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ORIGINAL PAPER

In Situ Regulated Dopamine Transporter Trafficking: There's No Place Like Home

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Abstract

Dopamine (DA) is critical for motivation, reward, movement initiation, and learning. Mechanisms that control DA signaling have a profound impact on these important behaviors, and additionally play a role in DA-related neuropathologies. The presynaptic SLC6 DA transporter (DAT) limits extracellular DA levels by clearing released DA, and is potently inhibited by addictive and therapeutic psychostimulants. Decades of evidence support that the DAT is subject to acute regulation by a number of signaling pathways, and that endocytic trafficking strongly regulates DAT availability and function. DAT trafficking studies have been performed in a variety of model systems, including both in vitro and ex vivo preparations. In this review, we focus on the breadth of DAT trafficking studies, with specific attention to, and comparison of, how context may influence DAT's response to different stimuli. In particular, this overview highlights that stimulated DAT trafficking not only differs between in vitro and ex vivo environments, but also is influenced by both sex and anatomical subregions.

Keywords Dopamine transporter · Membrane trafficking · Striatum

Introduction

Dopamine (DA) is a modulatory neurotransmitter that plays a central role in a variety of complex, evolutionarily conserved behaviors. Midbrain DA neurons in the substantia nigra project to the dorsal striatum (DS), where DA is required for motor control and habit formation. DAergic neurons in the ventral tegmental area (VTA) project primarily to the prefrontal cortex and ventral striatum (VS), where DA critically influences reward, motivation, anxiety, and predictive cue conditioning [1, 2]. DA neurons fire tonically with phasic bursting, and rewarding stimuli drive enhanced bursting [3]. Once released, DA's extracellular half-life is strictly limited by presynaptic reuptake, mediated by the Na^+/Cl^--dependent DA transporter (DAT). DAT is potently inhibited by addictive and therapeutic psychostimulants, such as cocaine, methylphenidate (Ritalin), and amphetamines, which are competitive antagonists (cocaine, methylphenidate) and substrates (amphetamines), and their binding to DAT is requisite to elicit rewarding behaviors [4–6]. Multiple DAT coding variants have been identified in patients with attention-deficit/hyperactivity disorder, ADHD [7–10], autism spectrum disorder, ASD [9, 11, 12], and Parkinson's-like neurodegenerative disorders [13–15], illustrating that DAT dysfunction has a marked impact on DAergic homeostasis.

Decades of effort from multiple investigators support that DAT is not static in the plasma membrane, but is dynamically regulated by endocytic trafficking. Multiple signaling pathways modulate DAT endocytic trafficking, which ultimately impacts DAT surface expression. Given the profound impact that DAT dysfunction imparts on baseline DAergic tone and function, regulated DAT surface expression is mechanistically well poised to likewise influence DA signaling and DA-dependent behaviors. The majority of investigations into the individual mechanisms that mediate regulated DAT trafficking, and their potential impact on DAergic function, have primarily been conducted outside the context of DAergic terminals. Recent technical advances in conditional gene expression and delivery, as well as in optical and ex vivo approaches, have facilitated examining DAT regulation and trafficking in its appropriate context, and have

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raised the possibility that the complex circuitry inherent to DAergic terminal regions may converge to dynamically regulate DAT. Here, we review regulated DAT trafficking studies to date, with an emphasis on how context may influence DAT trafficking. It is our hope that viewing DAT trafficking studies in this light may set the stage for where future DAT regulatory studies may be aimed.

