide in 5% H_3PO_4) and 0.3 mL of culture medium from treated cells were mixed. After 30 min incubation at 45 °C, the absorbance at 550 nm was determined. The NO_2^- concentration (pmol/1.0 × 10^5 cells) was determined from a standard curve using $NaNO_2$ (Sigma) at a range of 0−10 μM. Nitrite levels originating from the chemical oxidation of DA (due to its instability in the absence of reducing agents) were measured in the absence of cells in parallel wells (processed in a similar manner as the wells used for drug treatment) and were subtracted from the nitrite levels induced by the DA cell treatment. Cell death was determined by the MTT cell viability assay. Briefly, treated cells were carefully washed twice with D-PBS and incubated for 3 h at 37 °C and 5% CO_2 in DMEM without serum containing 0.5 mg/mL MTT. After two washes with D-PBS, formazan salts, derived from the reduction of MTT by cellular dehydrogenases of viable cells, were solubilized with pure ethanol, and the absorbance at 564 nm was determined by visible spectrophotometry against an ethanol blank.

[^{3}H]Dopamine Uptake. Dopamine uptake was measured by incubation of cells with 20 nM [^{3}H]DA in uptake buffer for 10 min as described by Lee et al. (*38*). For kinetic analysis, cells were preincubated with unlabeled DA (10^{-11}−10^{-4} M) for 5 min prior to addition of [^{3}H]DA strictly as described by Lee et al. (*38*); 10 μM INDT was used to define nonspecific uptake. An aliquot of cells was collected for cell counting using trypan blue, and radioactivity incorporated into the remaining cells was measured by scintillation counting after hydrolysis of cellular proteins by 0.1 N NaOH for 1 h at 37 °C.

Coimmunoprecipitations and Western Blotting. Cotransfected L*tk*$^-$ cells [(1−2) × 10^7] were solubilized with 1% sodium cholate in lysis buffer [50 mM Tris buffer, pH 7.6, containing 500 mM NaCl, 250 mM sucrose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 200 μM sodium orthovanadate, 5 μg/mL each of leupeptin and pepstatin, and 500 μM PMSF], as previously described (*47*, *48*). To the soluble extracts (400 μL/assay tube, 1−1.5 mg/mL protein) was added the following antiserum: DAT polyclonal Ab (1:100, Chemicon AB5802) or α-synuclein polyclonal Ab (1:100, Chemicon AB5334P) or nonimmune sera (4 μg of protein) or the heat-inactivated (100 °C for 10 min) specific antisera (used at 1:100). After overnight incubation (12 h), immune complexes were precipitated with protein A−Sepharose beads (50% slurry, CL-4B; Pharmacia), and pellets were washed and subjected to SDS−PAGE and Western blots. Blots were probed with antibodies for either hDAT (1:1000, Chemicon MAB 369 monoclonal antibody) or α-synuclein (1:500; BD Transduction Labs monoclonal antibody 610787). Proteins were visualized using peroxidase-conjugated secondary antibodies (1:7500; Santa Cruz) and enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Biotinylation of Plasma Membrane Proteins. Two days after transfection, L*tk*$^-$ cells were washed in ice-cold PBS, pH 8.5, containing 1 mM $MgCl_2$ (PBS-M) and incubated on ice for 30 min with gentle shaking with a freshly prepared solution of the water-soluble, cell-impermeable biotin analogue, EZ-link NHS-biotin (Pierce; 0.5 mg/mL in PBS-M), as previously described (*47*). The reaction was stopped by removing the biotin solution, washing the cells with ice-cold PBS-M, and incubating for 10 min on ice with gentle agitation in 50 mM glycine (in PBS-M). After being rinsed with PBS-M, the cells were collected in ice-cold lysis buffer [50 mM Tris buffer, pH 7.6, containing 500 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, 5 μg/mL each of leupeptin and pepstatin, 500 μM PMSF, and 1% (w/v) sodium cholate] and rocked for 1 h at 4 °C. The lysates were cleared by centrifugation at 18500*g* at 4 °C and assayed for protein content by the method of Lowry (*49*). Lysate volumes were adjusted to equalize protein concentration with dilution buffer (20 mM Tris buffer, pH 7.5, containing 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 μg/mL each of leupeptin and pepstatin, 200 μM sodium orthovanadate, and 500 μM PMSF). To the soluble diluted extracts (400 μL/assay tube, 1−1.5 mg/mL of protein) was added the following antiserum for immunoprecipitation as described above: DAT polyclonal Ab (1:100, Chemicon AB5802), α-synuclein polyclonal Ab (1:100, Chemicon AB5334P), or nonimmune sera (4 μg of protein). After an overnight rocking at 4 °C, 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 blots. Blots were blocked for 1 h with 5% (w/v) BSA in TTBS (10 mM Tris buffer, pH 7.4, containing 150 mM NaCl and 0.05% Tween-20) and probed for 1 h with HRP-conjugated avidin (1:1000 in TTBS plus 5% BSA; Pierce) to quantify biotin-bound hDAT, a reliable index of the amount of hDAT present at the cell surface. In parallel, reverse experiments were carried out, and solubilized protein samples (∼1 mg/mL protein) were affinity purified with Sepharose beads conjugated to NeutrAvidin (Pierce), according to the manufacturer's protocol. After an overnight rocking at 4 °C, beads were washed five times, and bound

proteins were solubilized by heating the beads for 10 min at 65 °C in 2× Laemmli buffer, followed by SDS−PAGE and Western blots. Blots were probed with antibodies for either hDAT (1:1000, Chemicon MAB 369 monoclonal antibody) or α-synuclein (1:500, BD Biosciences monoclonal antibody 610787). Proteins were visualized after incubation with peroxidase-conjugated secondary antibodies (1:7500; Santa Cruz) at room temperature for 1 h using enhanced chemiluminescence (Amersham, Arlington Heights, IL). To ensure that equal expression levels of either hDAT or α-synuclein protein were present in each sample (biotinylated plus nonbiotinylated protein), we verified that similar levels of hDAT or α-synuclein protein could be immunoprecipitated from each sample by their specific antisera, as described above, or by directly analyzing the whole cell lysates on Western blots, as described above.

