Dopamine Transporter Activity Is Modulated by α-Synuclein*

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Background: DAT regulates dopamine neurotransmission in the brain.

Results: α-Synuclein influences DA efflux and membrane microdomain distribution of DAT.

Conclusion: DAT activation recruits α-synuclein to the membrane, which in turn influences dopamine neurotransmission.

Significance: Understanding the mechanisms associated with α-synuclein regulation of DAT may reveal disease-modifying targets for the treatment of pathologies associated with DA dysregulation.

The duration and strength of the dopaminergic signal are regulated by the dopamine transporter (DAT). Drug addiction and neurodegenerative and neuropsychiatric diseases have all been associated with altered DAT activity. The membrane localization and the activity of DAT are regulated by a number of intracellular proteins. α-Synuclein, a protein partner of DAT, is implicated in neurodegenerative disease and drug addiction. Little is known about the regulatory mechanisms of the interaction between DAT and α-synuclein, the cellular location of this interaction, and the functional consequences of this interaction on the basal, amphetamine-induced DAT-mediated dopamine efflux, and membrane microdomain distribution of the transporter. Here, we found that the majority of DAT-α-synuclein protein complexes are found at the plasma membrane of dopaminergic neurons or mammalian cells and that the amphetamine-mediated increase in DAT activity enhances the association of these proteins at the plasma membrane. Further examination of the interaction of DAT and α-synuclein revealed a transient interaction between these two proteins at the plasma membrane. Additionally, we found DAT-induced membrane depolarization enhances plasma membrane localization of α-synuclein, which in turn increases dopamine efflux and enhances DAT localization in cholesterol-rich membrane microdomains.

Dysregulation of dopamine neurotransmission is implicated in the pathogenesis of drug addiction and neuropsychiatric and neurodegenerative diseases (1–4). The dimension and duration of synaptic dopamine (DA) levels are regulated by the dopamine transporter (DAT) (5). DAT is a presynaptic membrane protein (6, 7) essential in removing DA from the synaptic cleft, thus terminating DA signaling.

Proteomic analysis and functional studies have identified a number of proteins that interact with DAT to regulate its activity and its membrane distribution, one being α-synuclein (8–11). Although the exact stoichiometry of DAT and α-synuclein interaction is not known, by using the yeast two-hybrid system, Lee et al. (12) first demonstrated that DAT directly interacts with α-synuclein, a neuronal protein enriched at presynaptic terminals (13, 14) implicated in neurodegenerative diseases (15–18) and drug addiction (19, 20).

Sucrose gradient fractionation and differential centrifugation assays suggest α-synuclein behaves as a soluble cytosolic protein (21, 22). More than 80% of the protein is membrane-bound and can be Triton-extracted (23, 24).

Post-mortem studies of human tissue and animal studies have shown that exposure to amphetamines and cocaine increases α-synuclein levels in the striatum (19), a brain region implicated in drug addiction and neurodegenerative diseases, with the highest level of DAT protein (25). It has been shown that amphetamines regulate DAT-mediated dopamine efflux, cell surface redistribution of the transporter, and ionic conductance of the transporter (8). However, the cellular distribution of α-synuclein and its role on the baseline and AMPH-induced modulation of dopamine neurotransmission are less understood. The results of this study suggest that the majority of DAT-α-synuclein protein complexes are found at the plasma membrane of dopaminergic neurons, and α-synuclein overexpression enhances basal and AMPH-stimulated DAT-mediated DA efflux at resting membrane potential.

Materials and Methods

Plasmids and Cell Lines—Dr. Jonathan Javitch, Columbia University, generously provided the CHO-YFP-DAT. The plasmid cDNAs for YFP-DAT and YFP-HA-DAT were obtained from Dr. Alexander Sorkin, University of Pittsburgh (26). The plasmid cDNA for α-synuclein was obtained from Dr. Ted Dawson, Johns Hopkins University (27). YFP-GL-GPI was a...
cytochemistry. Following the induction of with either external solution alone, vehicle, or external solution as described by Saha et al. (31); midbrain tissues were dissected from C57Bl/6 mice on postnatal days 0–3 (P0–3) (The Jackson Laboratory) and were isolated and grown on a monolayer of glial cells on poly-D-lysine-treated glass-bottom dishes in neuronal media 10 containing 25 mg/ml insulin, 100 mg/2 ml of GBSS transferring, 45% glucose, and minimum Eagle’s medium (Gibco) as we have described previously (9, 30).

Preparation of Primary Culture of Midbrain Dopaminergic Neurons—The animal protocols were approved by the University of Florida Institutional Animal Use and Care Committee. Data were adapted from previous methods by Saha et al. (31); midbrain tissues were dissected from C57Bl/6 mice on postnatal days 0–3 (P0–3) (The Jackson Laboratory) and were isolated and grown on a monolayer of glial cells on poly-D-lysine-treated glass-bottom dishes in neuronal media 10 containing 25 mg/ml insulin, 100 mg/2 ml of GBSS transferring, 45% glucose, and minimum Eagle’s medium (Gibco) as we have described previously (9, 30).

Cell Culture—For imaging experiments tracking the mobility of DAT in real time, we used a fluorophore-transporter fusion protein, specifically a YFP-tagged DAT stably expressed in a parental CHO cell line (CHO-YFP-DAT). The CHO-YFP-DAT cells were grown and maintained as described previously (31). Human embryonic kidney (HEK) cells stably expressing a FLAG-tagged DAT or YFP-tagged DAT were used for imaging and biochemical analysis when applicable. These constructs and the maintenance protocol are described previously (32). As needed, the cells were transfected with the desired construct using Lipofectamine 2000 (Invitrogen).

