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Dopamine transporter trafficking: rapid response on demand

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Abstract

The dopamine transporter (DAT) is a primary determinant of the concentration of dopamine in the synapse and is involved in a number of psychiatric and neurological diseases. The transporter actively takes up its physiological substrate, dopamine, when it is on the surface of the plasmalemmal membrane, but the concentration of DAT in the membrane is highly regulated by substrate. Substrates initially, and very rapidly, recruit more DAT into the membrane for greater function, but continued presence of substrate downregulates the activity of DAT and even membrane DAT content. This biphasic regulation is orchestrated by numerous signal transduction mechanisms, including a palette of protein kinases. Understanding the mechanisms of rapid regulation of DAT could provide new therapeutic strategies to improve transporter function and modulate responses to its more notorious substrates, amphetamine and methamphetamine.

Keywords

amphetamine; endocytosis; PKC β ; recycling; trafficking

Dopamine (DA), a monoaminergic neurotransmitter, is involved in the control of movement, reward, motivation, emotion, learning, and pituitary and hypothalamic function. The DA transporter (DAT), which removes DA from the synapse through reuptake, is crucial for the regulation of dopaminergic signaling and homeostasis in many areas of the brain. DAT (*SLC6A3*) belongs to the *SLC6* Na⁺/Cl⁻-dependent transporter family, which also consists of serotonin transporter (SERT) and norepinephrine transporter (NET). DAT tightly controls the duration and strength of dopaminergic neurotransmission at the synapse by taking up extraneuronal DA into presynaptic terminals, thus terminating DA action at pre- and post-synaptic sites [1]. Dysfunctional dopaminergic signaling related to DAT has been implicated

in multiple neurodegenerative and psychiatric disorders, including Parkinson's disease, schizophrenia, bipolar disorder, attention-deficit/hyperactivity disorder and drug addiction [2–4]. Recently, the molecular basis of infantile parkinsonism-dystonia was traced to a homozygous loss-of-function mutation in *DAT* [5]. Furthermore, the expression density of DAT strongly correlates with the extent of DA cell loss in Parkinson's disease [6]. Coding variants of DAT have been identified in some patients with attention-deficit/hyperactivity disorder and bipolar disorder [7]. DAT is a target of abused psychostimulants, such as methamphetamine and cocaine, and therapeutic drugs, such as amphetamine (AMPH; Dexedrine and Adderall) and methylphenidate (Ritalin), as well as the antidepressant and antismoking drug, bupropion (Wellbutrin and Zyban).

The primary function of DAT is to remove DA from the synaptic cleft; the newly taken-up DA then re-enters the synaptic vesicle through the vesicular monoamine transporter (VMAT)-2 for storage until it is released again by exocytosis. DAT functions as a gated channel, in that it is open to either the extracellular or intracellular milieu, but not both. These conformations are delineated as outward-facing and inward-facing, respectively (see Figure 1). It is presumed that when extracellular substrate, together with sodium and chloride, bind to DAT, they promote change in the conformation of DAT from one that is primarily outward-facing to one predominantly inward-facing. Recently, a molecular model was proposed to illustrate these conformational changes of DAT [8]. In this model, a salt bridge between Arg-60 in the N-terminus close to the cytoplasmic end of transmembrane (TM)-1 and Asp-436 at the cytoplasmic end of TM8 of DAT was demonstrated to be stabilized by a cation- π interaction between Arg-60 and Tyr-335 at the cytoplasmic end of TM6. The continuous interruption and reformation of the salt bridge was hypothesized to determine the inward- and outward-facing state. However, the mechanism governing the conformational transition of DAT is not understood.

Both AMPH and cocaine, through distinct mechanisms at DAT, inhibit DA reuptake and increase extracellular levels of DA. Cocaine is a DAT blocker, while AMPH is a substrate of DAT and is taken up into the nerve terminal. AMPH competitively inhibits uptake of DA into the terminal at DAT and into the synaptic vesicle at VMAT2. Thus, AMPH prevents proper storage of DA. AMPH is also a weak inhibitor of monoamine oxidase activity. Once inside the terminal, AMPH promotes DA efflux through a functional reversal of the transporter (see Figure 1). While there are several models describing AMPH-stimulated DA efflux (for review see [9,10]), it is generally thought that AMPH translocation through DAT results in an altered electrochemical gradient and a DAT conformation (inward-facing), which favors DA efflux [11].