Immunofluorescence and Confocal Microscopy. Forty-eight hours after transfection, cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% (v/v) Triton X-100 in D-PBS as described previously (*50*). Cells were incubated with primary anti-hDAT polyclonal antiserum (1:500, Chemicon AB5802) and with primary anti-α-synuclein monoclonal antiserum (1:200, BD Transduction Labs monoclonal antibody 610787) diluted in D-PBS plus 0.05% (v/v) Triton X-100 plus 1% (w/v) BSA for 12 h at 4 °C and for 2 h at room temperature with Alexa Fluor 568- and Alexa Fluor 488-conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR) diluted in D-PBS plus 0.05% (v/v) Triton X-100 plus 1% (w/v) BSA. Cells were analyzed with a Nikon Eclipse E800 inverted fluorescent microscope or a Leica TCS4D fluorescent confocal microscope. For the latter, successive fluorescence acquisitions were performed using the 488 and the 568 nm laser lines to excite Alexa488 and Alexa568. The fluorescence was selected with appropriate double fluorescence dichromic mirrors and band-pass filters and measured with blue-green sensitive and red-side sensitive photomultipliers. The absence of cross detection between the fluorescent emissions was carefully checked.

Data Analysis. Kinetic parameters of dopamine 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). Statistical significance of the experimental results was obtained by variance analysis with Fisher's test using Instat Statistical Software (Graphpad, Sorrento Valley, CA). $p < 0.05$ was considered to denote statistical significance.

RESULTS

Mild Trypsinization Reverses Attenuation of hDAT Activity Induced by wt and A30P a-Synucleins. L*tk*$^-$ cells were cotransfected with 1 μg of DNA each/10^5 cells of hDAT and either wt α-synuclein or pcDNA3.1 (used as a control) DNAs; at these concentrations and ratios, the expression levels of α-synuclein and DAT seem to be similar to that seen in the endogenously expressing rat substantia nigra, that were assessed by Western blots using increasing amounts of DNA in transfected cells (Wersinger, Banta, and Sidhu, unpublished observations). Following mild trypsinization (0.1%, 30 s), the activity of hDAT was assessed by [^{3}H]DA uptake studies. In nontrypsinized cotransfected cells, the presence of wt α-synuclein decreased hDAT activity by 30% as shown previously (*39*, *41*, *47*), but mild trypsinization relieved this inhibition with full reversal of [^{3}H]DA uptake to levels observed in cells expressing only hDAT (Figure 1A). This reversal in hDAT function was not due to changes in K_m of the transporter for its preferred ligand but was solely due to an increase in the translocation velocity of uptake (V_{max}) of DA (Table 1). The effect of trypsin on hDAT was indirect, since trypsin did not directly change the functional properties of hDAT itself; in trypsinized L*tk*$^-$ cells transfected with hDAT DNA alone, there were no changes in the kinetics of [^{3}H]DA uptake relative to nontrypsinized cells (Table 1). That the increase in [^{3}H]DA uptake proceeded entirely through the functional actions of hDAT and not through passive diffusion or increased permeability of the plasma membrane, which may have been compromised by such treatments, is demonstrated by the ability of the hDAT blocker, INDT, to completely prevent [^{3}H]DA uptake in a specific manner in treated cells (Figure 1A). Moreover, plama membrane permeability was not significantly modified by such treatments, as assessed by trypan blue or neutral red staining of trypsinized cells (data not shown). In all instances, [^{3}H]DA basal efflux was negligible in all experimental conditions used in this study and unaffected by the coexpression of hDAT with wt α-synuclein or its two familial PD-linked mutants (data not shown).

We next examined the effects of mild trypsinization in L*tk*$^-$ cells cotransfected with the A30P or A53T mutants of α-synuclein. Similar to the wt α-synuclein, trypsin reversed the A30P variant-mediated inhibition of hDAT activity, with full restoration of functional activity (Figure 1B). Moreover, the increase in hDAT function was also due to increased V_{max}, without changes in K_m (Table 1). In cells cotransfected with hDAT and the A53T mutant, there was no significant ($p > 0.05$) change in [^{3}H]DA uptake (Figure 1B), whatever cells were subjected to mild trypsinization or not, consistent with the inability of this mutant to modulate hDAT function (*41*).

The reversal of the wt- and A30P α-synuclein-induced modulations of hDAT-mediated [^{3}H]DA uptake by increasing amounts of trypsin (30 s) in cotransfected L*tk*$^-$ cells was dose-dependent (Figure 1C). Concentrations as low as 0.01% (v/v) trypsin were sufficient to significantly ($p < 0.05$) show a reversal of the effects of wt α-synuclein or its A30P mutant on hDAT activity. Again, trypsin did not affect hDAT activity in cells coexpressing hDAT with the A53T mutant of α-synuclein.

These findings were also confirmed in two other cell lines, HEK293 (Figure 1D) and SK-N-MC (Figure 1E) cells, where specific relief of hDAT inhibition seen with both wt α-synuclein and the A30P mutant upon mild trypsinization was noted. In cells cotransfected with the A53T mutant, trypsin failed to cause any changes in hDAT function, and [^{3}H]DA uptake remained at levels similar to that of cells expressing only hDAT, indicating a complete lack of effect of this variant on hDAT function, regardless of the experimental condition.

To ascertain whether these effects of trypsin were also detectable in endogenously expressing cells and were not merely an artifact of transfected cells, rat primary mesencephalic neurons were also similarly subjected to mild trypsinization, and [^{3}H]DA uptake studies were conducted (Figure 1F). When compared to nontrypsinized neurons, there