Immunocytochemistry—For immunocytochemistry experiments in DA neurons, AAV2-α-synuclein (untagged α-synuclein) was used to overexpress α-synuclein. Neuronal cultures (DIV 5) were incubated with 1 μl of virus for 3 days to allow optimum expression of α-synuclein verified by immunocytochemistry. Following the induction of α-synuclein expression, neuronal cultures were washed three times with the external solution as described by Saha et al. (31) and then pretreated with either external solution alone, vehicle, or external solution containing 10 μM AMPH for 15 min prior to antibody labeling. The neurons were then labeled with rabbit anti-DAT (1:1000, Millipore) followed by chicken anti-rabbit conjugated to Alexa 633 (1:1000, Invitrogen). The neurons were washed with 1% bovine serum albumin (BSA)/PBS solution followed by labeling with mouse anti-α-synuclein (1:1000) and chicken anti-mouse conjugated to Alexa 488 (1:1000, Invitrogen). Neurons were then mounted on the glass slides using Fluoromount G (Southern Biotech).

For experiments in heterologous expression system, the cells (CHO and HEK cells as specified above) were co-transfected with YFP-DAT and α-synuclein or α-synuclein only. The experiments with CFP-α-synuclein are described above and below. The cells were grown on glass coverslips at 37 °C using a 12-well plate. The cells were then washed twice with 1× PBS containing CaCl₂ and MgCl₂ and fixed in 3.7% paraformaldehyde diluted in PBS for 15 min at room temperature at a volume of 0.5 ml/well, followed by two gentle washes in PBS. The cells were then incubated with blocking/permeabilization solution containing PBS, 10% fetal bovine serum, and 0.1% Triton X-100 for 30 min at room temperature. Cells were incubated with 250 μl/coverslip primary antibody that had been diluted in blocking/permeabilization solution rabbit anti-α-synuclein (1:1500, Invitrogen) overnight and then washed three times for 5 min with PBS. The cells were then incubated with secondary antibody solutions diluted in blocking/permeabilization solution (250 μl/coverslip) Alexa 546 donkey anti-rabbit and Alexa 488 chicken anti-mouse (1:1000, Invitrogen). Treated cells were washed three times for 5 min each with PBS, and coverslips were inverted on mounting media prior to running experiments. Data were analyzed by Student’s t test, and a value of p < 0.05 was considered significant (n = 17–20, from five independent experiments).

Fluorescence Resonance Energy Transfer (FRET)—FRET is a commonly used all or none microscopy technique that can detect association of proteins within a complex (33). If two fluorescently tagged molecules are within 100 Å of each other and have an optimum orientation, then following the excitation of the donor fluorophore the energy of the donor fluorophore transfers and excites the acceptor fluorophore. Primary neuronal culture containing dopamine neurons were prepared as described and transfected using Ca²⁺-phosphate DNA precipitation with the YFP-DAT and eCFP-α-synuclein, eCFP-α-synuclein alone, or the relevant empty vectors. Images were captured using the Nikon Eclipse Ti confocal microscope. To bleach the acceptor protein, YFP-DAT, the 514-nm laser was used at 100% power for 1 s followed by an acquisition phase. Filter combinations were configured as follows: cerulean fluorescent protein (eCFP) (donor: excitation 457 nm; emission, 464–499 nm) and yellow fluorescent protein (YFP) (acceptor: excitation, 514 nm; emission, 552–617 nm). Background fluorescence was subtracted from all images. FRET values were calculated from the mean fluorescence intensities for each region of interest (plasma membrane) and are shown in Table 1.

FRET Experiments in CHO Cells—CHO-YFP-DAT cells were transfected with eCFP-α-synuclein. CHO parental cells not expressing DAT were transfected with eCFP-α-synuclein and YFP-GL-GPI using Lipofectamine 2000, and experiments were conducted identically to that of dopamine neurons described above. YFP-GL-GPI is a membrane-anchored protein (34). Additional control experiments were performed when the cells expressed empty YFP and/or eCFP vectors, eCFP-α-synuclein, or YFP-DAT, respectively. Normalized FRET values for individual regions of interests were calculated according to Equation 1,

\[
\text{FRET efficiency} = \frac{(\text{donor}_{\text{post}} - BkGrd_{\text{post}}) - (\text{donor}_{\text{pre}} - BkGrd_{\text{pre}})}{(\text{donor}_{\text{post}} - BkGrd_{\text{post}})} 
\]

where the donorpost is the fluorescent value of CFP after bleaching, and donorprie is the fluorescent value of CFP prior to bleaching. BkGrd is the background fluorescence at the respective frame. FRET images are presented in pseudo color mode. FRET intensity is shown between the low and high renormalization values. There
was no FRET signal in control experiments (the cells expressing the empty vectors or YFP-GL-GPI).