DRD2- and Gi-Mediated DAT Plasma Membrane Delivery

The D2 DA receptor subtype (DRD2) is a G_i-coupled receptor expressed widely throughout the striatum. Presynaptically, DRD2 is an autoreceptor on DAergic terminals. Post-synaptically, DRD2 is expressed in striatal glutamatergic terminals, cholinergic interneurons, and GABAergic medium spiny neurons of the indirect pathway, which project either (1) from the dorsal striatum to the globus pallidus, or (2) from the nucleus accumbens to the ventral pallidum [16]. DRD2 is a member of the DRD2-like receptor subfamily, which is comprised of DRD2, DRD3, and DRD4. Multiple lines of evidence, both from ex vivo and transfected cell line studies, support that DRD2 activation increases DAT function and plasma membrane expression. Initial studies in rat striatal synaptosomes revealed that the DRD2-like agonist, quinpirole, increased DA uptake as measured by rotating disk voltammetry [17]. Moreover, in vivo chronoamperometry demonstrated that DA clearance decreased following systemic injection with the broad-spectrum DRD antagonist, haloperidol [17]. Subsequent kinetic studies in *Xenopus* oocytes co-expressing DAT and DRD2 observed both increased DA uptake V_{max} and [^{3}H]WIN35,428 whole cell binding B_{max}, suggesting that DRD2 activation may increase DAT activity via enhanced surface expression [18]. DRD2-mediated DAT functional upregulation was further confirmed by Liu and colleagues [19], who reported that DRD2 associates with DAT in isolated protein complexes from rat striatal lysates, and that DAT residues 1–26 were sufficient to recover DRD2 in vitro. One potential confound in studies using [^{3}H]DA uptake to measure how DRD2 activation impacts DAT function, is that the inherent addition of DA to the assay will also activate DRD2. To eliminate this potential pitfall, Shippenberg and colleagues leveraged the fluorescent DAT substrate, 4-[4-(diethylamino)-styryl]-*N*-methylpyridinium iodide (ASP^+), which is taken up by DAT, but does not activate DRD2 [20]. Using ASP^+ uptake, these studies found that DRD2-mediated increases in DAT function required ERK1/2, but not PI3-kinase, activity [20] in HEK and N2a cells. Further, using BRET they confirmed the DRD2-DAT association, but found that DAT N-terminal residues 1–55 were not required for the DRD2-DAT association by co-immunoprecipitation. Taken together, these initial studies clearly demonstrated that DRD2 increases DAT activity, and were consistent with the hypothesis DRD2-mediated DAT upregulation was likely due to enhanced surface expression.

DRD2-mediated DAT surface delivery was first directly demonstrated by Gnegy and colleagues, using a surface biotinylation approach in ex vivo mouse striatal synaptosomes, prepared from total striatum that included both DS and VS [21]. Moreover, using both PKCβ-specific inhibitors and $PKC\beta^{-/-}$ mice, they found that DRD2-mediated DAT surface delivery requires PKCβ [21, 22]. These landmark results have opened the door to a variety of new potential questions regarding DRD2-mediated DAT trafficking: Is DRD2-activated DAT trafficking mediated by DRD2 autoreceptors, or is there a retrograde signaling contribution via DRD2 receptors expressed throughout the striatum? Are there regional differences in DRD2-mediated DAT surface delivery? Blakely and colleagues recently reported that DRD2-dependent DAT trafficking differs between DS and VS in ex vivo slices, where the DRD2 agonist, quinpirole, significantly increased DAT surface expression in DS, but had no effect on DAT surface levels in VS [23]. The mechanisms governing these regional differences remain unknown. However, it should be noted that quinpirole can activate all D2-like receptors (i.e. DRD2, DRD3, DRD4; Ki ~ 4.8, 24, and 30 nM, respectively), as well as DRD1 (1.9 µM). Since their study used 1 µM quinpirole, there is a possibility that region-specific effects reported may reflect a net integrated signal from multiple DRDs, which would be equally interesting to discern. Alternatively, region-specific, DRD2-mediated DAT trafficking could arise from distinct DRD2 signaling, which is differentially sensitive to DA in the DS vs. VS [24].

Does DRD2-dependent DAT trafficking occur in vivo? In vivo chronoamperometric studies revealed that hypoinsulinemic rats exhibit reduced DA clearance, due to decreased insulin receptor-mediated PI3K/Akt signaling [25]. Interestingly, DAT activity in hypoinsulinemic rats was restored in a DRD2-dependent manner by treating with AMPH [26], which drives DA efflux through the DAT [27, 28]. These results strongly suggest that DRD2-mediated DAT membrane insertion occurs in vivo, in response to elevated extracellular DA.

Do other G_i-coupled GPCRs promote DAT surface expression? Studies from Shippenberg and colleagues found that kappa opiate receptor (KOR) activation increased DA uptake and DAT surface expression in cell lines and synaptosomes, and likewise found that KOR activation increased DA uptake in minced striatal preparations, using rotating disk voltammetry [29]. Given that KOR activation has aversive properties, KOR-mediated DAT trafficking is poised as a pivotal interaction point between the opiate and reward circuitry, and may have future therapeutic potential [30].