The importance of DAT in maintaining DA homeostasis is exemplified in mice lacking the *DAT* gene. *DAT*-knockout mice have increased basal locomotor activity compared with wild-type mice and high levels of extracellular DA [12]. The *DAT*-knockout mice also have reduced DA tissue content, reduced DA release and reduced DA receptors relative to wild-type controls, underscoring the importance of DAT in the maintenance of normal DA levels. Although *DAT*-knockout mice were unresponsive to the locomotor effects of cocaine and AMPH, they still exhibited cocaine- and amphetamine-induced conditioned place preference, resulting from neuroadaptation in the lifetime absence of the *DAT* gene. However, cocaine-induced locomotor activity and conditioned place preference were abolished in mice in which mutations on DAT residues abrogated cocaine binding to DAT [13], giving emphasis to this protein as the major site of psychostimulant reward function.

Considering that the primary function of DAT is the reuptake of synaptic DA, the protein will only be effective when it is inserted into the plasmalemmal membrane. Similar to most other membrane-bound proteins, DAT is not static in the membranes, but traffics between the

plasmalemmal membrane and subcellular organelles in a regulated fashion. DAT trafficking is subjected to regulation by DAT substrates, inhibitors and a complicated protein network, including protein kinases, DAT-interacting proteins, oligomerization and glycosylation [14]. Numerous reviews have covered facets of DAT trafficking, particularly the internalization of DAT [1,15]. However, rapid DAT trafficking to the surface has not been fully investigated and understood. In this review, we summarize the recent findings in this field, focusing on the characterization and potential mechanisms of a time-dependent, biphasic effect of DAT substrates and PKC on DAT trafficking. Since DAT plays such a critical role in normal dopaminergic transmission, and because its location at the surface of the neuron is required for normal function, strategies to regulate the trafficking of DAT could be therapeutically useful in a number of disease states.

Constitutive DAT trafficking

The trafficking of newly synthesized DAT from the endoplasmic reticulum and Golgi through to lysosomes has been succinctly described in a recent review by Zahniser and Sorkin [1]. As shown in Figure 2, DAT undergoes constitutive internalization [16–21] and recycling to the surface [16,18,20,22], with a surface half-life of approximately 13 min when measured in heterologous systems [16,23]. Constitutive bidirectional movement of DAT labeled with fluorescent cocaine analogs has been demonstrated by fluorescence recovery after photobleaching analysis in neurites and varicosities of cultured live midbrain dopaminergic neurons [20]. These studies indicated that DAT was highly mobile in both extensions and varicosities of the neurons. DAT constitutively internalized into vesicular structures and partially colocalized with transferrin. Dynamin-dependence of DAT constitutive endocytosis has also been demonstrated in heterologous cell systems using either overexpression of a dominant-negative dynamin I mutant (K44A) or knockdown of clathrin heavy chain [18,20, 24–26].

Internalized DAT colocalizes with the small GTPases Rab-5 and -11, which are markers for recycling endosomes [20,27], demonstrating that DAT internalizes into recycling endosomes. Rab11 has recently been demonstrated to be involved in constitutive DAT recycling. Overexpression of the constitutively active Rab11 mutant in N2A neuroblastoma cells stably expressing human DAT (hDAT) resulted in increased surface DAT expression, whereas the dominant-negative Rab11 mutant led to reduced surface DAT expression [28]. Although DAT lacks conventional endocytic signals, basal DAT trafficking is directed by a constitutive motif in the C-terminus of DAT (the amino acid residues 589–598), demonstrated by the observation that mutations in this motif increase the endocytic rate of DAT, and impair DAT exit from the endoplasmic reticulum and recycling to the surface [17,18,29,30]. Residues in the N-terminus of DAT (amino acids 60–65) also play an important role in constitutive DAT endocytosis because mutations in these residues or removal of the entire N-terminus of DAT results in retention of the DAT mutant in early and recycling endosomes [24]. Furthermore, the N-terminus is necessary for the trafficking of newly synthesized DATs to the plasma membrane, as demonstrated by an intracellular accumulation of a DAT mutant lacking the first 60 residues of the N-terminus [31]. The retention of the DAT mutant in the endosomes is primarily attributed to accelerated constitutive endocytosis, rather than retarded recycling since monensin, a recycling inhibitor, results in greater endosomal accumulation of the DAT mutant than that of controls [24].