Co-immunoprecipitation—CHO-YFP-DAT or HEK-YFP-DAT cells transiently transfected with wild type or eCFP-tagged α-synuclein were plated on poly-d-lysine-coated 6-well plates 3–4 days before experiments. The cells were solubilized by scraping with a Cell Scraper 5 (TPP) in lysis buffer (50 mM NaCl, 2 mM MgCl₂, 20 mM HEPEs, pH 7.2, 10% glycerol, 10 mM dithiothreitol (DTT), 1 mM EGTA, 1 mM EDTA, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 544 μM iodoacetamide, 10 μg/ml aprotinin, 1% Triton X-100, 1% sodium deoxycholate) and by further incubating for 10 min at 4 °C. The lysates were then cleared by centrifugation for 10 min at 16,000 g and then denatured by heating the sample buffer supplemented with 100 mM NaCl, washed once without NaCl, and then denatured by heating in sample buffer for 5 min at 95 °C. The immunoprecipitates and the aliquots of cell lysates were resolved on 10% SDS-PAGE, and the proteins were transferred to Hybond-Ptm membranes. Western blotting was performed with antibodies to DAT or α-synuclein followed by goat anti-rat (1:10,000, Invitrogen) and goat anti-mouse (1:10,000, Invitrogen) species-specific secondary antibodies conjugated with horseradish peroxidase. The enhanced chemiluminescence kit was purchased from Bio-Rad. To confirm equal protein loading, blots were stripped and re-probed with an anti-α-actin antibody (1:3000, Sigma) for 2 h at room temperature. Signal intensity was measured using densitometric analysis and quantified using ImageJ software (National Institutes of Health). Data were analyzed by Student’s t test, and a value of p < 0.05 was considered significant.

Fluorescence Recovery after Photo-bleaching (FRAP)—CHO cells expressing CFP- or YFP-DAT and/or eCFP-α-synuclein, ΔNAC-eCFP-α-synuclein, or YFP-GL-GPI were grown on glass coverslips and subjected to FRAP analysis using Nikon Eclipse Ti confocal microscope. For fluorophore imaging, a ×60 oil-immersion objective was used with the 514-nm laser line for excitation of YFP and the 457-nm line was used for excitation of eCFP. An area of 5 μm in diameter was bleached for 1 s at 100% transmission (as in Sorkina et al. (35)). Time-lapse image acquisition of FYP-tagged proteins was performed for 300 s post-bleaching, whereas eCFP-tagged proteins were imaged 60 s post-bleaching. To compare recovery curves for different cells, data were normalized to the pre-bleach fluorescent values to correct for variations in protein expression levels, background fluorescence, and loss of fluorescence during bleaching (36). For each experimental group, 50 normalized FRAP curves from three independent experiments were analyzed using GraphPad Prism5. Data were analyzed by Student’s t test, and a value of p < 0.05 was considered significant.

Surface Biotinylation—CHO-YFP-DAT or HEK-YFP-DAT transiently transfected with wild type, eCFP-tagged α-synuclein, or empty vector were grown to 80% confluency. The cells were washed twice with cold phosphate-buffered saline containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS) and incubated for 20 min on ice with 1 mg/ml sulfooxacinnimidyl-2-(biontinamido)ethyl-1,3-dithiopropionate (EZ-LinkTM sulfo-NHS-S-S-biotin, Pierce) in PBS, followed by a second incubation with fresh NHS-S-S-biotin. After biotinylation, the cells were washed twice with cold PBS and then incubated for 20 min on ice with 100 mM glycine in PBS, washed with PBS again, and solubilized in the lysis buffer (see above) supplemented with 10 mm Tris-HCl, pH 6.8, in which DTT was omitted. The cell lysates were then cleared by centrifugation at 16,000 × g; the biotinylated proteins were precipitated with NeutrAvidin™ beads (Pierce), washed five times with the lysis buffer, incubated in the sample buffer supplemented with 100 mM DTT for 10 min to cleave disulfide bonds, and denatured by heating the beads in sample buffer at 95 °C for 5 min. The supernatants from the NeutrAvidin precipitation and the aliquots of the cell lysates were further subjected to immunoprecipitation with rat anti-DAT (1:1000, Millipore) or mouse anti-α-synuclein (1:1000). NeutrAvidin beads and immunoprecipitates were analyzed by electrophoresis and Western blotting, and densities were quantified as described above.

Cholera Toxin-B (CTX-B) Labeling—Dopamine neurons were plated on glass coverslips in 6-well plates at 37 °C and transfected with eCFP-α-synuclein using CaCl₂ phosphate DNA precipitation when required. After 48 h, neuronal cultures were fixed using 3.7% paraformaldehyde diluted in PBS for 10 min. The neurons were then labeled with rabbit anti-DAT antibody (1:500, Millipore) overnight at 4 °C, followed by secondary antibody labeling with chicken anti-rabbit-Alexa 633 (1:1000, Invitrogen). Finally, the neurons were labeled with Alexa 555-conjugated CTX-B (Invitrogen) at 4 μg/ml for 30 min on ice and washed three times with ice-cold 1× PBS. HEK-YFP-DAT cells, expressing eCFP-α-synuclein, were treated with vehicle, or 10 μM AMPH, when stated. The cells were labeled with Alexa 647-conjugated CTX-B (Invitrogen) at 4 μg/ml for 30 min and washed three times with ice-cold PBS. Coverslips were then mounted using Fluoromount G (Southern Biotech). Confocal imaging was performed on the Nikon Eclipse Ti Microscope. Laser output was adjusted to ensure that all fluorescence monitored was non-saturating with response to fluorophore emission. Regions of interests at the plasma membrane were auto-detected by NIS Element image analysis software (Nikon). NIS Elements Co-localization Analysis software was used to obtain Pearson’s correlation coefficient to assess the co-localization of DAT and CTX-B. The coefficient values can range from 1 to −1, with 1 standing for complete positive correlation, −1 for a negative correlation, and zero standing for no correlation. Data were analyzed by one-way ANOVA followed by Tukey’s post hoc analysis with p < 0.05 considered significant.