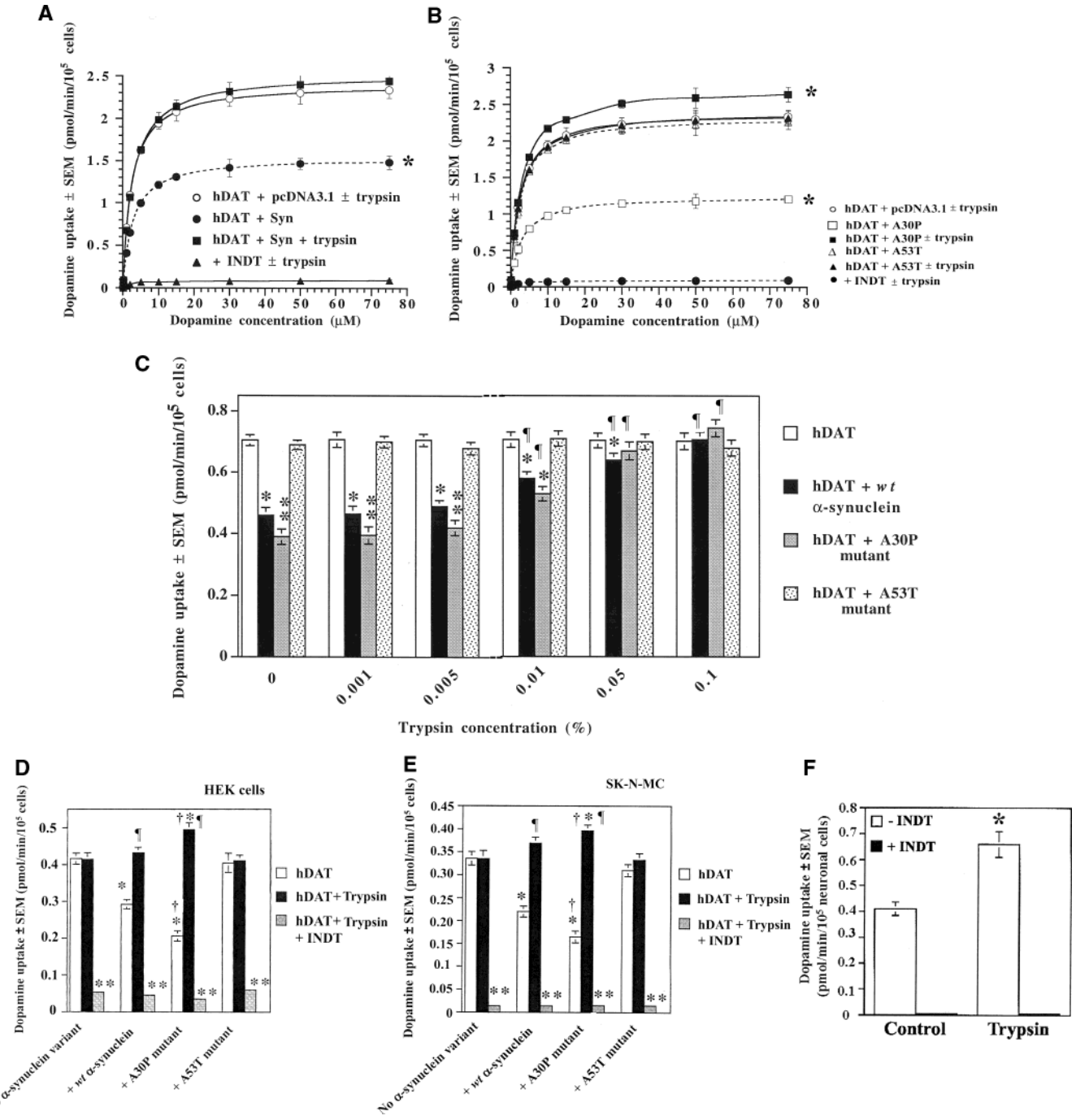

FIGURE 1: Reversal of wt α-synuclein- or A30P-mediated attenuation of hDAT functional activity upon trypsinization. The indicated cells were cotransfected with hDAT and either pcDNA3.1, wt α-synuclein, A30P DNA, or A53T DNA (1 μg of DNA each/1 $\times$ 10^5 cells). 24 h after transfection, cells were subjected to mild trypsinization (0.1%, 30 s; trypsin), and after 24 h of further growth, hDAT activity was measured in quadruplicate with 20 nM [^{3}H]DA, as described in Experimental Procedures. Panels A and B show the saturation curves of [^{3}H]DA uptake in hDAT plus either pcDNA3.1 control vector or wt α-synuclein (Syn) cotransfected L*tk*$^-$ cells (A) or in hDAT plus either pcDNA3.1 control vector or A30P or A53T variants of α-synuclein cotransfected L*tk*$^-$ cells (B) after preincubation of cells with the indicated concentrations of unlabeled dopamine for 5 min. Panel C shows the dose-dependent reversal, in cotransfected L*tk*$^-$ cells, of wt α-synuclein- or its A30P mutant-, but not the A53T mutant-, induced modulation of hDAT-mediated [^{3}H]DA uptake (20 nM) by various concentrations of trypsin. Cotransfected cells were subjected to mild trypsinization (30 s) with various concentrations of trypsin (in % v/v). In panels D and E, uptake of [^{3}H]DA (20 nM) was measured in hDAT plus either pcDNA3.1 control vector, wt α-synuclein, or A30P or A53T variants of α-synuclein cotransfected HEK293 (D) and SK-N-MC (E) cells, respectively. In panels A, B, D, and E, the specificity of the uptake was measured with 10 μM hDAT blocker INDT. Data shown in panels A−E are the mean ± SEM of three to four experiments performed in quadruplicate. Key: ** ($p < 0.01$) or * ($p < 0.05$), significantly different from cells expressing only hDAT; ¶ ($p < 0.05$), significantly different from cells not subjected to trypsinization; † ($p < 0.05$), significantly different from hDAT plus wt α-synuclein expressing cells for each group (trypsinized or nontrypsinized). In panel F, mesencephalic neurons were either subjected or not subjected to mild trypsinization, and [^{3}H]DA uptake (20 nM) was measured in the presence or absence of 10 μM INDT, as described in Experimental Procedures. Data shown are the mean ± SEM of four determinations performed in triplicate. Key: * ($p < 0.05$), significantly different from nontrypsinized neurons.

Table 1: Effect of Mild Trypsinization on the Kinetic Parameters of [^{3}H]DA Uptake in Transfected L*tk*$^-$ Cells

	V_{max} [pmol min^{-1} (10^5 cells)$^{-1}$]		K_m (μM)	
transfection	−trypsin	+trypsin	−trypsin	+trypsin
hDAT + pcDNA3.1	2.34 ± 0.09	2.31 ± 0.07	2.55 ± 0.10	2.68 ± 0.13
hDAT + α-synuclein	1.55 ± 0.09[a]	2.53 ± 0.13	2.78 ± 0.09	2.81 ± 0.15
hDAT + A30P	1.28 ± 0.08[b,c]	2.79 ± 0.12[a]	2.79 ± 0.06	2.82 ± 0.12
hDAT + A53T	2.24 ± 0.10	2.40 ± 0.10	2.78 ± 0.09	2.75 ± 0.15

[a] $p < 0.05$, significantly different from hDAT + pcDNA3.1 cotransfected cells. [b] $p < 0.01$, significantly different from hDAT + pcDNA3.1 cotransfected cells. [c] $p < 0.05$, significantly different from hDAT + wt α-synuclein cotransfected cells.

was a significant ($p < 0.05$) increase of ~55% in [^{3}H]DA uptake in neurons that were trypsinized. This increase is similar in magnitude to that seen in cotransfected cells expressing hDAT and wt or A30P α-synuclein (Table 1). That this increase in [^{3}H]DA uptake by neurons was due to the hDAT and not due to changes in membrane permeability or efflux of the radioligand was confirmed through the use of INDT. These data show that the trypsinization effect can also be demonstrated in neurons and that trypsinization may be a useful tool for analyzing α-synuclein−hDAT interactions and their functional outcome.