DAT PDZ Domain-Dependent Surface Expression

Multiple DAT domains have been identified that are required either (1) to maintain DAT surface expression, or (2) to promote biosynthetic (i.e. "forward") DAT trafficking. The final carboxy terminal amino acids of DAT, "LKV", constitute a PDZ-binding domain, and are required for DAT binding to the PDZ protein, PICK1 (protein interacting with C kinase 1) [31]. Initial studies in HEK293 cells and cultured DA neurons found that PICK1 potentiated DAT function in an LKV-dependent manner. Moreover, truncating the LKV residues from the DAT carboxy terminus substantially reduced DA uptake and DAT axonal targeting, suggesting that the PDZ domain, possibly through the PICK1 association, is required for DAT surface delivery [31]. A subsequent study by Gether and colleagues confirmed that truncating the LKV motif indeed resulted in DAT retention in the endoplasmic reticulum (ER). They further found that replacing the LKV motif with the β2-adrenergic receptor PDZ domain (SLL) sufficed to rescue DAT surface targeting, but not PICK1 binding, indicating that PDZ-dependent plasma membrane targeting may not be solely dependent upon the DAT-PICK1 interaction [32]. Moreover, using an alanine substitution mutant (DAT-AAA), our laboratory recently found that the LKV PDZ domain is required for retromer-dependent, DAT endosomal surface delivery in the rat mesencephalic cell line, AN27 [33]. However, DAT-AAA relative surface levels were comparable to wildtype DAT, indicating that the DAT LKV motif, per se, might not be required for DAT biosynthesis and forward trafficking in AN27 cells [33].

In order to address the role of the LKV motif in situ, Gether and colleagues generated a knock-in mouse expressing DAT-AAA, which had significantly reduced affinity for purified PICK1 protein [34]. The DAT-AAA mouse had a striking loss in striatal DAT protein. Furthermore, DAT-AAA was not retained in the ER in neuronal cultures made from the knock-in mouse, in agreement with their previous cell line report [32]. However, PICK1 was not required in vivo for proper DAT protein levels or axonal targeting, as demonstrated by the PICK1 knockout mouse [34]. In summary, these data indicate that (1) the DAT PDZ domain is required in vivo for DAT protein expression, but not for DAT's overall surface: intracellular distribution, and (2) PICK1, though initially thought to be required for PDZ-dependent DAT plasma membrane targeting, is not required in vivo for DAT protein expression. Together, these reports highlight the importance of investigating DAT trafficking mechanisms in DAergic neurons, especially if there are contradictory findings among various expression systems.

Constitutive DAT Endocytosis

Constitutive DAT internalization and recycling has been reported in a variety of heterologous expression systems [33, 35–40], as well as in primary DAergic neuronal cultures [38, 41], as measured using biochemical and imaging approaches [35, 36, 38–44]. Constitutively internalized DAT can reportedly target to several endocytic compartments, including those positive for EEA1, rab4, rab5, and the Vps35 retromer complex component. DAT also targets, albeit to a lesser extent, to rab11- and rab7-positive loci [33, 38, 41].

Despite these findings, constitutive DAT trafficking in intact DA terminals has proven difficult to assess. In cell lines, basal DAT endocytic trafficking can be readily measured using reversible biotinylation assays [45]. However, the rapid and dramatic temperature shifts required for this approach are not optimal for acute brain slice viability, creating a sizable obstacle in measuring DAT internalization in bona fide DAergic terminals. Using cultured rat midbrain DA neurons and the fluorescent cocaine analog JHC 1-64, which selectively labels DAT [46], Gether and colleagues found that native DAT indeed constitutively internalizes [38]. Hong and Amara further confirmed this finding, and found that internalized DAT co-localizes with $Rab11^+$ recycling endosomes in rat embryonic mesencephalic primary cultured neurons [41]. To track DAT internalization in DAergic terminals in situ, Sorkin and colleagues generated a DAT knock-in mouse, in which an HA epitope was engineered into the DAT extracellular loop 2 (HA-EL2-DAT), and used this mouse to monitor DAT internalization by tracking anti-HA antibody internalization in ex vivo striatal slices. They found only sparse intracellular HA immunoreactivity via electron microscopy [47], and therefore concluded that DAT undergoes little, if any, constitutive or regulated endocytosis in axon terminals. This result is in contrast to biochemical studies that demonstrate that various stimuli can modulate DAT surface expression in ex vivo striatal slices (elaborated below), and raises the possibility that technical obstacles may have impacted their study. For example, studies were performed in 800 µm brain slices, which are relatively thick in comparison to the standard 250–400 µm thickness typically prepared, which maximizes tissue oxygenation for ex vivo studies. Moreover, several recent reports demonstrated that large immunoglobulins cannot efficiently penetrate thick tissue slices beyond 50–100 µm [48, 49]. Similarly, our laboratory recently reported that although PRIME (PRobe Incorporation Mediated by Enzyme) labeling can efficaciously label surface DAT and track its internalization in monolayer culture, it cannot be used to successfully label DAT in 300 µm acute brain slices, presumably due to an inability of the lipoic acid ligase (LpIA) enzyme to effectively penetrate the slice [33]. Given that the HA-EL2-DAT mouse study did not present controls for either slice viability