Membrane Depolarization—CHO cells expressing YFP-DAT and/or eCFP-α-synuclein were treated with 10 μM AMPH to increase DAT activity or 50 mM KCl to induce membrane depolarization in DAT cells, as shown in Khoshbouei et al. (32). Time-lapse images on a Nikon Eclipse Ti confocal microscope were recorded every 10 s for 100 s before and 600 s after addition of drug or vehicle. Images were taken using a ×60 oil-immersion objective. The microscope was equipped with a Perfect Focus System, which is designed to combat axial focus fluctuations in real time during long term imaging. The eCFP-
α-synuclein fluorescence intensity was measured on the periphery of the cell at the plasma membrane. For the control groups, the experiments were repeated in the presence or absence of DAT expression and following vehicle treatment. For Na⁺ substitution experiments, the NMDG-Cl was isosmotically substituted for NaCl in the external solution.

**Amperometry**—Basal DA efflux was measured at resting membrane potential in the presence or absence of α-synuclein. The HEK-YFP-DAT cells plated at a density of ~50,000 in 35-mm dishes. The cells were transfected with eCFP-tagged α-synuclein or eCFP vector. All recordings were performed 48 h after transfection. More than 45% of the cells expressed eCFP-α-synuclein or eCFP vector. The cells were preloaded with DA as described previously (67). Briefly, the cells were washed twice with external solution (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, and 10 mM HEPES, pH 7.4) containing 10 mM D-glucose. The cells then were incubated with the external solution containing 1 μM DA, 100 μM pargyline, 10 μM tropolone, and 100 μM ascorbic acid for 20 min at 37°C. A carbon fiber electrode (ProCFF; fiber diameter is 5 μm; Dagan Corp.) was positioned opposed to the plasma membrane of a cluster of cells that co-expressed YFP-DAT and eCFP-α-synuclein or eCFP vector. The cells were held at +700 mV with respect to the bath ground (a potential greater than the oxidizing potential of DA) to measure DA efflux via oxidation reactions. The amperometric electrode measures the electrical currents (in picamperes (pA)) as a result of oxidation of DA molecules. Amperometric currents were recorded using Axopatch 200B with a low-pass Bessel filter set at 1000 Hz and digitally filtered off line at 10 Hz before analysis. The DAT-mediated DA efflux was defined as baseline amperometric current minus current after the bath application of 10 μM nomifensine. The AMPH-induced DAT-mediated DA efflux was defined as baseline amperometric current minus current after the bath application of 1 μM AMPH. Data were acquired by averaging a 10-s interval of current directly before nomifensine application (baseline current) and 20 min following nomifensine application (nomifensine current) and directly before AMPH application (AMPH-induced current).

**Statistical Analysis**—All data are shown as mean ± S.E. unless otherwise noted. Data were analyzed using a Student’s t test or one-way ANOVA with Tukey post hoc analysis. Levels of significance were set at p < 0.05.

**Results**

**Dopamine Transporter and α-Synuclein Co-localize at the Plasma Membrane**—The cellular location of DAT/α-synuclein association in dopaminergic neurons is unknown. DAT is localized primarily to the plasma membrane, but it can also be found in the cytoplasm. To identify the cellular localization of the association between DAT and α-synuclein, we used single neuron confocal microscopy to determine where α-synuclein is co-localized with DAT (Fig. 1). In DAT⁺ neurons obtained from midbrain primary culture, the majority of α-synuclein (green labeling) is found at or near the surface membrane of dopaminergic neurons and is co-localized with endogenous
DAT (red labeling) (Fig. 1A). Similar to the results observed in DAT+ neurons, we found α-synuclein primarily co-localizes with DAT in CHO cells expressing both proteins (Fig. 1B, top panel), whereas α-synuclein exhibits a diffuse distribution throughout the cell in the absence of DAT (Fig. 1B, middle panel). CHO cells expressing YFP-DAT only show a distinct localization of YFP-DAT primarily at the plasma membrane that is not altered in the presence of α-synuclein overexpression (Fig. 1B, bottom panel). The merged confocal image of CHO cells co-expressing YFP-DAT (green) and α-synuclein (red) demonstrates the co-localization YFP-DAT and α-synuclein (yellow) at or near the plasma membrane (Fig. 1B, upper right). The line analysis reveals an enrichment of α-synuclein at the cell surface (Fig. 1C), where the majority of the transporter is located. In the absence of the DAT, α-synuclein is uniformly distributed in the cell (Fig. 1D).

It has been shown in CHO and HEK cells that α-synuclein, if present, is below the detection limit of Western blot and immunocytochemistry assays (8). We found that in these cells the absence of detectable α-synuclein does not affect membrane localization of DAT (Fig. 1D).

**FRET Microscopy Reveals an Association between eCFP-α-Synuclein and YFP-DAT at the Plasma Membrane**—Next, we used FRET microscopy, a quantitative microscopy approach to examine the cellular localization of α-synuclein and its association with DAT. In our study, we investigated the FRET efficiency between eCFP-α-synuclein, the donor molecule, and YFP-DAT, the acceptor molecule. This FRET pair has been extensively used and characterized in the literature (33). As shown in Fig. 2, we measured a positive FRET signal between YFP-DAT and eCFP-α-synuclein in the dopaminergic neurons that was limited to the area at or near the membrane (Fig. 2C). We observed similar results in both heterologous model systems stably transfected with YFP-DAT and transiently expressing eCFP-α-synuclein (data not shown). No FRET signal was detected in control experiments shown in Table 1. The control experiments were performed as follows: 1) when cells co-expressed YFP and eCFP empty vectors; 2) when cells co-expressed YFP empty vector and eCFP-α-synuclein, or 3) when cells co-expressed eCFP empty vector and YFP-DAT (Table 1). Previous studies have demonstrated interactions between α-synuclein and other SLC6 transporters (37, 38); therefore, it is likely to measure positive FRET signal between other SLC6 proteins and α-synuclein. Therefore, to examine the specificity of the FRET signal between YFP-DAT and eCFP-α-synuclein, additional control experiments were performed using a membrane-anchored protein, GL-GPI tagged with YFP (Fig. 3C) (34). No FRET signal was detected between YFP-GL-GPI and eCFP-α-synuclein, supporting the specificity of FRET signal between YFP-DAT and CFP-α-synuclein at the membrane.