It should be noted that under all these experimental conditions trypsin did not cause any detachment of cells from the culture dishes, and both total cell counts, indexed by cell counting using the trypan blue exclusion method (*41*), and cell viability, indexed by the MTT cell viability assay, were similar in both control dishes not treated with trypsin and dishes that were trypsinized (data not shown).

To assess whether the reversal of α-synuclein-induced inhibition of hDAT function by tryspin was also seen using other proteases which perturb cell adhesion, cotransfected L*tk*$^-$ cells were treated with collagenase and Dispase (0.1%, 1 min each). Thus, upon treatment with either collagenase or Dispase, the inhibition of hDAT function by wt α-synuclein was significantly ($p < 0.5$) reversed by 78% and 76%, respectively (data not shown), similar to the near 100% reversal obtained with trypsin. That the increase in [^{3}H]DA uptake proceeded entirely through the functional actions of hDAT and not through passive diffusion or increased permeability of the plasma membrane, which may have been compromised by such treatments, is demonstrated by the ability of the hDAT blocker, INDT, to completely prevent [^{3}H]DA uptake in a specific manner in treated cells (data not shown). Moreover, plasma membrane permeability was not significantly modified by such treatments, as assessed by trypan blue or neutral red staining of collagenase- or dispase-treated cells, and basal [^{3}H]DA efflux was negligible and unaffected by the treatment with proteases (data not shown). By contrast, proteases which do not significantly affect cell adhesive properties, such as chymotrypsin, Pronase, and papain (0.1% each for up to 2 min of cell treatment), did not alter the attenuation of hDAT function by wt α-synuclein (data not shown). Similar to trypsin, hDAT activity was unchanged by pretreatment with the proteases in cells expressing only hDAT (data not shown). Together, these data show that the response seen with trypsin was also mimicked by other proteases that affect cell adhesion, implicating cellular adhesive properties in the modulation of hDAT function by α-synuclein. As trypsinization is the most widely used method for cell detachment and dissociation, and induces the most efficient reversal of α-synuclein-induced modulation of hDAT activity, trypsin (0.1%, 30 s) was used to analyze the contribution of cell adhesion to α-synuclein-induced attenuation of hDAT activity.

Relief of α-Synuclein-Mediated Inhibition of hDAT Activity Accelerates Dopamine-Induced Neurotoxicity. A consequence of the attenuation of hDAT activity by wt or the A30P α-synuclein is the reduction of dopamine-mediated cytotoxicity, indexed by both ROS production and cell death, consistent with reduced uptake of dopamine (*41*, *47*). Conversely, increased uptake of dopamine would imply increased intracellular dopamine-mediated cytotoxicity. We therefore assessed this in L*tk*$^-$ cells by treating both trypsinized and nontrypsinized cells with 200 μM dopamine for 24 h, followed by measurements of nitrite production, an indirect indicator of oxidative stress, and cell death. In cells transfected with hDAT alone, the intracellular autoxidation of dopamine caused an elevated production of nitrite (Figure 2A), which was also accompanied by decreased cell viability (Figure 2B). That this increase was due to hDAT-mediated uptake of dopamine was demonstrated with INDT, which abolished dopamine-mediated nitrite production (Figure 2A) and prevented cell death (Figure 2B).

In cells cotransfected with hDAT and wt α-synuclein, both nitrite production and cell viability were increased upon trypsinization to levels similar to that obtained in cells singly transfected with hDAT (Figure 2). Similar results were also seen with the A30P mutant of α-synuclein, although the production of nitrite and decrease in cell viability upon trypsinization were significantly ($p < 0.05$) higher than those of trypsinized cells transfected with hDAT alone. These indices were also somewhat higher compared to trypsinized cells transfected with wt α-synuclein, but the difference was not statistically significant. In both trypsinized and nontrypsinized A53T expressing cells, both nitrite levels and cell viability were nearly identical to those of cells cotransfected with hDAT alone, consistent with lack of attenuation of transporter function by this mutant. In all instances, INDT (and reduced vitamin C; data not shown) ablated the intracellular cytotoxicity of dopamine.

Trypsin Increases Associative Protein−Protein Interactions between α-Synuclein Variants and hDAT. We have previously shown that α-synuclein and hDAT form complexes, through protein:protein interactions, and that such complexes are essential for the attenuation of hDAT activity (*39*, *41*, *47*). We, therefore, speculated that the reversal of α-synuclein-induced inhibition of hDAT activity by trypsin may be due to disruption of such complexes. To test for this, co-IP studies were conducted using lysates from nontrypsinized and trypsinized L*tk*$^-$ cells, singly transfected with hDAT or α-synuclein variants, and from cotransfected cells, as described in Experimental Procedures. Surprisingly,

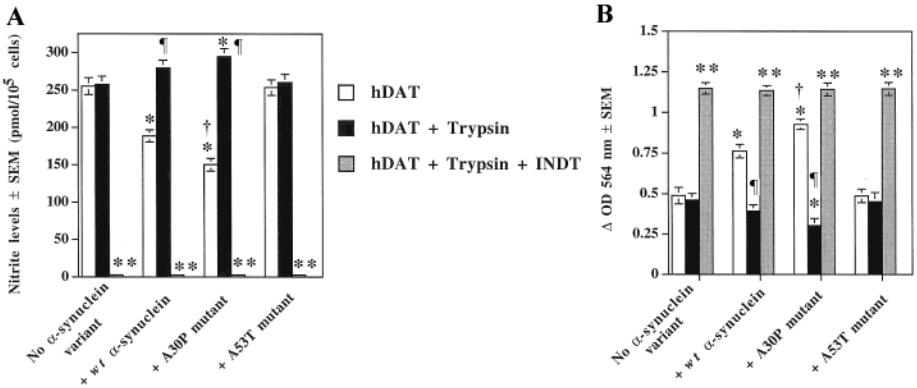

FIGURE 2: Relief of wt α-synuclein- or A30P mutant-mediated inhibition of hDAT activity accelerates dopamine-induced nitrite production and cell death in transfected L*tk*$^-$ cells. L*tk*$^-$ cells were cotransfected with hDAT and either pcDNA3.1, wt α-synuclein, A30P DNA, or A53T DNA (1 μg of DNA each/1 $\times$ 10^5 cells) and subjected or not subjected to mild trypsinization (0.1% trypsin, 30 s) 36 h after transfection as described in Experimental Procedures. 12 h after trypsinization, cultures were serum-starved in serum-free DMEM for 12 h and treated with 200 μM dopamine for 24 h in the presence or absence of INDT (10 μM), followed by measurement of nitrite production (A) by the Griess method or cell death (B) by the MTT cell viability assay as described in Experimental Procedures. Nitrite production and cell death observed in dopamine-treated, mock-transfected cells (78 ± 10.95 pmol of nitrite/10^5 cells and $\Delta OD_{564nm} = 0.155 \pm 0.012$) were subtracted from each group. Data shown are the mean ± SEM of four experiments performed in quadruplicate. Key: ** ($p < 0.01$) or * ($p < 0.05$), significantly different from cells expressing only hDAT; ¶ ($p < 0.05$), significantly different from cells not subjected to mild trypsinization; † ($p < 0.05$), significantly different from hDAT plus wt α-synuclein expressing cells for each group (trypsinized or nontrypsinized).

