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The Dopamine Transporter Recycles Via A Retromer-Dependent Post-Endocytic Mechanism: Tracking Studies Using A Novel Fluorophore-Coupling Approach

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The Dopamine Transporter Recycles Via A Retromer-Dependent Post-Endocytic
Mechanism: Tracking Studies Using A Novel Fluorophore-Coupling Approach

**Abbreviated Title: Dopamine Transporter Recycling**

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ABSTRACT

Presynaptic reuptake, mediated by the dopamine (DA) transporter (DAT), terminates DAergic neurotransmission and constrains extracellular DA levels. Addictive and therapeutic psychostimulants inhibit DA reuptake, and multiple DAT coding variants have been reported in patients with neuropsychiatric disorders. These findings underscore that DAT is critical for DA neurotransmission and homeostasis. DAT surface availability is acutely regulated by endocytic trafficking, and considerable effort has been directed towards understanding mechanisms that govern DAT’s plasma membrane expression and post-endocytic fate. Multiple laboratories have demonstrated DAT endocytic recycling and enhanced surface delivery in response to various stimuli. Paradoxically, imaging studies have not detected DAT targeting to classic recycling endosomes, suggesting that internalized DAT targets to either degradation or an undefined recycling compartment. Here, we leveraged PRobe Incorporation Mediated by Enzyme (PRIME) labeling to directly label surface DAT with fluorophore and track DAT’s post-endocytic itinerary in immortalized mesencephalic cells. DAT robustly targeted to retromer-positive endosomes following internalization, and DAT/retromer colocalization was observed in male mouse dopaminergic somatodendritic and terminal regions. shRNA-mediated Vps35 knockdown revealed that DAT endocytic recycling requires intact retromer. DAT also targeted rab7-positive endosomes with slow, linear kinetics that were unaffected by either accelerating DAT internalization, or binding a high-affinity cocaine analog. However, cocaine significantly increased DAT exit from retromer-positive endosomes. Finally, we found that the DAT carboxy-terminal PDZ-binding motif was required for DAT recycling and exit from retromer. These results define the DAT recycling mechanism, and provide a unifying explanation for previous, seemingly disparate, DAT endocytic trafficking findings.
SIGNIFICANCE STATEMENT

The neuronal DAT recaptures released DA and modulates DAergic neurotransmission, and a number of DAT coding variants have been reported in several DA-related disorders, including infantile parkinsonism, attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD). DAT is also competitively inhibited by psychostimulants with high abuse potential. Thus, mechanisms acutely impacting DAT availability will likely significantly impact both normal and pathological DAergic homeostasis. Here, we explore the cellular mechanisms that acutely control DAT surface expression. Our results reveal the intracellular mechanisms that mediate DAT endocytic recycling following constitutive and regulated internalization. In addition to shedding light on this critical process, these findings resolve conflict among multiple, seemingly disparate, previous reports on DAT’s post-endocytic fate.
INTRODUCTION

DA neurotransmission is required for vital functions in the central nervous system, including locomotion, reward and sleep/arousal (Iversen and Iversen, 2007). Dysregulated DA neurotransmission is linked to multiple neurological and psychiatric disorders such as Parkinson’s disease, schizophrenia, ADHD, ASD and bipolar disorder (Sawa and Snyder, 2002; Chen et al., 2004; Sulzer and Schmitz, 2007; Sharma and Couture, 2014). Following evoked DA release, extracellular DA is rapidly cleared via presynaptic reuptake, mediated by the plasma membrane DA transporter (DAT) (Gether et al., 2006; Torres and Amara, 2007). DAT is potently inhibited by addictive psychostimulants, such as cocaine and amphetamine, as well as by therapeutic drugs, such as methylphenidate (Ritalin) (Torres and Amara, 2007; Kristensen et al., 2011). Multiple DAT coding variants have been reported in ADHD, ASD and infantile Parkinsonism, and these DAT mutants exhibit either anomalous DA efflux, loss of function or DAT membrane instability due to endocytic dysfunction (Mazei-Robison et al., 2008; Sakrikar et al., 2012; Hamilton et al., 2013; Bowton et al., 2014; Cartier et al., 2015). Thus, DA homeostasis is exquisitely sensitive to altered DAT activity, and mechanisms that regulate DAT function and availability are predicted to exert impactful consequences upon DA-dependent processes.

Endocytic trafficking dynamically regulates DAT surface expression (Melikian, 2004; Eriksen et al., 2010; Bermingham and Blakely, 2016). DAT constitutively internalizes, and either acute PKC activation or amphetamine exposure accelerates DAT endocytosis, thereby decreasing DAT surface expression and function (Daniels and Amara, 1999; Melikian and Buckley, 1999; Saunders et al., 2000). Considerable effort has been directed towards understanding DAT’s post-endocytic fate. Multiple studies indicate that DAT recycles back to the plasma membrane under basal and regulated conditions (Loder and
Melikian, 2003; Boudanova et al., 2008a; Furman et al., 2009b; Chen et al., 2010; Richardson et al., 2016). Paradoxically, DAT post-endocytic tracking studies, using either antibody feeding or fluorescent cocaine analogs to label surface DAT, reported that DAT targets to pre-lysosomal and lysosomal pathways, and not to classical recycling endosomes (Eriksen et al., 2009; Eriksen et al., 2010; Vuorenpuu et al., 2016). In contrast, neuronal fractionation studies (Rao et al., 2011) and tracking studies in heterologous cells and primary cultured neurons suggest differential DAT targeting (Hong and Amara, 2013). Moreover, a recent antibody feeding study performed in acute mouse brain slices reported little surface DAT internalization, in either DAergic terminals or somatodendritic compartments (Block et al., 2015). These conflicting reports have raised considerable debate among transporter biologists, and have failed to identify the mechanism(s) that govern DAT’s post-endocytic fate.

The retromer complex mediates endosomal export of cargo destined for either retrograde transport to the trans-Golgi network (TGN), or for rapid recycling to the plasma membrane (Seaman, 2004; Burd and Cullen, 2014). Multiple neuronal proteins recycle via a retromer-dependent mechanism, including β2-adrenergic receptors, ionotropic glutamate receptors, wntless, and Alzheimer-associated sortilin-related receptor 1 (Rogaeva et al., 2007; Zhang et al., 2012; Choy et al., 2014; Varandas et al., 2016). Moreover, retromer disruption is closely linked to multiple neurological disorders, including Alzheimer’s and Parkinson’s diseases (Muhammad et al., 2008; Tsika et al., 2014; Dhungel et al., 2015; Small and Petsko, 2015).

Previous discrepancies in DAT post-endocytic mechanisms prompted us to 1) interrogate the DAT post-endocytic itinerary in absence of either bound ligands or antibodies, and 2) identify the molecular mechanisms governing DAT post-endocytic
sorting. In the current study, we took advantage of PRIME labeling (Uttamapinant et al., 2010) to covalently couple fluorophore to the DAT surface population, and subsequently track DAT's temporal-spatial post-endocytic itinerary. Our results reveal a critical role for retromer in DAT endocytic trafficking, and implicate the DAT carboxy-terminal PDZ-binding motif in retromer-mediated DAT recycling.

**MATERIALS & METHODS**

**Materials:** Picolyl azide (pAz) and Bis[(tertbutyltriazoyl)methyl]-[(2-carboxymethyltriazoyl)methyl]-amine (BTAA) were synthesized as previously described (Uttamapinant et al., 2012). Rat anti-DAT antibody (MAB369) was from EMD Millipore and mouse anti-actin antibody (sc-56459) was from Santa Cruz Biotechnology. Mouse anti-EEA1 (610456) and mouse anti-rab11 (610656) antibodies were from BD Transduction. Rabbit anti-rab7 antibody (D95F2) was from Cell Signaling Technology and goat anti-Vps35 antibody (NB100-1397) was from Novus Biologicals. Horseradish peroxidase-conjugated secondary antibodies were from EMD Millipore (goat anti-rat), Jackson ImmunoResearch (donkey anti-goat) and Pierce (goat anti-mouse). AlexaFluor-conjugated anti-rat, anti-mouse and anti-rabbit secondary antibodies were from Invitrogen, and donkey anti-goat Alexa488 (minimal cross-reaction to mouse serum proteins), and donkey anti-rat Alexa594 (minimal cross-reaction to mouse serum proteins) were from Jackson ImmunoResearch. Alkyne-Alexa594 was from Invitrogen. $[^{3}H]$DA (dihydroxyphenylethylamine 3,4-[ring-2,5,6-$^{3}$H]) was from Perkin Elmer. Sulfo-NHS-SS-biotin, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and streptavidin agarose were purchased from Thermo Fisher Scientific. Phorbol 12-myristate 13-acetate (PMA), AIM-100, β-CFT (WIN35,428) and GBR12909 were from Tocris Bioscience. All other
chemicals and reagents were from Thermo Fisher Scientific or Sigma-Aldrich and were of highest grade possible.