DAT recycling is regulated by PKC. PKC activation by phorbol 12-myristate 13-acetate (PMA) in PC-12 cells stably expressing hDAT increases the DAT endocytic rate and decreases the transporter recycling rate, thus reducing DAT surface levels [16]. Among all PKC isoforms, PKC β is potentially important in constitutive maintenance of the surface DAT (Figure 2). Compared with wild-type mice, PKC β -knockout mice have a reduced surface DAT expression

level and reduced [^3H]DA uptake with no difference in the total DAT expression in the striatum [32]. Importantly, treatment of striatal synaptosomes of wild-type mice with a specific PKC β inhibitor (LY379196) reduced basal surface DAT expression and DA uptake, mimicking the basal DAT trafficking pattern in PKC β -knockout mice [32]. It remains to be determined whether PKC β -dependent maintenance of normal surface DAT levels is due to reduced endocytosis or an enhanced recycling rate.

PKC-dependent internalization appears to be dependent upon ubiquitylation of three lysine groups in the N-terminus of DAT; mutation of these lysines resulted in a diminished internalization of DAT to PKC activation [33]. The NEDD4-2 (neural precursor cell expressed, developmentally downregulated 4-2) protein, a homologous to E6-associated protein C-terminus (HECT) domain-containing E3 ubiquitin ligase, was demonstrated to be a requisite component of PKC-dependent DAT internalization [22].

PKC-dependent regulation of constitutive DAT trafficking is readily apparent in various heterologous cell types [1,15], as well as in striatal dopaminergic nerve endings [21,34,35]. By contrast, PKC regulation of constitutive DAT trafficking was not observed in cultured live midbrain dopaminergic neurons because the PKC inhibitor (staurosporine) did not prevent constitutive DAT internalization [20]. It is unclear whether this is a characteristic of post-natal cell cultures or whether it is reflective of dopaminergic cell bodies. In addition to PKC, basal DAT surface expression is also mediated by the signaling of PKB (Akt). Overexpression of a dominant-negative mutant of Akt (K179R) or treatment with Akt inhibitors reduces the surface DAT expression [36].

Many of the studies on factors affecting constitutive or even substrate-induced trafficking of DAT have relied heavily on the use of heterologous cells, and caution should be used in interpretation of the data. Loder and Melikian found that internalization of DAT was insensitive to PKC in cells expressing high basal levels of DAT [16]. In addition to the effect of expression levels, the use of cell types that do not contain the normal contingent of proteins available in the DA terminal could also affect trafficking results. Therefore, findings from heterologous cells should be replicated in animal models, synaptosomes and/or cultured mesencephalic neurons.

DAT internalization induced by continuous exposure to DAT substrates

DAT shows a time-dependent, biphasic trafficking pattern *in vitro* and *in vivo* upon long- and short-term exposure to substrates such as AMPH and DA, as illustrated in Figure 2. It has been well documented that exposure to AMPH results in DAT internalization or reduced DA uptake in cultured cell lines stably expressing DAT [23,24,27,37,38] or in striatal synaptosomes [39, 40]. AMPH-induced DAT internalization is blocked by DAT inhibitors, such as cocaine, nomifensine and mazindol [37], indicating that AMPH must bind to the transporter to elicit internalization. Other DAT substrates, such as DA and methamphetamine, also significantly reduce surface DAT expression or DA uptake [37,40]. Similar to the *in vitro* AMPH treatment, *in vivo* injection of AMPH also reduces DAT V_{max} with no change in K_m from striatal synaptosomes [40–42]. In contrast to AMPH, administration of a high dose of cocaine *in vivo* has no effect on DAT activity [41], although long-term treatment of heterologous cells with cocaine increases surface DAT [43]. Increases in DAT binding have been demonstrated in brains from cocaine addicts upon autopsy [44] and studies using single photon emission computed tomography in cocaine addicts [45,46], which probably represent surface DAT.