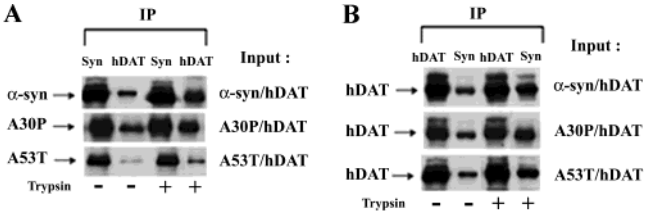

FIGURE 3: Mild trypsinization increases the associative protein−protein interactions between α-synuclein variants and hDAT in cotransfected L*tk*$^-$ cells. L*tk*$^-$ cells were cotransfected (input) with hDAT and either wt α-synuclein (α-syn/hDAT), A30P (A30P/hDAT), or A53T (A53T/hDAT) DNAs (1 μg of DNA each/1 $\times$ 10^5 cells) and subjected to mild trypsinization (+trypsin) or no trypsinization (−trypsin) as described under Experimental Procedures. Sodium cholate lysates were prepared, and IP studies were conducted using either hDAT (IP hDAT) or α-synuclein (IP Syn) antibodies, as described under Experimental Procedures. Proteins present in the immunopellets were probed by Western blots using antibodies against the α-synuclein variants (A) or hDAT (B). Blots were scanned to measure relative levels of immunoprecipitated proteins present in pellets obtained from trypsinized and nontrypsinized cells. Data shown are representative of three experiments.

trypsin did not interfere with the formation of protein−protein complexes but appeared to promote and/or stabilize such interactions. Thus, by using hDAT antibodies, a 3-fold *increase* in wt α-synuclein protein was found to be associated with the hDAT immunopellet upon treatment with trypsin as compared to the nontrypsinized cells (Figure 3A, top panel). The increased association of wt α-synuclein with hDAT in the coimmunoprecipitates was not due to any increase in expression of α-synuclein protein per se since the amount of α-synuclein detected by IP with its own antibody was identical in nontrypsinized and in trypsinized cotransfected cells (Figure 3A, top panel). Similarly, when α-synuclein antibodies were used in the reciprocal co-IPs, there was an ~3-fold increase in hDAT coimmunoprecipitated by these antibodies from lysates of trypsinized cells as compared to nontrypsinized cotransfected cells (Figure 3B, top panel). The increase in hDAT protein was also not due to any changes in protein, since the total amount of hDAT protein immunoprecipitated by its own antibody was identical in these lysates irrespective of trypsinization (Figure 3B, top panel).

We next analyzed the effect of trypsinization on protein−protein interactions between the A30P (Figure 3, middle panel) or the A53T (Figure 3, bottom panel) mutant and hDAT. In both instances, trypsinization increased by ~3-fold the association between the A30P or A53T variant and hDAT, similar to the results obtained with the wt α-synuclein. Moreover, the increased presence of either the α-synuclein variants or hDAT in the co-IPs after trypsinization was also not due to increases in protein expression levels. Of particular note is that the brief and weak trypsinization of cells also did not cause proteolysis of either hDAT or α-synuclein proteins, as indexed by a lack of change in both the M_r or the amount of protein obtained after IPs with their own specific antibodies. Rather, the combined data suggest that mild trypsinization somehow increases the *availability* of these proteins to physically interact with one another, such that there is now *increased* protein−protein interactions between hDAT and the α-synuclein variants. The relief of α-synuclein-induced negative modulation of hDAT activity by mild trypsinization is therefore not due to the disruption of hDAT−α-synuclein protein−protein complexes.

In all of the co-IPs studies, no coimmunoprecipitation between hDAT and either wt, A30P, or A53T variants of α-synuclein could be noticed when specific antibodies were replaced either by nonimmune sera or by heat-inactivated specific antisera (data not shown).

Trypsin Does Not Modify the Interactions between the Subdomains of α*-Synuclein Variants and hDAT.* Using DNA constructs encoding specific regions of wt α-synuclein or its familial PD-linked mutants and hDAT, we have previously shown that protein−protein interactions between these proteins occur through the NAC domain of the α-synuclein variants (aa58−107) and the last 22 amino acids of the CT tail of hDAT (aa598−620) (*41*, *47*). To test if the increased associations between α-synuclein variants and hDAT in the trypsinized cells were linked to the fact that different domains