cDNA Constructs and Mutagenesis: To generate LAP-hDAT-pcDNA3.1(+), hDAT was subcloned into pBS-SKII* as a shuttle vector, and degenerate mutations were introduced into hDAT codons corresponding to amino acids 193 and 204, adding BsaBI and Hpal sites (Quikchange mutagenesis kit, Agilent Technologies). Sense and anti-sense oligonucleotides encoding the LAP-peptide sequence, flanked by linkers (GSGSSGGEIDKVWHDFPAGSGSSG; LAP peptide sequence is underlined), were annealed and ligated into the blunt BsaBI/Hpal site, and the final LAP-DAT cDNA was excised subcloned into pcDNA3.1(+) at HindIII/XbaI sites. LAP-DAT-AAA-pcDNA3.1(+) was generated by mutating the last three amino acids of hDAT into alanines (LKV to AAA) using Quikchange mutagenesis (Agilent Technologies). All DNA sequences were determined by the dideoxy chain termination (Genewiz, New Jersey).

Cell Culture and Transfections: The rat mesencephalic cell line 1Rb3AN27 was kindly provided by Dr. Alexander Sorkin (University of Pittsburgh, Pittsburgh, PA) and was maintained in RPMI1640 supplemented with 10% fetal bovine serum, 2mM glutamine and 100 units/ml penicillin/streptomycin, 37°C, 5% CO₂. Pooled, stable AN27 cell lines expressing either wild-type hDAT, LAP-hDAT or LAP-hDAT-AAA, respectively, were generated by transfecting 2×10⁵ cells/well in 6-well culture plate with 1μg plasmid DNA using Lipofectamine 2000, at lipid:DNA ratio of 2:1 (w/w). Stably transfected cells were selected with 200μg/ml G418 (Invitrogen) and resistant cells were trypsinized, pooled and maintained under selective pressure in 80μg/ml G418. SK-N-MC cells were from American Type Culture Collection (ATCC) and were maintained in MEM (Sigma-Aldrich M2279) supplemented with 10% fetal bovine serum (Invitrogen), 2mM L-glutamine, 10² U/ml
penicillin/streptomycin, 37°C, 5% CO₂. Pooled #14 stable SK-N-MC cell lines expressing hDAT was generated by transfecting 1×10⁶ cells/well in 6-well culture plate with 3μg plasmid DNA using Lipofectamine 2000, at lipid:DNA ratio of 2:1 (w/w). Stably transfected cells were selected in 500μg/ml G418 (Invitrogen) and resistant cells were pooled and maintained under selective pressure in 200μg/ml G418.

[^3H]DA Uptake Assay: Cells were seeded onto 96-well plates one day prior to performing assays, at 2×10⁴ or 7.5×10⁴ cells per well, for single point and kinetic studies, respectively. Cells were washed twice with KRH buffer (120mM NaCl, 4.7mM KCl, 2.2mM CaCl₂, 1.2mM MgSO₄, 1.2mM KH₂PO₄, 10mM HEPES, pH 7.4) and preincubated in KRH buffer supplemented with 0.18% glucose, 37°C for 30min in the presence of either vehicle or the indicated drugs. For single point assays, uptake was initiated by adding 1μM[^3H]DA in KRH buffer supplemented with 0.18% glucose and 10μM each pargyline and sodium ascorbate (KRH/g/p/a). For uptake kinetics, 10x cocktails were prepared by diluting a 1/20th volume[^3H]DA into a 60μM unlabeled DA KRH/g/p/a. The 60μM[^3H]DA solution was serially diluted into KRH/g/p/a to generate[^3H]DA cocktails at 10X the indicated final substrate concentrations. Cells were preincubated (20 min, 37°C) with KRH buffer supplemented with 0.18% glucose (KRH/g) and uptake was initiated by adding a 1/10th volume of 10x concentrated[^3H] substrate cocktails. Non-specific uptake at each DA concentration was defined in parallel in the presence of 10μM GBR12909. Assays proceeded for 10 min, 37°C and were terminated by rapidly washing cells thrice with ice-cold KRH buffer. Cells were solubilized in scintillation fluid, and accumulated radioactivity was measured by liquid scintillation counting in a Wallac Microbeta plate counter.

Endocytic Rate Measurements by Reversible Biotinylation: Cells were plated at 3×10⁵ cells per well in 6-well plate 24 hours prior to conducting experiments. Cells were washed three
times with ice-cold PBS\textsuperscript{2+} (phosphate-buffered saline, pH 7.4, supplemented with 1mM MgCl\textsubscript{2} and 0.1mM CaCl\textsubscript{2}), surface proteins were biotinylated twice, 15 min, 4°C, with 2.5 mg/mL sulfo-NHS-SS-biotin, and were quenched twice, 15 min, 4°C with 100mM glycine/PBS\textsuperscript{2+} (quench solution) Internalization was initiated by washing cells rapidly with three times with pre-warmed (37°C) PBS\textsuperscript{2+} supplemented with 0.18% glucose/0.1% IgG/protease free BSA (PBS2+/g/BSA) containing either vehicle or the indicated drugs, and incubating 10 min, 37°C. Zero timepoint and strip controls were kept at 4°C in parallel. Endocytosis was arrested by rapidly washing cells with ice-cold NT buffer (150mM NaCl, 20mM Tris, pH 8.6, 1.0mM EDTA, pH 8.0, 0.2% protease free/IgG free BSA). Residual surface biotin was stripped by reducing twice with 100mM TCEP in NT buffer, 25 min, 4°C, followed by three washes with PBS\textsuperscript{2+}. Cells were lysed in RIPA containing protease inhibitors, 20 min, 4°C with constant shaking and insoluble cellular debris was pelleted, 14,000 x g, 10 min, 4°C. Lysate protein concentrations were determined by BCA protein assay (Thermo), compared to BSA standards, and biotinylated proteins were isolated from equivalent amounts of total cellular protein by batch streptavidin-agarose affinity chromatography as previously described (Loder and Melikian, 2003; Holton et al., 2005; Boudanova et al., 2008a; Boudanova et al., 2008b; Gabriel et al., 2013; Wu et al., 2015). Samples were resolved by SDS-PAGE and DAT was detected by immunoblot using a monoclonal rat anti-DAT antibody (MAB369; EMD Millipore). Non-saturating DAT bands were detected using a VersaDoc gel documentation system and were quantified using Quantity One software (Bio-Rad). Relative internalization rates were calculated as %biotinylated DAT internalized as compared to DAT surface levels at t= 0.

\textit{W37VLplA expression and purification}: His\textsubscript{s}-tagged \textit{W37VLplA} was expressed and purified as previously described (Uttamapinant et al., 2013). Briefly, pYFJ16-His\textsubscript{s}-\textit{W37VLplA} plasmid
was transformed into BL21 *E. Coli* and bacteria were incubated at 37°C, with shaking until they attained log phase growth. Protein expression was induced with 100μg/ml isopropyl-β-D-thiogalactopyranoside (IPTG), and proceeded for 8 hours at room temperature. Bacteria were lysed and His<sup>W37V</sup>LpLA was purified by nickel-affinity chromatography. Eluted protein was dialyzed for 8 hours, twice, against 20mM Tris base, 1mM DTT, 10% (v/v) glycerol, pH 7.5, at 4 °C, and protein concentrations were determined by A<sub>280</sub> absorbance (NanoDrop, Thermo Scientific) using an extinction coefficient of 46250 M<sup>-1</sup>cm<sup>-1</sup>. Ligase aliquots were stored at -80°C.