It is possible that reduction of surface DAT in response to continuous substrate exposure is a protective mechanism against the build-up of toxic levels of DA. Excessive levels of cytosolic DA and its oxidative products can be toxic to dopaminergic neurons and are postulated to contribute to the development of Parkinson's disease [47–49]. AMPH and methamphetamine

both cause toxic effects on DA neurons, most likely owing to their ability to deplete synaptic vesicles of DA, thus increasing cytosolic DA. Although AMPH abuse ultimately results in depletion of brain DA, toxic effects of AMPHs are not manifested as Parkinson's disease because the deficits are more pronounced in the caudate than in the motor function-related putamen [50]. PET studies demonstrate that chronic methamphetamine use in humans leads to a reduction in the density of striatal DAT. This may not be due to permanent damage, but rather to neuroadaptive changes, since there is no change in other presynaptic markers, such as VMAT2, upon autopsy in methamphetamine abusers [51].

Potential mechanisms underlying substrate-induced DAT endocytosis

Acceleration of DAT endocytosis

The reduced surface DAT expression upon long-term substrate exposure is due to accelerated DAT endocytosis rather than impeded DAT recycling [23]. Pretreatment with concanavalin A, a stabilizer for cell surface integrity, prevents AMPH-induced loss of surface DAT [37], further supporting the notion that acceleration of DAT endocytosis underlies long-term AMPH-induced loss of surface DAT. The accelerated DAT endocytosis is dynamin-dependent. Co-expression of hDAT with a dominant-negative mutant of dynamin I (K44A) inhibits AMPH-mediated loss of surface DAT compared with co-expression with wild-type dynamin I [37].

DAT conformational change

AMPH treatment results in DA efflux through transporter-like and channel-like pathways, in which intracellular sodium is required [52–54]. Therefore, it is hypothesized that AMPH-induced DAT endocytosis is a consequence of the change to an inward-facing conformation, which is driven by an inward current and intracellular sodium. Conformation-destabilizing mutations, which promote an inward-facing state, display a similar pattern of DAT endocytosis and distribution to that of the wild-type DAT exposed to AMPH [24].

Intracellular AMPH

An increase in intracellular AMPH is important for long-term AMPH-induced DAT internalization [38]. A mutant DAT (Y355A) displaying normal substrate binding but impaired substrate transport does not internalize when exposed to extracellular AMPH. However, the mutant DAT does internalize when AMPH is applied intracellularly [38]. This mutation shifts the conformational equilibrium of DAT away from a predominantly outward-facing state. Considering the fact that DAT blockers abrogate the ability of AMPH to induce internalization [37], it would appear that AMPH must be taken up by the wild-type DAT and act intracellularly to promote endocytosis. By contrast, direct intracellular application of DA did not induce DAT internalization [38], indicating that the cellular mechanisms of intra-cellular AMPH- and DA-induced endocytosis might differ.

PKC-dependent mechanism

Numerous studies have demonstrated that direct activation of PKC leads to downregulation of DAT activity with an accompanying redistribution of DAT from the cell surface [1,15]. Similarly, there is some evidence that PKC may mediate DAT substrate-stimulated downregulation of DAT activity. Continuous exposure to AMPH reduces DA uptake in rat striatal synaptosomes, which can be blocked by pretreatment with a PKC inhibitor, bisindolylmaleimide I (BIM I) [40]. BIM I was also shown to block DA- and methamphetamine-induced downregulation of DA uptake in rat DAT (rDAT)-LLC-PK1 cells [55,56]. However, Boudanova *et al.* reported that the AMPH-induced reduction in surface DAT in hDAT-PC12 cells was not inhibited by BIM I [23] and did not necessitate structural determinants required for PKC-dependent internalization signals [21]. Although all three groups utilized different

cells, somewhat different drugs and different incubation times, the differences among the results may partially lie in the outcome measure [1,23,55]. Inhibition of the psychostimulant action by the PKC inhibitor BIM I was detected when a reduction of DA uptake was measured [1,55], while no effect of BIM I was detected when reduction of surface DAT was measured [23]. In fact, a DAT substrate-induced reduction in DA uptake occurs long before a reduction in surface DAT in rat synaptosomes [1]. However, recent evidence strongly indicates that AMPH and PKC activation induce internalization of DAT through independent pathways [57].