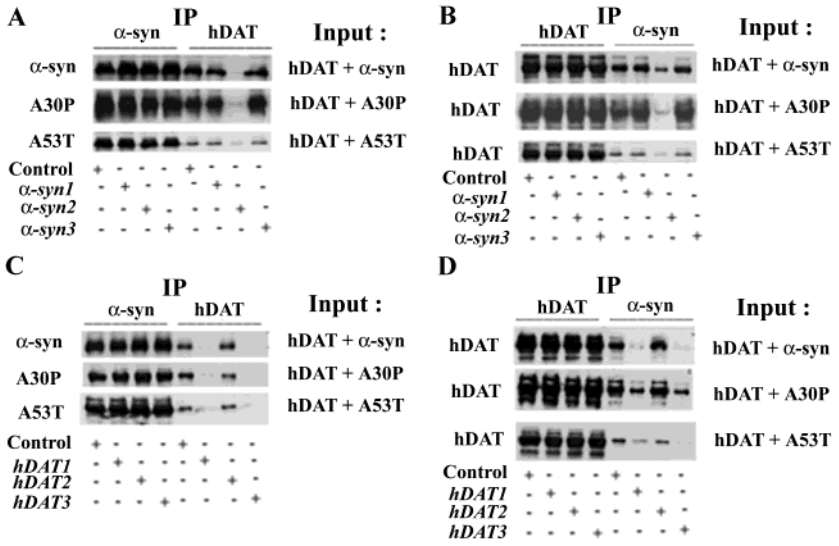

FIGURE 4: Trypsin does not alter interactions between specific subdomains of α-synucleins and hDAT. In addition to DNA encoding full-length hDAT and full-length α-synuclein variants (1 μg of DNA each/1.0 × 10^5 cells), L*tk*$^-$ cells were cotransfected with DNA constructs (1 μg of DNA each/1 × 10^5 cells) encoding specific regions of either the α-synuclein variants (panels A and B; α-*syn1*, aa1−57; α-*syn2*, aa58−107; α-*syn3*, aa108−140) or hDAT (panels C and D; *hDAT1*, aa579−620; *hDAT2*, aa579−597; *hDAT3*, aa598−620). 48 h after transfection, cells were subjected to mild trypsinization, and sodium cholate-solubilized cells were used in IP or co-IP studies using antibodies directed against either hDAT (IP hDAT) or the α-synuclein variants (IP α-syn). Proteins present in the immunopellets were analyzed by Western blots as described under Experimental Procedures. Panels A and C show the immunoreactivity for the α-synuclein variants whereas panels B and D show the hDAT immunoreactivity in the three cotransfection conditions (input). Data shown are representative of three experiments.

of both proteins are interacting compared to nontrypsinized cells, hDAT and α-synuclein variants coexpressing cells were additionally cotransfected with constructs encoding different subdomains of the α-synuclein variants or hDAT. Lysates were prepared from such cotransfected cells after trypsinization, and co-IPs were conducted with either α-synuclein or hDAT antibodies. The data show that, in these trypsinized cells, only the coexpression of α-*syn2*, encoding the NAC domain (residues 58−107), prevented the formation of a complex between the wt α-synuclein and hDAT, while constructs α-*syn1* or α-*syn3* did not (Figure 4A,B). Similar results were also obtained using lysates from trypsinized cells coexpressing hDAT with either the A30P or the A53T mutant, and in all instances the NAC domain encoded by α-*syn2* prevented interactions between these mutant proteins and hDAT. In all studies, we verified that the construct proteins did not prevent the interaction between the α-synucleins with its own antibody by conducting IP studies with the α-synuclein antibody wherein similar levels of full-length α-synuclein proteins were immunoprecipitated (Figure 4A,B).

We next conducted studies by cotransfecting cells with hDAT and α-synuclein variant DNAs in the additional presence of hDAT constructs encoding the carboxy-terminal (CT) tail of hDAT (Figure 4C,D). We found that *hDAT1* and *hDAT3*, those sequences encoding residues 579−620 and 598−620, respectively, of the CT tail of hDAT, blocked the interaction between the α-synuclein variants and hDAT, while the construct *hDAT2* did not, testifying to the participation of the terminal amino acids of the CT tail of hDAT in the α-synuclein variant/hDAT interactions, even after mild trypsinization of the cells. These hDAT constructs also did not block the interactions between the full-length hDAT with its own antibody as evidenced by IP studies. These combined results indicate that trypsinization does not alter the association of α-synuclein and hDAT to form protein complexes and that these proteins continue to interact with one another through the same amino acid motifs identified earlier in nontrypsinized cells (*41*, *47*).

Trypsin Increases Recruitment of hDAT to the Plasma Membrane Surface in Ltk$^-$ *Cells Coexpressing hDAT and the wt* α-*Synuclein.* We next conducted confocal microscopy studies to examine whether there were any changes in hDAT localization upon trypsinization. In hDAT plus wt α-synuclein cotransfected nontrypsinized cells, there was substantial overlap in hDAT and wt α-synuclein labeling, evidenced at both the plasma membrane and intracellular reticular compartments, indicating partial colocalization of these proteins within the cell (Figure 5, panels A−C). However, wt α-synuclein appeared to be predominantly distributed along the plasma membrane, while hDAT appeared to be less well located at the plasma membrane. Upon treatment with low amounts of trypsin, an initiation of retraction of the plasma membrane was observed, marked by numerous spicules seen all around the cells (Figure 5, panels E and F, arrowheads). This was accompanied by a relative enhancement of hDAT labeling in the spicules, with a conspicuous lack of wt α-synuclein labeling in these spicules (Figure 5, panels D−F, arrowheads). In particular, at the plasma membrane there appeared to be enhanced localization of hDAT highlighted by the general observation of a red border of hDAT labeling at the periphery of the cells (Figure 5, panels D−F, arrows). There was also substantial hDAT immunoreactivity seen in the cytoplasm of the cell after mild trypsinization, indicating that not all of the hDAT was relocated to the plasma membrane, consistent with our observation that trypsinization causes an increase of ∼30% of hDAT activity in cells coexpressing hDAT with wt α-synuclein. Together, these data suggest that trypsinization may cause a discrete reorganization of the cellular distribution of hDAT and wt α-synuclein, resulting in increased recruitment of hDAT to the plasma membrane in cells coexpressing hDAT with wt α-synuclein. Such

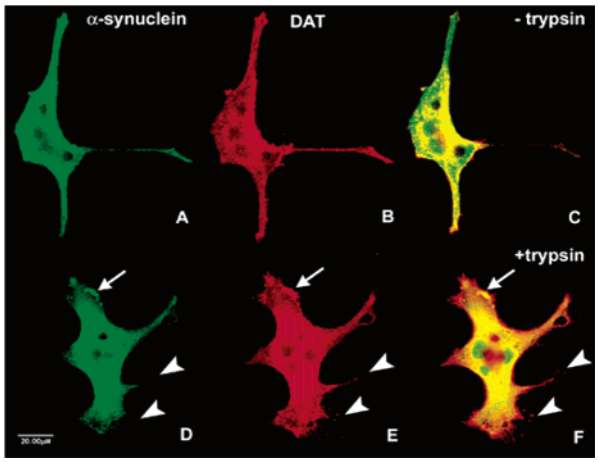

FIGURE 5: Trypsinization increases recruitment of hDAT to the plasma membrane surface in hDAT plus wt α-synuclein cotransfected L*tk*⁻ cells. L*tk*⁻ cells cotransfected with hDAT and α-synuclein DNAs (1 μg of DNA each/10^5 cells) were either subjected (panels D−F) or not subjected (panels A−C) to mild trypsinization (0.1% trypsin; 30 s) and analyzed by confocal microscopy to assess the subcellular distribution of hDAT (red labeling) and α-synuclein (green labeling) immunoreactivities as described under Experimental Procedures. Confocal analysis of trypsinized cotransfected cells shows that the hDAT immunostaining at the plasma membrane becomes relatively stronger and tends to go beyond the α-synuclein labeling, especially when plasma membrane tends to retract (arrow in panels D−F). Note the enhancement of hDAT labeling in spicules of trypsinized cells (arrowheads in panels D−F). Data shown are representative of four independent studies.