**PRoBE Incorporation Mediated by Enzymes (PRIME) Labeling and Post-Endocytic Tracking Studies:** Live AN27 cells stably expressing the indicated LAP-DAT constructs were covalently labeled with alkyne-AlexaFluor as described previously (Uttamapinant et al., 2010; Uttamapinant et al., 2013). Briefly, cells were seeded onto glass coverslips in 24-well plates at a density of 8x10<sup>4</sup> cell/well, one day prior to assaying. Cells incubated with a ligation mixture containing 10μM<sup>W37V</sup>LpLA, 200μM picolyl azide (pAz), 1.0mM ATP and 5.0mM MgCl<sub>2</sub> in PBS/3%BSA, 20min, room temperature, followed by three washes with PBS<sup>2+</sup>. A low Cu<sup>2+</sup> click labeling solution containing 10mM CuSO<sub>4</sub>, 50mM BTTAA and 100mM sodium ascorbate and was prepared and incubated at room temperature, 10min in a closed tube. The labeling solution was diluted 200X with PBS<sup>2+</sup> (producing final concentrations of 50μM CuSO<sub>4</sub>, 250μM BTTAA and 500μM sodium ascorbate), supplemented with alkyne-Alexa594 to a final concentration of 20μM, and incubated with cells for 10 min, room temperature. Cells were washed three times with room temperature PBS<sup>2+</sup> and internalization was initiated by rapidly washing cells and incubating in pre-warmed (37°C) PBS<sup>2+</sup>/g/BSA, containing the indicated drugs. Cells were fixed at the indicated post-endocytic timepoints in 4% paraformaldehyde for 10 min, room temperature.
and were subsequently blocked, permeabilized and stained with indicated primary antibodies and Alexa488-conjugated secondary antibodies as previously described (Navaroli et al., 2011). Note that all of the antibodies directed against endosomal markers were carefully vetted for specificity: 1) By their ability to recognize native and GFP-tagged proteins via immunoblot, and 2) by their ability to label GFP-tagged endosomal markers in situ. Dried coverslips were mounted in ProLong Gold with DAPI to stain nuclei, and were thoroughly cured prior to performing imaging.

**Wide Field Microscopy:** Cells were visualized with a Zeiss Axiovert 200M microscope using a 63X, 1.4 N.A. oil immersion objective and 0.2μm optical sections were captured through the z-axis with a Retiga-1300R or Regita-R1 cooled CCD camera (Qimaging). 3-D z-stack images were deconvolved with a constrained iterative algorithm using measured point spread functions for each fluorescent channel using Slidebook 5.0 software (Intelligent Imaging Innovations). All representative images shown are single, 0.2μm planes through the center of each cell.

**Image Processing for Co-localization:** Given an unprocessed, multi-color, 3-D image stack, the DAT image was used to identify: 1) an extracellular background region-of-interest (ROI) for background fluorescence estimation, and 2) the range of contiguous optical sections (z-axis planes) containing in-focus DAT signal (i.e. the cell). The sample fields imaged were chosen to contain at least one extracellular region at least 5μm across. This background region was automatically determined by first taking the maximum intensity projection (in Z) of the DAT stack. Then the (x,y) position of the 2-D region (x±radius, y±radius) having the lowest average intensity within this projection was saved. The radius used was nominally 20 pixels (±2μm). For each of the color image stacks, at each z plane the average intensity of this 2-D region was subtracted from all pixels of the plane, leaving as positive signal the
fluorescence greater than the extracellular background. The outline of the box containing
this background region was superimposed on the DAT maximum projection image, as well
as the maximum projections of the other color stacks, for visual inspection and verification
before proceeding with the analysis.

The in-focus DAT data planes were also automatically determined, by first calculating the
normalized total energy $\hat{E}$ of each Z plane, defined as

$$E(z) = \frac{\sum \sum I(x,y,z)^2 - n(z) \bar{I}(z)^2}{n(z) \bar{I}(z)^2}$$

and

$$\hat{E}(z) = \frac{(E(z)-E_{\text{min}})/(E_{\text{max}}-E_{\text{min}})}.$$

where $I(x,y,z)$ is a pixel intensity at position $[x,y]$ in plane $z$, $\bar{I}(z)$ is the average intensity of
plane $z$, and $n(z)$ is the total number of pixels in plane $z$. Starting from the bottom of the
stack (1st $z$ plane) and moving up, the first in-focus plane $z_{\text{bot}}$ was defined as the $z$ plane
where $\hat{E}(z)$ exceeds $\hat{E}_T=0.5$. Similarly, starting from the top (last $z$ plane) and moving down,
the last in-focus plane $z_{\text{top}}$ was where $\hat{E}(z)$ exceeds the threshold $\hat{E}_T$. This was generally a
conservative threshold, keeping a few out-of-focus planes at the top and the bottom. The
planes $z_{\text{bot}}$ to $z_{\text{top}}$ were extracted from the background-corrected multi-color stacks for
deconvolution. The point-spread function of the microscope system was determined from
images of slides of 4-color, 175nM diameter beads (PS-Speck Microscope Point Source
Kit, Thermo Fisher Scientific Inc.) All images were subjected to regularized, constrained,
iterative deconvolution as previously described (Carrington et al., 1995) with the same
smoothness parameter ($\alpha=5\cdot10^{-5}$) and integrated until the algorithm reached convergence
(0.001 level). All image processing was performed using custom software.
Co-localization analysis: All image analysis was performed using custom software. Restored images were first segmented via a manually set threshold to identify the signal in each independent channel. Each 3-D restored image was projected (via maximum intensity projection) to 2-D and then displayed to maximize contrast between signal and background. Three independent thresholds were then chosen for each image, at low, medium and high levels. The criteria used to define these thresholds were as follows: "low" allowed for some diffuse background signal within the cells, "medium" removed all the diffuse cellular signal, and "high" eliminated the margins of labeled structures. To avoid biasing the analysis, thresholds were chosen without knowledge of the experimental conditions of the image. All pixels above threshold in the 3-D images were retained, while those below threshold were set to 0. DAT co-localization with the indicated proteins was calculated by counting the number of positive (>0) pixels in the 3-D DAT image that were also positive in the corresponding position of the indicated 3-D protein image, and dividing by the total number of positive DAT pixels. Co-localization percentages were calculated at three different thresholds for each channel, however the percentages calculated using the medium threshold were chosen for statistical analysis and data presentation. The low and high thresholds were used to evaluate whether conclusions drawn from the co-localization results at the medium thresholds varied dependent upon the threshold chosen. Comparison among these conditions revealed that relative changes in DAT/protein co-localization across a given time course were not dependent upon the threshold level chosen; rather, only the baseline (t=0) %co-localization was impacted by a given threshold level.

Brain Slice Immunohistochemistry and Confocal Microscopy: All animals were handled according to University of Massachusetts Medical School IACUC protocol A1506 (H.E.M.).
Adult male C57/Blk6 mice were transcardially perfused with 4% paraformaldehyde and brains were removed and post-fixed for one day at 4°C, followed by dehydration in PBS/30% sucrose, 4°C, 2-3 days. 25μm coronal sections were taken through the striatum and midbrain using a sliding microtome (Leica) and slices were blocked in PBS with 0.2% Triton-X-100, 5% normal donkey serum and 1% H₂O₂. For DAT and Vps35 immunofluorescence, sections were co-incubated overnight with rat anti-DAT (1:2000) and goat anti-Vps35 (1:500) in PBS with 0.2% TritonX-100, 5% normal donkey serum and 1% H₂O₂. Slices were rinsed in PBS, and incubated with donkey anti-goat and donkey anti-mouse AlexaFluor (1:2000 each) for 1hr at room temperature. Unbound secondary antibodies were washed in PBS and slices were mounted onto glass slides, dried and coverslipped in Prolong Gold mounting medium containing DAPI (Invitrogen). Images were acquired with a Leica TCS SP5 II laser scanning confocal microscope (Cell and Developmental Biology Core, University of Massachusetts Medical School) using either a 20X, 0.7 N.A. (HCX PL APO CS 20.0x0.70 IMM, Leica) or 63X, 1.4 N.A. oil immersion (HCX PL APO CS 63.0x1.40 OIL, Leica) objective. 0.4μm optical sections were captured through the z-axis and 3-D z-stack images were imported into ImageJ using Bio-Format Importer plugin. All images shown are single, representative 0.4μm planes.

**shRNA, Lentiviral Production and Transduction:** Human Vps35-targeted short hairpin RNAs (shRNA), cloned into the pGIPZ lentiviral vector, were from GE Healthcare Dharmacon, and were purchased from University of Massachusetts Medical School RNAi Core.