**Co-immunoprecipitation and Cell Surface Membrane Biotinylation Suggest DAT and α-Synuclein Associate at the Plasma Membrane**—Co-immunoprecipitation assays demonstrate DAT and α-synuclein co-immunoprecipitate in total cell lysate.
(Fig. 3A) confirming previous reports (12). To validate the specificity of the DAT antibody, we immunoprecipitated DAT and then probed for α-synuclein and DAT. Only a signal for DAT was detected. The immunoprecipitation of IgG beads in YFP-DAT-expressing cells or cells co-expressing YFP-DAT and eCFP-α-synuclein showed no signal when probed with anti-DAT or anti-α-synuclein, further confirming the specificity of the DAT antibody and immunoprecipitation assay in Fig. 3A (Fig. 2B). The surface biotinylation (Fig. 3C) of CHO cells co-expressing YFP-DAT and eCFP-α-synuclein suggests that DAT and α-synuclein protein complexes exist at the membrane (Fig. 3C).

Interaction between DAT and α-Synuclein Is Transient—To quantify the mobility of DAT and α-synuclein individually and when they are co-expressed, we used FRAP. FRAP experiments provide quantitative analysis of DAT and α-synuclein interaction in living cells, without disruption of their cellular localization or microenvironment (Fig. 4, normalized FRAP curves from 25–30 cells for all experimental groups). This experimental approach identifies how alterations in protein mobility may be a result of protein-protein interactions (36). For example, small proteins such as Ras or empty YFP or CFP vectors recover rapidly with a high mobile fraction due to the increased amount of protein able to move back into the initial bleached region of interest (see the recovery profile of eCFP empty vector in Fig. 4B) (39). In contrast, larger membrane proteins such as dopamine transporter have significantly slower mobility and decreased mobile fraction due to size (compare the recovery profile of YFP-DAT with eCFP vector and eCFP-α-synuclein in Fig. 4B). However, when a large protein interacts with a small protein, be it a stable or transient interaction, the mobility of the smaller protein is expected to decrease in both the lateral mobility and mobile fraction, although the larger protein may experience little to no change in mobility (40, 41). We aimed to test this hypothesis with DAT (>75 kDa) and α-synuclein (14 kDa). FRAP analysis shows there is a significant decrease in the lateral mobility of eCFP-α-synuclein in the presence of YFP-DAT suggesting the interaction between DAT and α-synuclein reduces the diffusion rate of α-synuclein (Fig. 4, B and C). Notably, the recovery profile of eCFP-α-synuclein in the presence of DAT is significantly different from the recovery profile of YFP-DAT, indicating the interaction between these two proteins is not stable (Fig. 4, B and D). Within the region of interest, the recovery profile of eCFP vector is nearly 100%, which is consistent with the diffusion profile of small cytoplasmic unbound proteins (42–44). The recovery profile of eCFP vector is not altered by the presence or absence of YFP-DAT (Fig. 4, B and C), eliminating the possibility of a nonspecific effect of YFP empty vector. In addition to vector control groups, additional control groups substantiated the specificity of these results. There was no change in the recovery profile or τ1⁄2 of either DAT or α-synuclein in YFP- or CFP-DAT cells expressing the following: 1) CFP-α-synuclein protein lacking the non-Aβ component (NAC) region, which is the DAT interacting domain of α-synuclein (12); 2) DAT cells expressing YFP-GL-GPI (34), a transmembrane protein, respectively (data not shown). These results suggest that the slowed recovery profile of α-synuclein in the presence of DAT, without affecting the recovery profile of DAT, is a consequence of dynamic and specific interaction between DAT and α-synuclein.

**TABLE 1**

| FRET pair | Cellular compartment | FRET value       |
|-----------|----------------------|------------------|
| YFP-DAT/eCFP | Plasma membrane   | 5.554 ± 0.5812   |
| YFP-DAT/eCFP-α-synuclein | Plasma membrane | 3.379 ± 0.3847   |
| YFP-DAT/eCFP-α-synuclein + AMPH | Plasma membrane | 7.024 ± 0.6956   |
| eCFP-α-synuclein/YFP-GL-GPI | Plasma membrane | -1.173 ± 1.398   |
| YFP-DAT/eCFP-vector | Plasma membrane | 1.340 ± 1.34     |
| eCFP-α-synuclein/YFP-vector | Plasma membrane | -0.08806 ± 1.237 |