or antibody access to deep tissue loci, it is not clear whether the approach used was able to accurately measure endogenous DAT trafficking events.

Recent studies using super-resolution microscopy techniques such as PALM (photoactivated localization microscopy) and STORM (stochastic optical reconstruction microscopy) have allowed researchers to more precisely measure DAT surface dynamics in cultured DA neurons [50], however this type of high-resolution approach has not yet been employed to study basal or stimulated DAT trafficking in DA terminals. Thus, it remains unclear whether DAT undergoes constitutive internalization in the striatum.

PKC-Stimulated DAT Endocytosis

Early studies in *Xenopus* oocytes, COS cells, and striatal synaptosomes demonstrated that the V_{max} of DA uptake rapidly decreases in response to acute protein kinase C (PKC) activation with phorbol esters [51–53], suggesting that DAT may be subject to either PKC-mediated catalytic inactivation, decreased surface expression, or both. Subsequent studies in heterologous expression systems demonstrated that acute PKC activation decreases DAT surface expression [54, 55], and that the shift in DAT from the cell surface to endosomal loci is mediated by increased DAT internalization combined with decreased plasma membrane delivery [35, 41, 53]. PKC-stimulated DAT surface downregulation has been demonstrated in both neuronal and non-neuronal cell culture models (for review see: [56]). We further reported that PKC activation decreases DAT surface expression in ex vivo acute (total) striatal slices, demonstrating that PKC activation impacts DAT surface expression in bona fide DAergic terminals [40]. More recently, we further explored whether there are region-specific differences in the ability of PKC to drive DAT internalization. Surprisingly, PKC activation in DS had no effect on DAT surface expression, whereas in the VS, PKC activation significantly decreased DAT surface levels in male and female mice [57]. Given that PKC activation decreases surface DAT in total striatal slices (i.e. that include both DS and VS) [40], these results suggest that any PKC-mediated effects on DAT trafficking observed in total striatum were driven solely from VS.

We recently reported that in vivo, conditional Rit2 (AKA: Rin) knockdown (Rit2-KD) in DAergic neurons decreased DAT protein levels in total striatum of male mice [58]. Given that Rit2 is required for PKC-stimulated DAT internalization in cell lines [43], we subsequently leveraged shRNA-mediated DAergic Rit2-KD to directly test whether Rit2 is required for the PKC-mediated DAT surface loss in DAergic terminals [57]. In male and female VS, Rit2 was indeed required for PKC-mediated DAT internalization. Surprisingly, following Rit2-KD, PKC activation *increased* DAT surface expression in male DS and had no effect on DAT surface expression in female DS. The mechanism(s) through which PKC increases DAT surface expression in the absence of Rit2 in male DS are not yet known. These results emphasize the importance of studying DAT endocytic mechanisms not only the specific context where DAT is endogenously expressed, but also in both male and female subjects, as the mechanisms are not necessarily the same, and should not be assumed to be so.

As described above, PKCβ is required for D2-dependent DAT insertion (see "DRD2- and Gi-Mediated DAT Plasma Membrane Delivery"), however it remains unknown which PKC isoform(s) are required for PKC-stimulated DAT internalization in response to phorbol ester treatment. PMA activates two diacylglycerol (DAG)-sensitive PKC isozyme subtypes: classical (DAG- and Ca^{2+}-dependent) and novel (DAG-dependent, Ca^{2+}-independent) PKCs [59]. Candidate PKCs can be further narrowed, as PMA-stimulated DAT internalization is blocked by the PKC inhibitor, bisindolylmaleimide (BIM I, GF 109203X, Gö 6850) [54, 60, 61], which is selective for α, βI, δ, ε, and ζ PKC isozymes. However, PKCζ is not DAG-dependent, and therefore not activated by PMA. Thus, PKC-stimulated DAT internalization likely requires either PKCα, βI, δ, or ε.