Full-length hairpin sequences were as follows:

Non-silencing (Luciferase 693):
Initial shRNA efficacies were determined by immunoblotting cell lysates obtained from HEK293T cells transiently transfected (Lipofectamine 2000) with the indicated pGIPZ-shRNA vs. control plasmids. Replication incompetent lentiviral particles were produced as previously described by our laboratory (Wu et al., 2015) and titers were determined 48 hrs. post-transfection by counting GFP-positive cells in transduced HEK293T cells.

For Lentiviral Transduction: 2.5x10^5 DAT SK-N-MC cells/well were seeded into 12-well plates one day prior to viral transduction and were infected with 3ml of the indicated crude lentivirus supplemented with 8μg/ml polybrene. Virus was removed 24 hours post-infection and replaced with media supplemented with 1μg/ml puromycin to select transduced cells. Cells were assayed 96 hours post-infection.

Endocytic Recycling Assay: DAT delivery to the cell surface from endocytic compartments was measured using a continuous biotinylation assay, as previously described (Boudanova et al., 2008a). DAT-SK-N-MC cells were treated with the indicated lentiviral particles, 96 hours, 37°C and surface DAT was biotinylated at 4°C as described above. Endocytic trafficking was initiated by rapidly by washing cells thrice in pre-warmed PBS^2+/g/BSA (37°C) and incubating cells in PBS^2+/g/BSA containing 1.0 mg/ml NHS-SS-biotin, 37°C for the indicated times to covalently couple biotin to proteins newly delivered to the plasma membrane. To compensate for NHS group hydrolysis, the biotinylation reagent was replenished following the initial 20 min incubation for the 30 and 45 min
timepoint samples. Labeling was terminated by washing thrice with ice cold quench solution and residual biotinylation reagent was quenched by incubating twice with quench solution, 15 min, 4°C. Parallel samples were maintained at 4°C to define the baseline biotinylation level at t=0. Cells were lysed and biotinylated proteins were separated from non-biotinylated by batch streptavidin affinity chromatography, as described above. Supernatants were concentrated using Amicon Ultra spin concentrators with a 30kDa molecular weight cutoff (Millipore), and both biotinylated and supernatant DAT fractions were detected by immunoblot. % biotinylated DAT at each timepoint was calculated as:

\[
\frac{\text{Biotinylated DAT}}{(\text{biotinylated DAT}) + (\text{non-biotinylated DAT})} \times 100
\]

Biotinylated DAT values at each timepoint were normalized to the baseline biotinylated DAT value determined at t=0 for each independent experiment. Timecourse data were fitted to a one-phase association kinetic equation:

\[
Y = Y_0 + (Y_{max} - Y_0) \times (1 - e^{-kt}),
\]

where K is the rate constant and \( \tau \) is the time constant, defined as \( 1/k \).

Cell Surface Biotinylation: DAT surface levels in SK-N-MC cells were determined by steady state biotinylation as previously described (Navaroli et al., 2011; Gabriel et al., 2013; Wu et al., 2015). Briefly, following infection with the indicated lentiviral particles, cells were labeled twice, 15 min, 4°C with 1.0 mg/ml sulfo-NHS-SS-biotin in PBS\( ^{2+} \) and excess biotinylation reagent was quenched twice, 15 min, 4°C with PBS\( ^{2+} /100\text{mM glycine} \). Excess glycine was removed by washing three times in ice-cold PBS\( ^{2+} \) and cells were lysed in RIPA buffer (10mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 0.1%SDS, 1%Triton-X-100, 1% sodium deoxycholate) containing protease inhibitors. Lysates were cleared by centrifugation and protein concentrations were determined by BCA protein assay kit.
Biotinylated proteins from equivalent amount of cellular protein were recovered by batch streptavidin affinity chromatography (overnight, 4°C) and bound proteins were eluted in denaturing SDS-PAGE sample buffer, 30 min, at room temperature with rotation. Samples were analyzed by SDS-PAGE and indicated proteins were detected by immunoblotting with the indicated antibodies. Immunoreactive bands were detected with SuperSignal West Dura (Pierce) and were captured using the VersaDoc Imaging station (Biorad). Non-saturating bands were quantified using Quantity One software (Biorad).

**Statistical Analysis:** Data are presented as means of results from each independent experiment, ±S.E.M, as indicated in figure legends. For experiments in which two conditions were compared, data were analyzed using an unpaired, two-tailed Student’s t-test. For experiments in which three or more conditions were evaluated, statistical significance was calculated using either a one-way or two-way ANOVA, followed by post-hoc multiple comparison tests, as indicated in the figure legends. The initial slope for each timecourse was determined for data points between t=0 and the plateau, defined as the point after which no significant differences were detected. Data from independent experiments were analyzed by linear regression, and slope values were averaged. All statistical analyses were performed using GraphPad Prism 6.0 software.

**RESULTS**

**DAT expression, function and trafficking tolerate LAP peptide incorporation into extracellular loop 2**

Previous studies investigating DAT post-endocytic trafficking relied primarily on either antibody feeding or DAT bound to fluorescent cocaine analogs, either of which have potential to artifactually target DAT’s post-endocytic destination(s). We aimed to track DAT
endocytic trafficking by covalently labeling the DAT cell surface population with small fluorophores, thereby creating a “fluorescent DAT”. To accomplish this, we took advantage of the recently reported PRIME labeling approach (Uttamapinant et al., 2013). This method covalently couples fluorophore to cell surface proteins that encode an extracellular ligase acceptor peptide (LAP), which is a substrate for bacterial lipoic acid ligase (LplA). To generate a DAT construct compatible with extracellular LAP labeling, we replaced hDAT 2nd extracellular loop (EL2) residues 193-204 with a twenty-seven amino acid peptide that encodes the 13 amino acid LAP sequence, flanked by linkers of 7 amino acids in length (LAP-DAT). We first tested whether the LAP peptide insertion had any deleterious effect on DAT expression, function, functional downregulation or regulated endocytosis when stably expressed in the rat mesencephalic cell line, 1Rb3AN27 (AN27). LAP-DAT exhibited robust, saturable [3H]DA uptake in these cells (Fig. 1A) and kinetic analyses revealed no significant difference between LAP-DAT and wildtype DAT (WT-DAT) V_{max} values (WT-DAT: 99.3±18.2 vs. LAP-DAT: 94.4±19.7 pmol/min/mg; p=0.86, Student’s t test, n=6), or affinity for DA (K_{m} - WT-DAT: 2.8±0.4 vs. LAP-DAT: 4.8±1.3μM; p=0.17, Student’s t test, n=6). Immunoblot analysis revealed that LAP-DAT expressed at levels comparable to WT-DAT, with a similar ratio of mature (~75kDa) to immature (~55kDa) biosynthetic species (Fig. 1B, top). LAP-DAT was also subject to PKC-induced function downregulation comparable to WT-DAT. PKC activation with PMA significantly decreased WT-DAT and LAP-DAT function to 68.7±3.4% and 58.2±7.5 %control levels, respectively (Fig. 1B, bottom). We next tested whether LAP-DAT constitutively internalizes and is subject to stimulated endocytosis in response to Ack1 inhibition as we previously reported (Wu et al., 2015). Basal LAP-DAT internalization rates were not significantly different from WT-DAT (p=0.20) and AIM-100 treatment significantly increased both LAP-DAT
(134.5±5.3 % control levels) and WT-DAT (162.6±16.8 % control levels) internalization (Fig. 1C). Thus, appending the DAT EL2 domain with the LAP peptide did not deleteriously impact DAT biosynthesis, function or regulated endocytosis, consistent with previous studies in which epitopes were engineered into EL2 (Sorkina et al., 2006; Navaroli et al., 2011). It should be noted, however, that although LAP-DAT expression and function was comparable to WT-DAT in AN27 cells, we observed marked LAP-DAT proteolytic cleavage when expressed in either SK-N-MC or SH-SY5Y cells (data not shown), precluding use of these cells for LAP-DAT studies.