**FIGURE 3.** Co-immunoprecipitation and cell surface membrane biotinylation suggest DAT and α-synuclein associate at the plasma membrane. A, co-IP of YFP-DAT in CHO cells expressing vector or eCFP-α-synuclein show co-immunoprecipitation of DAT and eCFP-α-synuclein (α-syn). B, control experiments demonstrate the specificity of the DAT antibody and the signals detected in Fig. 3A. C, biotinylation of CHO cells expressing YFP-DAT and eCFP vector, eCFP-α-synuclein, or untagged α-synuclein reveals an interaction between DAT and α-synuclein at the plasma membrane. IP, immunoprecipitation.
We used live cell confocal microscopy to examine the modulatory role of AMPH-induced DAT activity on the cellular localization of α-synuclein. We monitored the membrane localization of eCFP-α-synuclein in CHO and HEK cells before and after exposure to AMPH (10 μM), KCl (50 mM) (45, 46), or DA (10 or 20 μM). In YFP-DAT-expressing cells, AMPH exposure (10 μM) gradually increased eCFP fluorescent intensity at or near the surface membrane (Fig. 5A, n = 20–25 cells from three independent experiments, *p < 0.05, Student’s t test). Varying concentrations of DA had no effect on localization of eCFP-α-synuclein at the plasma membrane in YFP-DAT cells (data not shown). To examine the mechanism, we iso-osmotically substituted NMDG-Cl for NaCl in the extracellular solution. Iso-osmotic substitution of NMDG-Cl eliminated the effect of AMPH on the membrane localization of eCFP-α-synuclein, suggesting AMPH-induced DAT-mediated increase in Na⁺ conductance and membrane depolarization influences cellular distribution of α-synuclein (Fig. 5, C and D, n = 20–25 cells from three independent experiments). In control experiments, we found that AMPH exposure does not affect the membrane localization of eCFP-α-synuclein in the absence of DAT or when the cells express eCFP empty vector (Fig. 5A and data not shown, n = 20–25 cells from three independent experiments). Similar to AMPH, the KCl-induced membrane depolarization produced a robust and rapid increase in the membrane localization of α-synuclein in HEK cells expressing only eCFP-α-synuclein. As predicted, KCl induces a large and rapid membrane depolarization (Fig. 5B) (47). Therefore, as compared with the AMPH treatment, KCl recruits a greater level of α-synuclein to the membrane even in the absence of DAT expression due to its robust increase in membrane depolarization. In contrast to the response measured following AMPH treatment, when DAT- and eCFP-α-synuclein-expressing cells and eCFP-α-synuclein-expressing cells only are treated with DA (10 or 20 μM), there is no change in the membrane localization of α-synuclein. We and others have shown that AMPH induces a greater inward depolarizing Na⁺ current compared with DAT’s native ligand, DA (30, 32, 48–50), which might explain the measured differences. Collectively, these findings support the interpretation that membrane localization of α-synuclein depends on membrane depolarization and DAT ligand specificity.

Amphetamine Exposure Enhances the DAT and α-Synuclein Association at the Plasma Membrane—AMPH is a potent substrate of the transporter that has been shown to regulate the
transporter’s function and its surface membrane distribution (51, 52). Because we observed a robust increase in the membrane localization of α-synuclein following AMPH exposure but not DAT’s native substrate dopamine, we hypothesized that AMPH enhances the association between α-synuclein and DAT. We used the following three complementary approaches to examine whether AMPH-induced membrane recruitment of α-synuclein influences the interaction between DAT and α-synuclein: 1) co-immunoprecipitation assay (co-IP); 2) immunocytochemistry; and 3) FRET microscopy in the dopaminergic neurons and heterologous cells. We found that treatment with AMPH significantly enhanced the co-immunoprecipitation of DAT with α-synuclein as compared with vehicle-treated cells (n = 3, p < 0.05) (Fig. 6, A and B). These co-IP results were confirmed in dopaminergic neurons with physiological levels of endogenous DAT (red) and elevated levels of α-synuclein (green) (Fig. 6C). We measure a marked increase in DAT (red) and α-synuclein (green) co-localization at the surface membrane (yellow) following AMPH treatment as compared with vehicle treatment (p < 0.01) (Fig. 6, C and D). For these experiments, we identified DAT using a DAT antibody against the 2nd extracellular loop of the transporter. Furthermore, FRET studies following AMPH treatment revealed a greater FRET efficiency between the two proteins (Fig. 6, E and F). AMPH or vehicle treatment did not affect the lack of FRET efficiency between CFP-DAT/YFP-GL-GPI or CFP-α-synuclein/YFP-GL-GPI (Table 1). These results suggest that the association between DAT and α-synuclein at the neuronal membrane is increased by AMPH exposure. Taken together, our data suggest that in the dopaminergic neurons the presence of DAT influences the cellular localization of α-synuclein (Fig. 2) and that the DAT-mediated membrane depolarization, or its conformational state, further stabilizes α-synuclein at the neuronal plasma membrane. This DAT-mediated increase in the membrane localization of α-synuclein in turn may alter DAT activity. Therefore, we next investigated whether α-synuclein overexpression influences DAT-mediated DA efflux.

α-Synuclein Overexpression Increases DAT-mediated DA Efflux—Using microdialysis, Lam et al. (53) have shown mice overexpressing α-synuclein exhibit an elevated extracellular dopamine concentration in the striatum that precedes dopamine loss in that region. The increase in the extracellular dopamine levels following α-synuclein overexpression can be due to a decrease in dopamine uptake, an increase in dopamine efflux, or a combination of both mechanisms. Recently, we have shown elevated α-synuclein decreases the initial slope of dopamine uptake that within 5 min reaches the uptake level of cells not expressing α-synuclein (8). Therefore, it is unlikely that the decrease in dopamine uptake alone is the sole underlying mechanism for the elevated extracellular dopamine levels following α-synuclein overexpression. We therefore examined the hypothesis that α-synuclein elevates extracellular dopamine concentration by increasing DAT-mediated DA efflux. To test this hypothesis, the YFP-DAT cells with or without α-synuclein overexpression were loaded with 1 μM DA for 20 min (see “Materials and Methods”). DA efflux in response to 10 μM AMPH was measured via in vitro amperometry with a carbon fiber electrode (see “Materials and Methods”). As shown in Fig. 7, at resting membrane potential, the elevation of intracellular α-synuclein significantly increased AMPH-induced DAT-mediated DA efflux. In addition, we found that increased intracellular α-synuclein levels enhanced the nomifensine-sensitive basal DA efflux at resting membrane potential (Fig. 7C).