What are the physiological means that drive PKC-stimulated DAT internalization? Conventional and novel PKCs are typically activated in response to stimulating Gq-coupled GPCRs (G-protein-coupled receptors), which activate PKC and release Ca^{2+} from intracellular stores, in parallel, downstream of phospholipase C activation. However, it still not clear whether activating endogenously expressed, Gq-coupled GPCRs stimulates DAT internalization in intact DA terminals. Studies in transfected HEK293 and N2a cells demonstrated that activating the Gq-coupled receptor neurokinin (NK)-1 with its endogenous ligand, substance P, reduced DAT surface expression in a PKC-dependent manner [62], providing a possible candidate for endogenous PKC-dependent DAT endocytosis. However, substance P-dependent DAT internalization has not yet been reported in DAergic terminals. The Gq-coupled, Group I metabotropic glutamate receptor 5 (mGluR5) has also been implicated in DAT functional downregulation. DHPG, a Group I selective mGluR agonist, decreased DAT function in rat striatal synaptosomes, which was blocked by the mGluR5-specific antagonist, MPEP, as well as the PKC inhibitor, Ro-31–8220 [63]. However, Ro-21-8220 was used at a relatively high concentration that can also inhibit other kinases (e.g. GSK3β, MAPKAP-K1β), raising the possibility that mGluR5-mediated DAT downregulation may be mediated via signaling pathways other than PKC. Fast-scan cyclic voltammetry studies from Alvarez and colleagues recently found that activating the muscarinic receptor M5, a Gq-coupled GPCR selectively expressed in DA neurons [64, 65], significantly decreased DA clearance rates in VS [66]. Given

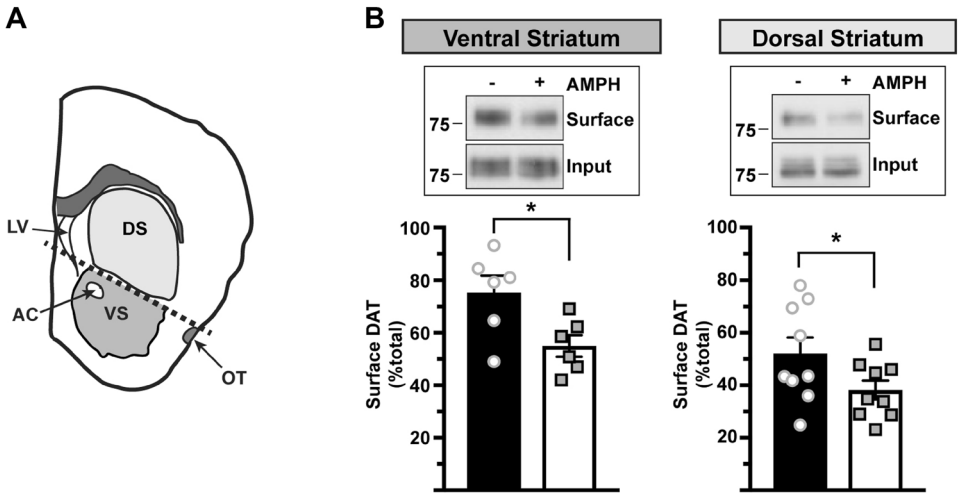

Fig. 1 AMPH stimulates DAT internalization in mouse dorsal and ventral striatum. Ex vivo *striatal slice biotinylation.* Acute striatal slices were prepared and treated ± 10 µM AMPH, 30 min, 37 °C. Surface proteins were biotinylated and isolated by streptavidin pull-down, and DAT was detected by immunoblot using rat αDAT (Millpore MAB369), as previously described [40, 58]; Wu, 2015 #35}. **a** *Striatal slice subdissection.* Slices including VS were identified by presence of the anterior commissure (AC). Prior to lysis, slices were subdissected to enrich for dorsal and ventral striatum, by cutting from the lateral ventricle (LV) to the olfactory tract (OT). **b** *Top* representative blots of surface and total (input) DAT following the indicated treatment(s). *Bottom* average DAT surface levels, expressed as %total DAT input. *Significantly less than vehicle-treated control, $p < 0.05$, one-tailed Student's t test, n = 6–9

that mGluR5, and possibly M5, receptors are expressed on other cell types throughout the striatum, such as cholinergic interneurons and medium spiny neurons, it is unclear whether or not mGluR5 and M5-mediated DAT downregulation occur cell autonomously. Thus, whether activating a Gq-coupled GPCR expressed on DA terminals can stimulate PKC-dependent DAT internalization, and whether this mechanism is subject to regional differences, remains to be tested.