We next tested whether LAP-DAT could be covalently coupled to fluorophore using the PRIME strategy (see schematic; Figure 1D), by coupling alkyne-Alexa594 to LAP-DAT AN27 cells, and subsequently fixing, permeabilizing and co-staining cells with an anti-DAT antibody. Alkyne-Alexa594 signal was detected at the surface of cells expressing LAP-DAT, whereas nearby non-transfected cells (identified using DAPI staining) were not labeled (Fig. 1E). Moreover, the alkyne-Alexa594 signal overlapped with the anti-DAT antibody signal at the cell surface. These results demonstrate that PRIME labeling is both highly efficacious and specific for LAP-DAT. As a negative control, we performed PRIME labeling on cells expressing WT-DAT, and observed no labeling (data not shown). Of note, we observed that a linker between the DAT polypeptide backbone and the LAP peptide was absolutely required in order to achieve high efficiency labeling, and earlier attempts at labeling LAP-DAT constructs, either without linkers or with shorter linkers, failed to efficiently label cells (data not shown).
We next used LAP-DAT to temporally track DAT’s post-endocytic fate. We labeled the DAT surface population at room temperature (18-22°C; conditions of minimal endocytosis), stimulated internalization by shifting cells to 37°C, and then fixed/stained cells at various post-endocytic time points to quantify DAT co-localization with several endosomal markers (see schematic, Fig. 2A). As a proof of concept, we first characterized DAT trafficking from the plasma membrane to early endosomes, given consistent reports that DAT co-localizes to EEA1/rab5-positive vesicles shortly following internalization (Daniels and Amara, 1999; Melikian and Buckley, 1999; Eriksen et al., 2009). At the zero-minute timepoint, 4.0±0.2% of the LAP-DAT signal co-localized with EEA1 (Fig. 2B-C). This minimal co-localization was observed between DAT and each of the endocytic markers investigated throughout our study, and we attributed this baseline signal to low-level DAT internalization that occurred during the room temperature labeling procedure. Under basal conditions, DAT/EEA1 co-localization rapidly and significantly increased over the first 10 minutes of internalization, and peaked at 21.3±1.4 %co-localization, which translates to a 430.9% enhancement over baseline. DAT/EEA1 co-localization plateaued at subsequent timepoints, although there was a trend for decreased DAT/EEA1 co-localization at the 60-minute timepoint (Fig. 2C; p=0.08). We also observed significant, time-dependent DAT/EEA1 co-localization upon stimulating DAT internalization with 20μM AIM-100 (Fig. 2C). However, we observed a significant decrease in the initial slope of DAT/EEA1 co-localization over time in cells treated with AIM-100, as compared to vehicle-treated cells (initial slope = vehicle: 1.7±0.4 vs. AIM-100: 0.87±0.1, p=0.02, Student’s t test, n=30) (Fig 2C). These results demonstrate that the PRIME labeling strategy is effective for tracking DAT from the cell surface to endosomal destinations. Note that in the interest of
space, we have only presented DAT internalization images under control conditions, as any AIM-100-induced differences are not discernible by visual inspection alone.

Following internalization and localization to early endosomes, proteins are targeted either to degradative, recycling, or retrograde (i.e. TGN) pathways. Given that DAT recycling and rapid delivery to the plasma membrane has been reported by our laboratory and others (Loder and Melikian, 2003; Lee et al., 2007; Gabriel et al., 2013; Richardson et al., 2016), we next asked whether DAT targets to the classic rab11 recycling endosome (Welz et al., 2014). Under basal conditions, we observed no significant DAT/rab11 co-localization over baseline at either the 5- or 10-minute timepoints (Fig. 3A-B). A small, but statistically significant, increase in DAT/rab11 co-localization (7.4±0.42 %co-localization) was observed at 20 minutes post-endocytosis, which translated to 46.3% over baseline.

During AIM-100 treatment, we observed significant increases in DAT/rab11 co-localization at 45- and 60-minute timepoints, and significantly more LAP-DAT co-localized with rab11 during AIM-100 treatment, as compared to control cells at the 60-minute timepoint (Fig. 3B). These results indicate that although the majority of DAT does not traverse the conventional recycling pathway, a small but significant DAT population enters rab11-positive endosomes, and this is increased when DAT endocytosis is stimulated by acute Ack1 inhibition.

Given that the majority of internalized DAT did not co-localize with rab11-positive endosomes, we next asked whether DAT targeted to degradation by staining late endosomes, using rab7 as a marker. Under basal conditions, DAT exhibited a slow, linear ($r^2=0.95$) increase in rab7 co-localization over time, with 22.1±0.97% DAT/rab7 co-localization observed at the 45-minute timepoint, which translated to a 295.5% elevation over baseline (Fig. 3C-D). AIM-100 treatment had no effect on the rate of DAT/rab7 co-
These results suggest that a fraction of DAT moves slowly into rab7-positive endosomes following internalization, with no kinetic difference in late endosome targeting under basal vs. stimulated DAT endocytic conditions.

**DAT targets to retromer-positive endosomes**

Given that DAT expression is quite stable, we questioned whether DAT post-endocytic sorting to a rab7-positive compartment was indicative of immediate post-endocytic targeting to the degradative pathway, or to another rab7-positive endosomal population. Recent studies indicate that rab7 is also part of the cargo-selective trimer (Vps35-Vps29-Vps26) that recruits proteins to the retromer complex from the endosomal membrane (Rojas et al., 2008; Seaman et al., 2009). Therefore, we hypothesized that DAT may recycle to the plasma membrane via a retromer-mediated mechanism. To test this possibility, we examined whether internalized DAT entered a retromer-positive endosome, as indicated by co-localization with Vps35, a retromer core protein required for cargo recruitment into retromer (Seaman, 2004; Burd and Cullen, 2014). Under basal conditions, we observed DAT/Vps35 co-localization that rapidly and significantly increased over the first 15 minutes of internalization, and which peaked at 24.0±0.88 %co-localization, which translates to 413.0% enhanced co-localization over baseline (Fig. 4A-B). We further observed a significant loss in DAT/Vps35 co-localization at 45 minutes, as compared to the 15-minute peak, likely reflecting DAT exiting from Vps35-positive endosomes over time.

During AIM-100 stimulation, DAT/Vps35 co-localization likewise increased significantly over time (Fig. 4B). However, AIM-100 treatment significantly diminished the rate of increase in DAT/Vps35 co-localization, as compared to vehicle-treated cells (initial slope = vehicle: 1.2±0.30 vs. AIM-100: 0.63±0.10, p=0.04, Student’s t test, n=24-30), and peaked
at 30 minutes post-internalization (Fig. 4B). We further observed no significant loss of DAT/Vps35 co-localization following the 30-minute peak. These results suggested that DAT is targeted to the retromer complex following either basal or stimulated endocytosis, and is recruited away from retromer-positive endosomes over time under basal conditions.

We next asked whether native DAT co-localized with Vps35 in bona fide DAergic neurons. Immunocytochemistry performed on coronal mouse brain slices revealed DAT/Vps35 co-localization in perinuclear regions of substantia nigra DAergic neurons (Fig. 4C). We also observed discrete DAT/Vps35-positive co-localized puncta in a subset of DAergic terminals in the dorsal striatum (Fig. 4D), consistent with DAT targeting to retromer complex. We also attempted to use PRIME labeling in ex vivo mouse striatal slices, to track DAT post-endocytic trafficking in native DAergic terminals. We engineered an AAV construct encoding a DIO-HA-tagged LAP-DAT, and packaged this construct into AAV2 particles. These were stereotaxically injected into DAT^{RES-Cre} mouse midbrain to facilitate HA-LAP-DAT expression exclusively in dopaminergic neurons. HA-LAP-DAT expressed and was robustly targeted to DAergic terminals in the dorsal striatum (data not shown). However, we were unable to efficiently label LAP-DAT in ex vivo striatal slices prepared from injected mice. Taken together with our cellular LAP-DAT studies, and mouse striatal immunocytochemistry, these results indicate that DAT targets to retromer in both AN27 cells and intact DA neurons, consistent with the premise that internalized DAT is sorted and recycled via a retromer-dependent mechanism.