AMPH treatment activates PKC, which results in N-terminal phosphorylation of DAT [55]. Although it has not been tested *in vivo* whether phosphorylation of DAT would induce DAT internalization, mutagenesis studies in cultured cell lines expressing mutant DAT have demonstrated that neither the N-terminus of DAT or the phosphorylation of serines within the N-terminus is required for AMPH-triggered DAT trafficking [55,58]. Recent work from Vaughan and coworkers indicates that PKC-induced DAT regulation may depend on the precise membrane localization of the transporter [59]. DAT that can be phosphorylated by PKC resides mostly in lipid rafts, whereas those that are internalized by PKC are in nonlipid rafts, suggesting that the membrane localization determines the fate of DAT phosphorylation or sequestration by PKC *in vitro*. Although the relationship between DAT phosphorylation and internalization is still not definitive, PKC phosphorylation of SERT has been linked to SERT internalization and consequently reduced SERT activity [60].

Other kinase-dependent mechanisms

In addition to PKC, Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) has also been implicated in AMPH-induced DAT internalization. AMPH increases intracellular Ca²⁺ [61, 62] and activates CaMKII activity in rat and mouse striatal synaptosomes and in overexpressed cells [63,64]. As discussed in this review, activation of PI3K and Akt, a protein kinase in the insulin pathway immediately downstream of PI3K, increase surface DAT [26,36]. Upon examining mechanisms of AMPH-induced internalization, Wei *et al.* demonstrated that AMPH inhibited Akt activity via activation of CaMKII [64]. However, the role of CaMKII in constitutive DAT internalization is unclear. CaMKII inhibitors blocked AMPH-induced DAT internalization, but the effect of CaMKII inhibitors on basal DAT levels was not presented.

Although these data strongly suggest that activation of CaMKII by AMPH leads to a down-regulation of DAT, there are other data suggesting that CaMKII contributes to DAT activity. CaMKII α binds directly to the C-terminus of DAT and facilitates *in vitro* phosphorylation of N-terminal serines [65]. The addition of activated CaMKII α increased AMPH-induced DA efflux, while inhibition of CaMKII decreased this activity in hDAT-EM4 cells and mouse midbrain neuronal cultures. Furthermore, the CaMKII inhibitor, KN93, reduced AMPH-stimulated DA efflux in perfused striatal slices. Other investigators have reported no effect of the CaMKII inhibitor on AMPH-stimulated DA efflux in striatal slices [66] or microdialysis of rat striatum in naive rats [67]. In addition, intra-accumbens KN93 had no effect on self-administration of AMPH in rats that had not previously been sensitized to AMPH, although it inhibited this activity in mice previously sensitized to AMPH [68]. Therefore, more studies need to be performed in the intact animal to investigate the role of CaMKII in AMPH-stimulated DA efflux. Whether CaMKII phosphorylation of DAT governs DAT trafficking also warrants further investigation.

Rapid DAT trafficking to the surface induced by short-term exposure to DAT substrates

In contrast to DAT internalization induced by long-term exposure to DAT substrates, an extremely short-term exposure to DAT substrates results in an increase in surface DAT expression (Figure 2). A rapid upregulation in surface DAT expression elicited by AMPH was demonstrated by 30 s in rat striatal synaptosomes and in rDAT-HEK 293 cells [39]. The AMPH-induced increase in surface DAT was blocked by cocaine, demonstrating that AMPH had to interact with DAT in order to elicit the increase. Furthermore, reverse biotinylation studies suggested that AMPH was actively stimulating the recycling of DAT-containing vesicles to the surface, and not reducing endocytosis.