Amphetamine-Stimulated DAT Endocytosis

Amphetamine (AMPH) is an addictive psychostimulant that increases extracellular DA concentrations via multiple actions at the DA terminal. AMPH is a competitive substrate for DAT, thus increases DA by blocking reuptake through DAT [67]. AMPH also depletes vesicular DA content and induces DA efflux through DAT, further enhancing DA levels at the synapse [27, 67–71]. Moreover, AMPH exposure induces DAT internalization from the plasma membrane, thus decreasing surface DAT availability [41, 61, 72–78].

AMPH-stimulated DAT surface loss was originally characterized in HEK293 cells treated with AMPH [72]. This result was later replicated in synaptosomes made from whole rat striatum [75] and in primary DA neuronal cultures [77]. AMPH-induced DAT internalization was further demonstrated in ex vivo mouse midbrain slices, and was shown to be dependent on Rho GTPase activity downstream of the trace amine-associated receptor (TAAR) 1 [77, 78]. However, it is still unknown whether AMPH stimulates DAT internalization in bona fide DA terminals. Using the HA-αEL2-DAT and electron microscopy techniques, Block and colleagues found that i.p. AMPH injection did not subsequently affect DAT surface distribution in axon terminals or DA cell bodies, however it is unclear whether the DAT labeling method employed was sufficient to detect drug-induced changes (see "Constitutive DAT Endocytosis") [47]. To directly test this possibility, we performed ex vivo striatal slice biotinylation (as previously described [40, 44, 58], and found that AMPH treatment (10 µM, 30 min, 37 °C) induced significant DAT surface loss, both in DS and VS (Fig. 1). These results confirm that AMPH drives DAT surface loss in DAergic terminals. However, whether the mechanisms required for AMPH-stimulated endocytosis in DAergic terminals are similar to those in somatodendritic regions remains to be tested.

Receptor Tyrosine Kinase-Mediated DAT Trafficking

DAT surface expression is also regulated by receptor tyrosine kinases (RTKs). Broad-spectrum tyrosine kinase inhibitors, such as genistein, tyrphostin 23, and tyrphostin 25, significantly decreased DAT function in DS synaptosomes and *Xenopus* oocytes [79, 80]. Additional studies indicate that direct RTK activation modulates DAT surface expression [81–84]. Insulin-like growth factor receptor (IGFR-1) activation increased DAT function and surface expression in transfected cell lines, and was dependent on PI3-kinase and Akt activity, as defined with PI3-kinase and Akt inhibitors [81, 82]. Moreover, hypoinsulinemia induced either by

streptozotocin treatment or high fat diet significantly reduced DA clearance rates, DA reuptake, and DAT surface expression compared to controls, as measured in rat striatal synaptosomes [25, 85, 86], consistent with the results obtained in cell lines.

Glial cell line-derived neurotrophic factor (GDNF) also regulates DAT surface expression through receptor Ret activation and downstream signaling [84]. *GDNF*$^{+/-}$ mice exhibited increased DA uptake in the VS, but not DS, as measured via in vivo chronoamperometry, and reduced striatal DA tissue content in both VS and DS [87]. Furthermore, a similar, regional-specific increase in DA levels and DAT function was observed in synaptosomes prepared from *Ret*$^{+/-}$ DS and VS [84]. GDNF/Ret-dependent negative regulation of DAT surface expression was demonstrated to require Vav2, a guanine exchange factor (GEF) that activates Rho and Rac GTPases [84]. In striatal synaptosomes prepared from *Vav2*$^{-/-}$ mice, DAT exhibited enhanced DA uptake and surface expression specifically in the VS, but not DS. Moreover, GDNF-dependent Ret activation increased Vav2 phosphorylation, and Ret co-expression increased the DAT-Vav2 interaction, suggesting that Ret RTK signaling may negatively regulate DAT surface expression through Vav2 activation [84].

In summary, regulated DAT trafficking occurs in response to multiple cellular signaling pathways, and is poised to significantly impact DAergic signaling, as well as DA-dependent behaviors and neuropathologies. The recent increase in DAT trafficking studies, carried out both in vivo and in ex vivo preparations, will undoubtedly glean mechanisms that impact DAT surface presentation, as well as how converging signaling pathways within DAergic terminal regions are integrated to impact DAT surface availability and function.

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