**Retromer complex is required for DAT recycling**

We next asked whether retromer activity was required to recycle DAT back to the plasma membrane. We reasoned that if DAT recycling were retromer-dependent, retromer disruption would decrease the DAT recycling rate and, potentially, decrease DAT surface
levels and target DAT to degradation in lieu of recycling. To test this hypothesis, we used shRNA to knockdown Vps35, an approach previously reported to disrupt retromer function (Choy et al., 2014; Varandas et al., 2016). For these studies, we turned to stably transfected DAT-SK-N-MC cells, in which DAT trafficking is indistinguishable from that observed in ex vivo mouse striatal slices (Gabriel et al., 2013; Wu et al., 2015). Moreover, this system enabled us to study WT-DAT (rather than LAP-labeled), and to make use of commercially available human-directed shRNA constructs targeting hVps35, whereas rat-directed shRNAs for use in AN27 cells are not commercially available. We screened several human Vps35-targeted shRNA constructs and found that shRNA#32 significantly reduced Vps35 expression to 35.2±4.8 %control Vps35 levels in DAT-SK-N-MC cells (Fig. 5A). Vps35 knockdown significantly decreased both absolute DAT surface levels (normalized to actin, 54.9±6.4 %control; Fig. 5B) and total DAT levels (54.4±4.7 %control; Fig. 5C), whereas non-silencing lentiviral particles had no effect on DAT expression. To test whether DAT loss in response to retromer disruption was due to impaired endocytic recycling, we used a continuous biotinylation assay to measure DAT recycling rates. We observed robust DAT recycling in cells infected with control lentiviral particles (Fig. 5D-E). In contrast, Vps35 knockdown significantly attenuated DAT recycling, with a significant increase in the recycling tau value (vector: 10.1±1.9 vs. shRNA#32: 15.7±2.4, p<0.05, one-tailed Student’s t test, n=7-8; Fig. 5E). Interestingly, although total DAT levels were diminished, the ratio of surface:intracellular DAT was significantly higher following Vps35 knockdown (Fig. 5E), suggesting that retromer disruption caused total DAT loss via enhanced post-endocytic degradation. Indeed, we observed a low molecular weight DAT fragment in intracellular fractions (Fig. 5D) that was significantly enhanced following Vps35 knockdown, as normalized to mature intracellular DAT at the zero-minute timepoint (vector:
Taken together, these results demonstrate that retromer is required to maintain DAT surface levels, and supports that retromer is a major DAT recycling mechanism.

Effect of cocaine and cocaine-analogs on DAT targeting and recycling

Given that previous reports found that cocaine acutely increases DAT surface expression (Daws et al., 2002; Little et al., 2002), and also that previous studies used high affinity, fluorescent cocaine analogs to track DAT trafficking (Eriksen et al., 2009; Eriksen et al., 2010), we next tested whether either cocaine or high-affinity tropane analogs influence DAT trafficking to either pre-lysosomal or retromer-positive endosomes. As seen in Figure 6A, incubation with saturating concentrations of the high affinity cocaine analog, WIN35,429, had no significant effect on the slope of DAT targeting to rab7-positive endosomes (vehicle: 0.19±0.05; WIN35429: 0.20±0.06, p=0.75, Student's t test, n=30 cells sampled over 3 independent experiments), suggesting that high-affinity tropanes do not enhance DAT targeting to degradation. We next tested whether cocaine binding to DAT influenced either targeting to or recruitment from retromer-positive endosomes. Under vehicle-treated conditions, and consistent with our initial findings (Fig. 4), DAT-Vps35 colocalization increased significantly over time, from 0 to 20 minutes post-endocytosis, and then significantly decreased from 20 to 45 minutes as DAT exited retromer-positive endosomes (Fig. 6B). In the presence of 100μM cocaine, DAT-Vps35 colocalization significantly increased from 0 to 10 minutes post-endocytosis, with no significant difference in the initial rate of DAT entry into Vps35-positive endosomes as compared to vehicle (slopes measured from 0 to 10 minutes: vehicle=1.7±0.2; 100μM cocaine=1.9±0.4, p=0.55,
Student’s t test, n=3 independent assays with 28-30 total cells; Fig. 6B). However, during cocaine treatment DAT/Vps35 co-localization plateaued by 20 minutes, and then significantly decreased by 45 minutes (Fig. 6B). Moreover, DAT/Vps35 co-localization was significantly less than vehicle controls at the 20- and 45-minute post-endocytic timepoints (Fig. 6B). Given that cocaine did not alter targeting to degradative endosomes, these results suggest that cocaine may increase DAT recruitment out of retromer-positive endosomes and, possibly, increase DAT recycling, consistent with previous results demonstrating increased DAT surface expression following cocaine exposure (Daws et al., 2002; Little et al., 2002).

**DAT exit from retromer is dependent upon its C-terminal PDZ-binding motif**

We next asked whether a specific domain either targeted DAT to and/or recruited DAT from retromer. Many cargo proteins are recruited to the retromer complex via PDZ binding motifs (Lauffer et al., 2010; Clairfeuille et al., 2016; McGarvey et al., 2016). Interestingly, DAT encodes a C-terminal PDZ-binding motif (-LKV), and multiple reports indicate that DAT surface stability is dependent upon this motif (Torres et al., 2001; Bjerggaard et al., 2004; Rickhag et al., 2013). To test whether the DAT PDZ-binding motif is required for retromer-dependent recycling, we mutagenized the DAT C-terminus within the LAP-DAT background, converting the –LKV motif to alanine residues (DAT-AAA), and tested whether DAT targeting to retromer-positive endosomes was affected. As seen in Figure 7, both WT-DAT and DAT-AAA robustly targeted to Vps35-positive endosomes, and no significant differences were observed in their co-localization with Vps35 at either the t=0 or t=20 minute timepoints (Fig. 7A-B). However, we observed significantly less WT-DAT/Vps35 co-localization as compared to DAT-AAA at the 45-minute timepoint, consistent with exit of WT-DAT, but not the DAT-AAA mutant, from retromer-positive endosomes.
endosomes. These data suggest that the DAT –LKV sequence may contribute to DAT exit from retromer-positive endosomes. To further test this possibility, we compared WT-DAT vs. DAT-AAA recycling in AN27 cells (Fig. 7C-D). Interestingly, we observed a significant difference in recycling between WT-DAT and DAT-AAA, and in several experiments DAT-AAA recycling failed to fit one-phase association kinetics, consistent with disrupted endocytic recycling.

DISCUSSION

Following biosynthesis in midbrain dopaminergic soma, DAT is forward trafficked to distant presynaptic boutons. Given the considerable energy expenditure that neurons invest to generate DAergic terminals, it is evolutionarily advantageous to maintain these complex structures. Endocytic recycling rapidly modulates synaptic function, bypassing the need for de novo biosynthesis. Multiple laboratories report that DAT undergoes constitutive and regulated internalization (Melikian, 2004; Bermingham and Blakely, 2016). However, DAT’s post-endocytic fate has long been debated, with numerous reports demonstrating functional DAT recycling (Loder and Melikian, 2003; Boudanova et al., 2008a; Chen et al., 2013; Richardson et al., 2016), whereas tracking studies observe DAT targeting to degradative vesicles (Miranda et al., 2005; Eriksen et al., 2010). These disparate findings may arise from methodological differences among the studies, which use either antibodies (Hong and Amara, 2013; Block et al., 2015) or fluorescent ligands (Eriksen et al., 2009) to interrogate DAT targeting. Although our approach using PRIME labeling is not likely to either perturb the DAT substrate-binding pocket, or impose large steric hindrance on DAT, there is still the possibility that appending DAT with a small fluorophore may influence its post-endocytic fate.
Consistent with previous results, we observed robust, rapid DAT entry into EEA1-positive endosomes within 5-10 minutes post-endocytosis. Rather than observing biphasic entry into and exit from these vesicles, we observed enhanced DAT/EEA1 co-localization that plateaued. This suggests that the DAT cell surface population did not internalize en masse. Rather, discrete DAT populations may differentially internalize over time, eventually re-distributing labeled DAT throughout the endocytic pathway (Fig. 2C). Alternatively, DAT internalization may be a stochastic process; however, single-particle tracking studies would be required to test this possibility. Surprisingly, we observed a significantly slower initial rate of DAT/EEA1 co-localization during AIM-100-stimulated DAT internalization (Fig. 2C). Basal DAT internalization is clathrin- and dynamin-independent, whereas PKC- and AIM-100 stimulated DAT internalization are clathrin and dynamin-dependent (Gabriel et al., 2013; Wu et al., 2015). Thus, a DAT subpopulation may target to EEA1-negative early endosomes (Hayakawa et al., 2006; Lakadamyali et al., 2006; Kalaidzidis et al., 2015) following stimulated endocytosis. Alternatively, stimulating DAT internalization may saturate the early endosomal machinery, and stall entry kinetics into EEA1-positive early endosomes.