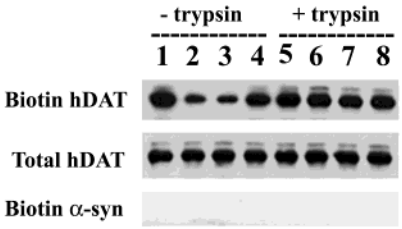

FIGURE 6: Biotinylation of hDAT in L*tk*⁻ cells coexpressing hDAT and the variants of α-synuclein and subjected or not subjected to mild trypsinization. L*tk*⁻ cells were transfected with hDAT and either pcDNA3.1 control vector (lanes 1 and 5) or the wt (lanes 2 and 6), A30P (lanes 3 and 7), or A53T (lanes 4 and 8) variants of α-synuclein DNAs (1 μg DNA each/1.0 × 10^5 cells), and cell surface proteins present in cells subjected (+trypsin) or not subjected (−trypsin) to mild trypsinization were labeled with EZ-link NHS-biotin as described in Experimental Procedures. Biotinylated hDAT levels were assessed by immunoprecipitating total hDAT and looking for the fraction of total hDAT that was biotinylated with avidin−HRP (upper gel). Total hDAT present in each fraction was measured by conducting IP studies using anti-hDAT antibodies, followed by Western blots (middle gel). Biotinylated α-synucleins (biotin−α-syn) were assessed by IP using anti-α-synuclein antibodies as described in Experimental Procedures, and blots were probed with avidin−HRP (bottom 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). Data shown are representative of three experiments.

increased localization of hDAT at the plasma membrane is likely to account for the enhanced dopamine uptake and cell toxicity measured in the same experimental conditions in cells coexpressing hDAT with wt α-synuclein.

Biotinylation Studies of hDAT in Transfected Ltk⁻ Cells. To confirm the qualitative findings obtained with the confocal immunocytochemistry experiments, quantitative biochemical studies were conducted whereby surface proteins were labeled with biotin, as described in Experimental Procedures. Biotinylated hDAT levels were assessed by probing with avidin−HRP after immunoprecipitation of total hDAT. In nontrypsinized L*tk*⁻ cells singly transfected with hDAT, there was clear and substantial biotinylation of hDAT, consistent with the presence of substantial amounts of the transporter at the plasma membrane of the cell (Figure 6, upper panel). In the presence of wt α-synuclein or the A30P mutant, there was a significant ($p < 0.05$) reduction (by 30% for wt α-synuclein and by 40% for the A30P mutant) in the levels of biotinylated hDAT, indicating the diminished presence of hDAT at the cell surface. In cells cotransfected with the A53T mutant, the level of biotinylated hDAT was not significantly different relative to cells transfected with hDAT plus pcDNA3.1 control vector. The decrease in surface labeling of hDAT in nontrypsinized cells coexpressing hDAT with wt α-synuclein or A30P was not related to decreases in hDAT protein levels, since total hDAT protein levels (biotinylated plus nonbiotinylated), assessed by anti-hDAT monoclonal antisera after immunoprecipitations with anti-hDAT polyclonal antibodies, were the same in singly transfected or cotransfected cells (Figure 6, middle panel).

Upon trypsinization of cotransfected cells, there was a significant ($p < 0.05$) increase (by 35% and 47% for cells coexpressing hDAT with wt α-synuclein and the A30P mutant, respectively) in levels of biotinylated hDAT, compared to nontrypsinized cells, consistent with increased localization of the protein at the plasma membrane. Moreover, the level of biotinylated hDAT was identical to that from cells expressing only hDAT, regardless of trypsinization. The increased presence of hDAT at the plasma membrane, compared to nontrypsinized cells, was also unrelated to changes in protein expression levels, as assessed by anti-hDAT monoclonal antibodies after immunoprecipitations of total hDAT protein levels (biotinylated plus nonbiotinylated) with anti-hDAT polyclonal antibodies (Figure 6, middle panel). In hDAT plus A53T cotransfected cells, there were no changes in the amount of biotinylated hDAT observed after trypsinization (Figure 6, upper panel).

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).

We also conducted parallel studies to assess the biotinylation of α-synuclein and its mutants (Figure 6, bottom panel). However, in all conditions tested, we failed to see any biotinylation of this protein, indicating that there are no parts of this protein which protrude into the extracellular space, consistent with its known intracellular localization. These combined data strongly support our findings obtained by confocal immunocytochemistry, indicating that trypsinization reverts the trafficking of the transporter to a membrane-bound state and, additionally, shows that both wt α-synuclein and its A30P mutant, but not the A53T mutant, diminish the presence of hDAT at the plasma membrane of the cell in nontrypsinized cells.

DISCUSSION

Using mild trypsinization as a tool, we have examined the mechanism by which α-synuclein and its familial PD-

linked mutants affect hDAT activity. Our results show that trypsinization reverses the negative modulation of hDAT activity by wt α-synuclein and its A30P mutant without affecting the preexisting inability of the A53T mutant to do so. The data show that trypsinization does not directly modify the hDAT itself, since transporter activity in the absence of any α-synuclein variants was identical in both untreated and trypsin-treated cells. Moreover, trypsin did not cause any noticeable proteolysis of either hDAT or the α-synuclein variants, nor did it cause any detachment of cells. Instead, the immunocytochemical and biotinylation data show that trypsinization caused increased trafficking of the hDAT protein to the cell plasma membrane only in cells that have been cotransfected with hDAT and wt α-synuclein or its A30P mutant. These data suggest that α-synuclein is implicated in the regulation of hDAT targeting both to and away from the plasma membrane.