α-Synuclein Alters Membrane Microdomain Distribution of DAT—DAT is distributed in the cholesterol-rich and cholesteryl-poor membrane microdomains termed membrane rafts (40, 54, 55). Previous studies support the idea that membrane microdomain distribution of DAT influences its conformational state and its function (54, 55). Recently, Cremona et al. (55) have shown that localization of the transporter in the cholesterol-rich membrane raft microdomains is essential for the AMPH-induced reversed transport of dopamine in dopaminergic neurons. GM1 (monosialoganglioside) is a well-characterized membrane ganglioside that is distributed in the cholesterol-enriched membrane microdomains (56) and has been shown to interact with and recruit α-synuclein to membrane rafts (57). We used confocal microscopy to examine the hypothesis that elevated α-synuclein affects the membrane microdomain distribution of DAT to regulate its activity. We
addressed this hypothesis by measuring DAT co-localization with Alexa 555-conjugated CTx-B in the presence of physiological (control group) or α-synuclein overexpression (experimental group) in dopaminergic neurons. The Alexa 555-conjugated CTx-B binds to GM1 gangliosides, identifying cholesterol-rich membrane microdomains. As shown in Fig. 8, A and B, as compared with dopamine neurons containing physiological levels of α-synuclein, α-synuclein overexpression increases co-localization of DAT and CTx-B at the plasma membrane. Similar results were obtained in YFP DAT cells (data not shown). Additionally, treatment with AMPH further increases DAT and CTx-B co-localization when α-synuclein is overexpressed. α-Synuclein overexpression in the presence or absence of AMPH has no effect on the membrane microdomain distribution of YFP-GL-GPI (data not shown). These data parallel the findings that α-synuclein’s enhancement of DAT mediated DA efflux. Taken together, our data suggest that increased α-synuclein levels in the dopaminergic neurons affect both the membrane microdomain distribution of DAT and function of DAT.

Discussion

Humans and rodents exposed to amphetamines have increased levels of α-synuclein in the striatum, which is a brain region with the highest level of dopamine transporter (19). Here, we found an increase in peri-plasma membrane localization of α-synuclein in DAT-expressing cells as compared with the cells not expressing the transporter (Fig. 1). In addition, as shown in Figs. 1, 3, and 4, A–C, the presence or absence of N-terminal CFP tag does not affect the membrane localization of α-synuclein in the presence of absence of DAT or its interaction with the DAT. The occurrence of FRET between YFP-DAT and eCFP-α-synuclein at the surface membrane (Fig. 2) further supports the idea that α-synuclein co-localizes with DAT at the plasma membrane. This is in agreement with the emerging consensus that local protein–protein interactions (58) or membrane charge (59) may lead to stabilization or even insertion of α-synuclein into the lipid bilayer that can consequently disrupt cellular homeostasis (60). Therefore, DAT recruitment and stabilization of α-synuclein at the cell membrane potentially provides a novel mechanism for the increased vulnerability of dopaminergic neurons expressing pathological levels of α-synuclein. Previously, Sorkina et al. (26) used FRET to examine DAT oligomerization. They measured on average a FRET value of 17 in HEK cells at the membrane. In our studies the FRET values obtained from DAT and α-synuclein interaction were significantly lower than those of oligomerized YFP-DAT/CFP-DAT, suggesting a less stable interaction between DAT and α-synuclein. This idea is supported by the FRAP data showing a dynamic interaction between DAT/α-synuclein (Fig. 4).

To test the hypothesis that DAT activation, which depolarizes the membrane, increases the membrane localization of α-synuclein, we used AMPH, a well characterized substrate for DAT that increases the activity of the transporter, induces a DAT-mediated inward depolarizing Na⁺ current, and induces an inward-facing conformation of the transporter (32). We
found that an AMPH-induced increase in DAT activity enhances localization of α-synuclein to the plasma membrane (Fig. 4A). This finding was further supported by co-immunoprecipitation assays (Fig. 5A), suggesting that AMPH-induced increase in amperometric current in YFP-DAT only and YFP-DAT cells overexpressing CFP-α-synuclein (p < 0.001 by Student’s t test; n = 6–8, from three independent experiments). To further test the hypothesis that DAT activity can regulate α-synuclein recruitment to the plasma membrane, we asked whether membrane depolarization affects α-synuclein regulation of DAT. The absence of extracellular Na⁺ ions decreases [³H]DA uptake (61–64) and AMPH-induced DAT-mediated inward current (32, 65). Therefore, NMDG⁺ substitution for Na⁺ ions is predicted to decrease the influence of AMPH on both DAT conformational state and membrane potential, decreasing α-synuclein recruitment to the plasma membrane. We found that NMDG⁺ substitution for Na⁺ ions in the external solution reduced the depolarizing Na⁺ current and blocked the AMPH-induced recruitment of α-synuclein to the membrane (Figs. 5C and 4D), further supporting the idea that transporter activity can regulate membrane localization of α-synuclein.