Although rab11-dependent DAT delivery to the plasma membrane has been reported (Loder and Melikian, 2003; Furman et al., 2009a; Sakrikar et al., 2012; Richardson et al., 2016), previous DAT tracking studies have not observed robust DAT entry into classic rab11-positive recycling endosomes (Eriksen et al., 2010; Hong and Amara, 2013). We observed a small, but significant, enhancement in DAT/rab11 co-localization over time under basal conditions, and significantly increased DAT targeting to rab11-positive endosomes following stimulated endocytosis (Fig. 3A-B). However, DAT/rab11 co-localization was relatively modest over time (~46%), with kinetics that
differed from DAT entry into early, EEA1-positive endosomes. In contrast, we observed robust and significant DAT co-localization in Vps35-positive vesicles, that peaked at 15-20 minutes post internalization (Fig. 4A, 4B), with 513% enhancement over baseline, comparable to that observed for DAT entry into early endosomes (~500% over baseline). These kinetic similarities suggest that the majority of internalized DAT targets to the retromer-positive endosomes. Retromer and rab11-dependence are not necessarily mutually exclusive, as several studies report that rab11 may function in consort with retromer to facilitate recycling (van Weering et al., 2012; Hsiao et al., 2015). Likewise, several reports indicate that rab7 is required for retromer recruitment, both in yeast and mammalian cells, and acts in consort with rab5 (Rojas et al., 2008; Seaman et al., 2009). Thus DAT trafficking through rab7-positive endosomes is also consistent with a retromer-mediated mechanism. However, DAT movement into rab7-positive vs. retromer-positive endosomes exhibited distinctly slower and linear kinetics, suggesting that a DAT subset consistently targets to a degradative pathway. Indeed, we observed a decrease in total and surface DAT in response to retromer disruption, consistent with targeted degradation. However, dissimilar to previous reports (Miranda et al., 2005; Hong and Amara, 2013), we did not observe enhanced DAT targeting to rab7-positive endosomes following stimulated DAT internalization (Fig. 3C-D).

It was not feasible to use PRIME labeling to track DAT’s endocytic fate in ex vivo striatal slices. One possible explanation for this obstacle is that the 38kDa LplA may not be able to efficiently penetrate a 300µm acute brain slice. A recent study similarly used antibody feeding to characterize DAT endocytic trafficking in a DAT knock-in mouse expressing an extracellular HA epitope, but found little antibody localization with endosomal markers in dopaminergic terminal regions (Block et al., 2015). It is possible that
this result is due to challenges in permeating a relatively thick brain slice (1.0mM) with a large (150kDa), globular antibody, similar to our results with PRIME labeling. Moreover, it is unclear whether DAT bound to large immunoglobulin would accurately reflect native DAT endocytic targeting, particularly in light of recent studies demonstrating that antibody-bound membrane proteins target to degradation, regardless of their endogenous endocytic routes (St Pierre et al., 2011; Bien-Ly et al., 2014). However, a recent antibody feeding study reported differential DAT targeting to degradation or recycling in response to PKC activation or AMPH exposure, respectively (Hong and Amara, 2013).

Although it was not technically feasible for us to track DAT trafficking in native preparations, robust perinuclear DAT/Vps35 co-localization was readily apparent in DAergic soma. This could indicate potential DAT recycling in somatodendritic compartments. Alternatively, this may simply reflect close opposition between retrograde-targeted proteins and TGN-localized DAT undergoing biosynthesis. Although DAT expression is robust in DAergic terminal regions, we only detected DAT/Vps35 co-localization in a small subset of DAT puncta. Interestingly, elegant recent work from Sulzer and colleagues revealed that only a small fraction of depolarized DAergic terminals actually release DA (Pereira et al., 2016). Thus, DAT recycling may only occur in the small subset of actively releasing terminals. Consistent with this premise, we observe somewhat modest, but significant, DAT surface expression loss following PKC activation in ex vivo mouse striatal slices (Gabriel et al., 2013), which might reflect larger losses in individual active boutons averaged across a more static DAT population in inactive DA terminals. Future studies should more clearly illuminate this possibility.

Although retromer complex was originally characterized as the machinery that recruits cargo destined for retrograde trafficking to the TGN (Seaman et al., 1997; Seaman
et al., 1998), recent studies demonstrate that retromer plays a key role in endosomal recycling (Seaman, 2012; Choy et al., 2014). Importantly, retromer disruption via shRNA-mediated Vps35 knockdown resulted in marked DAT depletion from the plasma membrane and a significant decrease in the DAT recycling rate (Fig. 5). However, retromer disruption did not completely deplete DAT surface expression over a 96-hour knockdown window, and did not completely block recycling. Given that we only achieved a partial Vps35 knockdown, there was likely still some active retromer complex available. Moreover, given that some DAT targets to rab11-positive endosomes, there may have remained some additional, compensatory retromer-independent recycling via a rab11-mediated mechanism.

Retromer-mediated cargo recruitment frequently requires interaction of sorting nexin 27 with a PDZ-binding motif on cargo proteins (Lauffer et al., 2010). DAT encodes a distal C-terminal PDZ binding motif (-LKV), and previous studies implicate this motif in stabilizing mature DAT in DAergic terminals (Torres et al., 2001; Bjerggaard et al., 2004; Rickhag et al., 2013). We found that DAT required the –LKV signal in order to exit from Vps35-positive endosomes and that mutagenizing this domain significantly altered DAT recycling, consistent with a role for this domain in retromer recruitment. Retromer-mediated endosomal fission in yeast requires Vps1, the yeast dynamin homologue (Chi et al., 2014; Arlt et al., 2015). Interestingly, we previously reported that DAT recycling relies upon a dynamin-dependent mechanism (Gabriel et al., 2013), also consistent with a retromer-mediated mechanism. Moreover, mutating the DAT carboxy-terminal PDZ binding motif reduced DAT surface expression in a mouse knock-in model, and the dynamin K44A dominant-negative mutant partially rescued the surface loss of this mutant (Rickhag et al., 2013), suggesting that DAT-AAA is post-endocytically degraded due to its inability to be
recruited by retromer for recycling. Consistent with this possibility, GluT1 encodes a PDZ-binding domain required for recycling, and retromer disruption drives GluT1 to degradation (Steinberg et al., 2013). Moreover, a recent interactome screen identified the DAT homolog, GLYT2 (SLC6A9), as a retromer-associated protein (McMillan et al., 2016), and GLYT2 also encodes a carboxy-terminal PDZ binding motif that is required for stable plasma membrane expression (Armsen et al., 2007). In contrast, serotonin transporter expression and stability are insensitive to C-terminal perturbations (Schmid et al., 2001; El-Kasaby et al., 2010). It is also possible that other nearby residues impact DAT recruitment by retromer. Of particular potential interest is R615, particularly since a coding variant at this residue (R615C) was recently identified in an ADHD proband (Sakrikar et al., 2012). When characterized in cell lines, the R615C exits marked endocytic dysfunction, which can be rescued by co-expressing constitutively active Ack1 (Wu et al., 2015). Future studies evaluating potential R615C recruitment by retromer should shed significant light onto whether retromer-dependent DAT recycling may contribute to neuropsychiatric disorders.
REFERENCES


Figure 1. DAT expression, function, downregulation and endocytosis tolerate LAP peptide incorporation into the 2nd extracellular loop. A. DA uptake kinetics. Saturable DA uptake was measured in stable WT-DAT AN27 or LAP-DAT AN27 cells as described in Material and Methods. Average data are presented ±S.E.M. n=6. (B) LAP-DAT expression and PKC-mediated downregulation. Top: Immunoblot analysis. Representative immunoblots characterizing WT-DAT and LAP-DAT expression in AN27 cells, and actin loading controls. Arrows indicated mature, fully glycosylated (m) and immature, partially glycosylation (i), DAT species. Bottom: [3H] DA uptake assay. Cells were treated ±1μM PMA, 30 min, 37°C and [3H]DA uptake was measured with 1μM DA, as described in Material and Methods. Average data are expressed as %vehicle-treated controls ±S.E.M. **Significant as compared to vehicle-treated control, p<0.01, Student’s t test, n=4. C. Internalization assay: WT-DAT and LAP-DAT internalization rates were measured in AN27 cells ±20μM AIM-100, as described in Material and Methods. Top: Representative immunoblots showing the total surface protein at t=0 (T), strip controls (S), and internalized protein during either vehicle (V) or AIM-100 (A) treatments. Bottom: Average internalization rates are presented as %vehicle-treated controls ±S.E.M, *Significantly greater than vehicle control, p<0.05, one-tailed Student’s t test, n=6-10. D. PRIME strategy for labeling surface DAT. A ligase acceptor peptide (LAP) was engineered into the DAT 2nd extracellular loop. Picolyl azide (pAz) was covalently bound to surface DAT encoding LAP peptide (LAP-DAT) via LplA-mediated coupling, followed by conjugation with alkyne-Alexa594 via low-Cu²⁺ click chemistry, resulting in a fluorescently labeled surface DAT population. E. LAP-DAT labeling. Stable LAP-DAT AN27 cells were labeled with alkyne-Alexa594 (red) as described in Materials and Methods, followed by fixation and staining.
with anti-DAT antibody (green). Two representative fields are shown, and all cells in the field are indicated by DAPI staining (blue).