The mechanism by which trypsin disrupts α-synuclein-mediated attenuation of hDAT activity is not clear, since trypsin could generate numerous effects in cells. Our data clearly show that this effect is not linked to a disruption of the ability of hDAT and the α-synuclein variants to physically interact, as the levels of hDAT−α-synuclein protein−protein complexes were increased after mild trypsinization of cotransfected cells. Moreover, trypsin did not change the domains of α-synuclein and hDAT mediating their binding in such protein complexes, as both proteins continued to interact through the NAC domain of the α-synuclein variants and residues 598−620 of the CT tail of hDAT. Trypsin may mediate changes in cell adhesive properties, causing subtle rearrangements of the plasma membrane. Indeed, these effects of trypsin were mimicked by collagenase I and Dispase, other proteases known to disrupt cell adhesion and used in cell detachment and/or dissociation in tissue culture, but not by chymotrypsin, Pronase, or papain, which do not significantly affect cell adhesion. Moreover, in cotransfected L*tk*$^-$ cells, trypsinization induced the production of numerous spicules that were enriched in hDAT, which were not colocalized with α-synuclein (Figure 5C). The increased presence of hDAT at the plasma membrane in turn would result in increased reuptake of dopamine, causing excessive accumulation of high intracellular levels of dopamine that contributes to and triggers a chain of cytotoxic events which culminate in cell death. Mild trypsinization may also induce subtle rearrangements and/or modifications of the cytoskeletal network, and participation of the cytoskeleton in the intracellular trafficking of hDAT has been previously hypothesized (*44*−*46*, *51*) although not studied or confirmed. In this regard, α-synuclein binds to and interacts closely with the cytoskeleton and with a variety of proteins which act to stabilize or organize the cytoskeleton (*24*, *31*−*37*). A physiological role for α-synuclein in axonal transport has been, therefore, hypothesized (*27*), entirely consistent with our observations that α-synuclein is implicated in the regulation of hDAT targeting both to (this study and ref *38*) and away (*39*, *41*, *47*) from the plasma membrane. Accordingly, α-synuclein is known to interact with tubulin (*31*−*33*), and tubulin monomers have been shown to initiate and promote α-synuclein fibrilization in vitro (*32*), through mechanisms that remain undefined. Moreover, α-synuclein increases the phosphorylation of soluble *tau* at serine residues 262 and 356 through a protein kinase A-dependent mechanism, thereby indirectly affecting the stability of axonal microtubules (*24*). Such studies and others showing colocalization of hyperphosphorylated *tau* and α-synuclein in LBs of both PD and dementia with LBs have led to the proposal that α-synuclein may cause collapse of the intraneuronal organization of microtubules (*52*). Our studies suggest that the converse may also occur, whereby a collapse, or minor perturbations in the structural integrity of the cytoskeleton, could trigger α-synuclein cytotoxicity. In particular, a dysregulated hDAT, which relocates to the plasma membrane, would enhance intracellular cytotoxicity through the increased reuptake of dopamine which autoxidizes to cytotoxic compounds inside the cells or, indeed, even of neurotoxins that cause Parkinsonian syndromes. Recent studies (*53*) showing the increased stabilization of α-synuclein protofibrils by oxidized dopamine are consistent with this hypothesis. In this regard it is also important to note that, in PD, the initial degeneration in dopamine-producing neurons is seen at distal nerve terminals. Emerging studies suggest that pre- and postsynaptic neurons are linked through molecules such as adhesion molecules, that are important to drive either synapse assembly or synaptic vesicle exocytosis, for the normal physiological functioning of both the synapse and the pre- and postsynaptic compartments (*54*, *55*). Disruption of such communications would be harmful to the normal functioning of neurons and synapses. Although it is unlikely that the initial degeneration of distal nerve terminals seen in PD is linked to a protease action on these terminals, it is tempting to speculate that the disruptive effects of trypsin on the negative modulation of hDAT activity by α-synuclein or its A30P variant may implicate similar events as the ones observed when such interneuronal communications between the synaptic compartments would be lost.

Another interesting aspect of our findings is with regard to the role of protein−protein interactions between α-synuclein and hDAT. We have previously shown that such interactions and the formation of a complex are mandatory prerequisites for observing the α-synuclein-induced reduction of hDAT function (*41*). The data presented in this paper, however, show that the formation of a protein−protein complex between the α-synucleins and hDAT does not in itself assure the manifestation of an inhibitory response. Thus, although trypsin reverses the inhibition mediated by wt α-synuclein and its mutants on hDAT activity, such treatment does not abrogate the protein−protein interactions between these proteins. Instead, there is a paradoxical increase in the coassociations between the α-synuclein variants to hDAT, as indexed by co-IP studies. This is also consistent with our overall hypothesis that rearrangements in the plasma membrane release any α-synuclein and hDAT that may have been bound to cytoarchitectural components, thereby increasing the intracellular pool of these proteins, resulting in increased protein−protein interactions. Together, these results imply that, in addition to protein−protein interactions, there is another trypsin-sensitive component which modulates both the protein−protein interactions and the functional outcome of such interactions.

In the studies presented here, we show that the effect transduced by the A53T mutant is mechanistically distinct from those of α-synuclein or the A30P mutant, as evidenced by its failure to regulate hDAT function at appreciable levels, in agreement with our previous observations (*41*). Moreover,

at least part of this lack of modulatory effects of the A53T mutant may be due to the inherent inability of the mutant to bind to and form a stable complex with hDAT due to weak protein−protein interactions (*41*). In this regard, even though trypsin appeared to increase the binding between hDAT and the A53T mutant, the overall magnitude of protein association in this complex was far below that seen with either the wt or the A30P mutant. Our data suggest that whereas the wt α-synuclein and A30P expressing cells require some cytotoxic event to trigger the onset of oxidative stress and cell death mediated by hDAT-dependent dopamine uptake, cells expressing the A53T mutant are constantly subject to a low-grade hDAT-dependent, dopamine-mediated, oxidative stress. Within this context, it should be noted that numerous studies have documented that the A53T mutant is much more cytotoxic than either wt α-synuclein or the A30P mutant (*40*, *41*, *56*−*58*), with an increased propensity to aggregate, and at a faster rate, than the other α-synuclein variants, both in vivo and in vitro (*53*, *58*−*63*). In addition to weak protein−protein interactions with hDAT, part of the A53T cytotoxicity against dopaminergic neurons may also be linked to its relative resistance to the effects of trypsin or to the absence of a trypsin-sensitive component which modulates its function. Consequently, tighter than normal binding of the A53T mutant to cellular components and/or the cytoskeleton may lead to weaker interactions with hDAT. Thus, the A53T mutant, by virtue of its inability to modulate dopamine homeostasis, may hasten the process of cellular degeneration, rendering this protein more toxic than either of the other variants. Regardless, our data clearly show that the A53T-mediated cytotoxicity occurs in a manner distinct from either the wt or the A30P mutant.

In conclusion, the dysregulation of hDAT function by wt α-synuclein and its A30P mutant upon trypsinization provides the impetus to search for additional factors which can reverse the effects of these synuclein variants with specific regard to the genesis of PD.

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