In contrast to the response measured following AMPH treatment, there is no change in the membrane localization of α-synuclein following DA. It has been shown that AMPH induces a greater inward depolarizing Na⁺ current compared with DAT’s native ligand, DA (30, 32, 48–50), which might explain the measured differences. In addition, the differential observations seen

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**FIGURE 7. Elevated intracellular α-synuclein increases basal and AMPH-induced dopamine efflux.** A, representative amperometric currents recorded from YFP-DAT only and YFP-DAT overexpressing eCFP-α-synuclein cells loaded with 1 μM dopamine for 20 min at 37 °C. Arrows indicate application of AMPH (10 μM). B, quantification of AMPH-induced increase in amperometric current in YFP-DAT only and YFP-DAT cells overexpressing CFP-α-synuclein (p < 0.001 by Student’s t test; n = 6–8, from three independent experiments). C shows analysis of nomifensine reduction (10 μM) of basal dopamine efflux (p < 0.001 by Student’s t test; n = 6–8, from three independent experiments). ***, p < 0.001.

**FIGURE 8. Elevation of intracellular α-synuclein increases DAT co-localization with the GM1 gangliosides in the cholesterol-rich membrane microdomains.** A, dopaminergic neurons labeled with CTxB-Alexa (green) and DAT (red) without (right) and with (left) eCFP-α-synuclein overexpression. CTxB labels GM1 gangliosides that are distributed in cholesterol-rich membrane microdomains. CFP-α-synuclein overexpression increases DAT/CTx-B co-localization at the membrane (yellow) compared with control group (see merged images). B, normalized bar graph shows CTxB-647 and YFP-DAT co-localization in HEK cells ± eCFP-α-synuclein following vehicle (VEH) or amphetamine treatment (p < 0.05, one-way ANOVA followed by Tukey post hoc analysis). ***, p < 0.001.
by DAT substrates could be a result of AMPH's ability to induce an inward-facing conformation of the transporter that might influence the association of DAT and α-synuclein. Additionally, it is important to note that redistribution of α-synuclein to the membrane may be a result of a number of mechanisms that occur following depolarization, such as altered protein phosphorylation or alterations in α-synuclein species formation.

FRAP is a powerful tool to study the nature of DAT and α-synuclein interaction in the living cell in a biologically relevant system, which complements biochemical data using isolated proteins. In general, the fluorescence intensity of small cytosolic proteins recovers faster than large membrane proteins. In the absence of protein–protein interaction, the fluorescence recovery profile of a smaller protein does not change when both proteins are expressed together or expressed individually. In contrast, when two proteins interact transiently, the proteins are partly bound and partly unbound at anytime, and FRAP of the smaller protein is slowed. When the two proteins interact stably, the fluorescence recovery of the smaller protein will be similar to the recovery profile of the larger protein (Fig. 4A). The overall diffusion rate of this stable complex could be slower than the diffusion rate of the larger protein by itself. With this in mind, the partially slowed recovery profile of α-synuclein in the presence of DAT without affecting the recovery profile of DAT suggests a dynamic association between DAT and α-synuclein. The dynamic “on” and “off,” interaction mode between DAT and α-synuclein provides an opportunity for other regulatory proteins to interact with the C terminus of DAT (66). For example, CaMKIIα and α-synuclein interact with the C terminus of DAT (residues 597–620 for α-synuclein (12) and 598–620 for CaMKIIα (9)) and influence the activity of DAT (9, 12). Therefore, at the physiological level of α-synuclein the balanced competition between CaMKIIα and α-synuclein for a shared binding domain on the C terminus of DAT regulates the function of DAT. At above physiological levels of α-synuclein, theoretically the frequency of CaMKIIα/DAT association decreases, which in turn can uniquely regulate the activity of the transporter.

DAT activity has been shown to be regulated by the stabilization or redistribution of the transporter in the cholesterol-rich membrane microdomains (40, 54, 55). Because α-synuclein has been shown to recruit the GM1 gangliosides to the plasma membrane (57), the measured increase in co-localization of DAT with the CTx-B-labeled GM1 gangliosides in Fig. 8, A and B, when α-synuclein is elevated may, in part, be responsible for regulation of DAT activity. Our data do not reconcile whether α-synuclein directly affects the membrane microdomain distribution of the transporter or whether it stabilizes the transporter in the GM1-rich membrane microdomain. Increased DAT localization in the GM1-rich membrane microdomains might be a mechanism by which α-synuclein elevation regulates DAT activity.

In addition to stimulating an inward Na+ current, AMPH exposure induces an inward-facing conformation of the transporter (32). Thus, the AMPH-induced enhancement of membrane localization of α-synuclein may, in part, be a consequence of increased accessibility of an α-synuclein favorable binding site on the DAT molecule when the transporter is in an inward-facing conformational state. Our results suggest, in DAT-expressing cells, at least three mechanisms may govern the membrane localization of α-synuclein as follows: 1) DAT/α-synuclein interaction (Figs. 1–3); 2) membrane depolarization-induced α-synuclein recruitment to the membrane (Fig. 5A); and 3) substrate-induced conformational state of the transporter (Fig. 7, A and B).

In summary, DAT recruitment and stabilization of α-synuclein at the membrane of dopaminergic neurons potentially provide a novel mechanism for dysregulation of DA neurotransmission and the increased vulnerability of DA neurons with pathological levels of α-synuclein.

Author Contributions—B. B., J. S. G., and H. K. designed the study and wrote the paper. K. S. conducted and analyzed the NMDG membrane depolarization experiments. J. S. G. and T. R. performed co-immunoprecipitation and cell surface membrane biotinylation experiments. J. P. B. conducted and analyzed the efflux experiments. D. S. conducted and analyzed the biochemistry experiments. P. D. analyzed the CTx-B data. All authors read and approved the final version of the manuscript.

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α-Synuclein Modulates DAT Activity

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