**Figure 2.** LAP-DAT labeling facilitates DAT post-endocytic tracking. A. **Strategy for tracking DAT post-endocytic routes using PRIME labeling.** Live LAP-DAT AN27 cells were labeled via PRIME labeling at room temperature, as described in Material and Methods. Internalization was induced by shifting cells to 37°C,±20μM AIM-100. Cells were then fixed at the indicated timepoints, permeabilized and stained for EEA1. B. **DAT trafficking to EEA1-positive early endosomes.** Representative images of LAP-DAT (red) and the early endosome marker EEA1 (green) are shown, at the indicated time points (Note: vehicle-treated cells are shown). C. Average data expressed as %DAT co-localized with EEA1±S.E.M. Asterisks indicate significant differences, **p<0.005, ***p<0.001, two-way ANOVA with Bonferroni’s multiple comparison test, n=19-49 cells at each timepoint, imaged across three independent experiments.

**Figure 3.** DAT targets to rab11- and rab-7 positive endosomes. **PRIME labeling studies.** Live LAP-DAT AN27 cells were labeled via PRIME labeling at room temperature, internalized for the indicated times, 37°C, and were probed for either rab7 or rab11 as described in Material and Methods. A and C. Representative images showing LAP-DAT (red) with recycling endosome marker rab11 (green, A) or late endosome marker rab7 (green, C) at the indicated time points. B and D. Average data expressed as %DAT co-localized with either rab11 (B) or rab7 (D) ±S.E.M. Asterisks indicate significant differences, *p<0.05, **p<0.005, ***p<0.001, two-way ANOVA with Bonferroni’s multiple comparison test, n=28-30 cells at each timepoint, imaged over three independent experiments.

**Figure 4.** DAT targets to retromer-positive endosomes. **A., B. PRIME labeling studies.** Surface DAT was coupled to Alexa594 via PRIME labeling, as described in Materials and
Methods, and DAT/Vps35 co-localization was determined at the indicated post-endocytic timepoints. A. Representative images showing LAP-DAT (red) and retromer marker Vps35 (green) at the indicated time points. B. Average data expressed as %DAT-positive objects that co-localized with Vps35 signal ±S.E.M. All later time points were significant different from baseline; Asterisks indicate significant differences, *p<0.05, **p<0.001, two-way ANOVA with Bonferroni’s multiple comparison test, n=24-30 cells imaged at each timepoint, over three independent experiments. C., D. DAT/Vps35 co-localization in mouse DAergic neurons. Coronal sections were prepared from mouse brains and were co-stained for DAT and Vps35 and imaged as described in Material and Methods. C. Representative single-plane confocal images showing DAT (red) and Vps35 (green) expression in somatodendritic region of the substantial nigra. Scale bar = 50μm (top) and 1μm (bottom). D. Representative confocal images showing DAT (red) and Vps35 (green) staining in dorsal striatum. Scale bar: 5μm. White arrowheads indicate DAT/Vps35 co-localized puncta.

Figure 5. Retromer complex is required for DAT recycling and to maintain DAT surface expression. Lentiviral-mediated hVps35 knockdown in SK-N-MC cells. DAT SK-N-MC cells were transduced with the indicated lentiviral particles, as described in Materials and Methods, and assays were performed 96 hours post-transduction. A. Vps35 knockdown efficiency. Top: Representative immunoblots of lysates from DAT-SK-N-MC cells transduced with lentiviral particles expressing either pGIPZ vector (vec), non-silencing shRNA (NS), or hVps35 #32 shRNA (#32), probed for Vps35 and actin. Bottom: Average hVps35 protein levels expressed as %vector-transduced hVps35 levels ±S.E.M. ****Significantly different from vector control, p<0.001, one-way ANOVA with Dunnett’s post-hoc analysis, n=7. n.s. indicates not significant. B. DAT surface levels. Cell surface
biotinylation. DAT surface levels were measured as described in Material and Methods, in DAT-SK-N-MC cells following transduction with the indicated lentiviral particles. Top: Representative immunoblots showing the biotinylated DAT surface fraction (DAT) and actin loading controls. Bottom: Average DAT surface levels (normalized to actin), expressed as %vector controls ±S.E.M. ***Significantly different from vector control, p<0.005, one-way ANOVA with Bonferroni’s multiple comparison test, n=6. C. Total DAT expression. Total DAT levels were quantified by immunoblotting, normalizing to actin loading controls. Top: Representative immunoblots. Bottom: Average total DAT levels (normalized to actin) expressed as %vector levels ±S.E.M. **Significantly different from vector control, p<0.01, one-way ANOVA with Bonferroni’s multiple comparison test, n=5.

D-E. Endocytic Recycling Assay. DAT endocytic recycling rates were determined by continuous biotinylation, as described in Materials and Methods. D. Representative DAT immunoblots of the biotinylated (biot) and non-biotinylated (non-biot) DAT fractions at the indicated timepoints, for vector-treated and Vps35 shRNA#32-treated cells. Arrow indicates a low molecular weight DAT fragment. E. Average recycling data. Values are expressed as %total DAT biotinylated over time, ±S.E.M. Two-way ANOVA indicates significant effect of shRNA, p<0.0001, **Significantly greater than vector control, p<0.0001, Sidak’s multiple comparison test, n=7-8.

Figure 6. Cocaine accelerates DAT exit from retromer-positive endosomes. PRIME labeling studies. The DAT surface population was coupled to Alexa594 via PRIME labeling, as described in Materials and Methods, and DAT co-localization with either rab7 (A, green), or Vps35 (B, green) was measured at the indicated post-endocytic timepoints, ±100μM WIN35,428 (A) or±100μM cocaine (B). Average data are presented. Two-way ANOVA revealed an interaction of both time (p<0.0001) and drug (p=0.0018). Asterisks denote
significant differences between the indicated samples, Tukey’s multiple comparison test, ***p<0.0001, *p<0.03, n=28-30 cells imaged over 3 independent experiments.

Figure 7. DAT exit from retromer and recycling require a C-terminal PDZ-binding motif. A., B. PRIME labeling studies. WT-DAT and DAT-AAA surface populations were coupled to alkyne-Alexa594 via PRIME labeling, as described in Materials and Methods, and DAT/Vps35 co-localization was determined at the indicated post-endocytic timepoints. A. Representative images showing WT-LAP-DAT or LAP-DAT-AAA (red) and retromer marker Vps35 (green) at the indicated time points. B. Average data expressed as %DAT-positive objects that co-localized with Vps35 signals ±S.E.M. Asterisks indicate significant differences, *p<0.04, **p<0.001, two-way ANOVA with Bonferroni’s multiple comparison test, n=29-30 cells imaged at each timepoint, over three independent experiments. C, D. Endocytic Recycling Assay. DAT endocytic delivery to the plasma membrane was measured by continuous biotinylation in AN27 cells stably expressing either WT-LAP-DAT or LAP-DAT-AAA, as described in Materials and Methods. C. Representative DAT immunoblots of the biotinylated (biot) and non-biotinylated (non-biot) DAT fractions at the indicated timepoints, for WT-DAT (top) vs. DAT-AAA (bottom). D. Average recycling data. Values are expressed as %total DAT biotinylated over time, ±S.E.M. Two-way ANOVA indicates a significant effect of DAT construct, p<0.0003, n=7.
Wu et al. Figure 2

A

B

C

%DAPI-dibutylized

0 10 20 30 40 50 60
time (min)

veh

AIM-100
Wu et al. Figure 3

A

B

C

D

% DAT colocalized with Rab11

% DAT colocalized with Rab7

0 10 20 30 40 50 60

0 10 20 30 40 50

**

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Wu et al. Figure 6

A

%DAT-mb7 colocalization

0 10 20 30 40 50 60

time (min)

vehicle

WIN55,428

B

%DAT/ps-8 colocalization

0 10 20 30 40 50

time (min)

vehicle

cocaine

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