Temporal and spatial resolution of the AMPH-induced ultra-rapid trafficking of DAT to the surface was demonstrated by live-cell imaging using total internal reflection fluorescence microscopy (TIRFM) [69]. TIRFM provides real-time resolution coupled with the ability to selectively detect and analyze cytosol to plasma-membrane movement of vesicles and granules. TIRFM has been successfully used to discern tethering of the glucose transporter 4 (GLUT4)-containing vesicles to the rat adipocyte membrane before fusion [70], as well as the multiple tethering states and significant motion of chromaffin granules immediately preceding exocytosis [71]. In N2A neuroblastoma cells stably expressing GFP-rDAT, AMPH was shown to dose-dependently induce movement of DAT-containing vesicles toward the plasmalemmal membrane within 2 min, as demonstrated by the increased surface intensity of GFP-rDAT in TIRFM analysis. A highly novel finding is that the physiological substrate, DA, also stimulated the movement of DAT toward the plasmalemmal membrane. It has been demonstrated that DA, acting on the D₂/D₃ subtype of DA receptors, will elicit a rapid movement of DAT toward the surface [72–74], but Furman *et al.* demonstrated that substrates could also influence trafficking of DAT to the surface in the absence of D₂/D₃ DA receptors [69]. D₂-type DA receptors were not detected in the GFP-rDATN2A cells; the D₂-type antagonist, sulpiride, did not block the effect of either AMPH or DA in increasing surface DAT, and the D₂/D₃ agonist, quinpirole, had no effect on DA uptake in those cells. Although an interaction of DA with D₂ DA receptors was not required for rapid trafficking of DAT to the surface, an interaction with DAT was required. The increased surface intensity induced by substrates was also blocked by cocaine and was reduced upon drug removal. These data suggest that the interaction of substrate with DAT triggers a further increase in surface DAT. Extracellular Na⁺ was required for the effect, suggesting that both binding and transport of the substrate is required.

The ultra-rapid increase in surface DAT elicited by substrates has physiological consequences. Incubation of cells or synaptosomes with AMPH or DA from 30 s to 2 min, followed by extensive washing, increases [³H]DA uptake [32,73] or AMPH-stimulated DA efflux [39, 69]. Moreover, the time frame of the substrate-elicited increases in surface DAT and DAT function are within the physiological time frame of DA uptake and DAT-mediated DA release. The half-life (T₅₀) of DA clearance in the striatum of a freely moving rat is 26–57 s [75]. The decay of the DA signal is mediated largely by reuptake. Furthermore, AMPH elicits the release of DA within seconds and efflux is maximal by 2 min [76]. This strongly suggests that the physiological substrate, DA, regulates its own uptake to limit excessive DA within the synapse.

Potential mechanisms underlying substrate-induced DAT trafficking to the plasmalemmal membrane

PKC β -dependence

Evidence that PKC β is involved in the regulation of DAT functional activity emerged from the finding that the specific PKC β inhibitor, LY379196, blocked the ability of AMPH to elicit

DA efflux in rat striatal synaptosomes [77]. A schematic illustrating rapid trafficking and the role of PKC β is shown in Figure 2. Moreover, PKC β co-immunoprecipitated with DAT, indicating that the two proteins are closely associated in the membrane. The ability of LY379196 to block AMPH-stimulated DA efflux may be due to a regulation of the ultra-rapid trafficking of DAT to the membrane following substrate stimulation. Thus, LY379196 blocked AMPH- and DA-stimulated ultra-rapid DAT exocytotic trafficking in rat and mouse striatal synaptosomes, and GFP-rDAT-N2A cells [32,69]. The role of PKC β in rapid DAT trafficking was further explored and validated using PKC β -knockout mice. PKC β -knockout mice on the C56BL/6 background exhibited less surface DAT, less [3 H]DA uptake, less AMPH-stimulated DA efflux and less AMPH-stimulated locomotor activity, compared with their wild-type counterparts [32]. Importantly, the regulation of DAT in response to AMPH was entirely opposite in the PKC β -knockout mice compared with wild-type mice; incubation with AMPH in striatal synaptosomes from PKC β -knockout mice rapidly decreased surface DAT, but after 1 h, surface DAT increased above vehicle levels [32].

Since transport of DAT to the surface is slowed, but not stopped, in the striata from PKC β -knockout mice, it appears that PKC β is not absolutely required for substrate-induced DAT exocytosis, but plays a major role in regulating the rate of DAT trafficking to the surface. This is consistent with the finding of Johnson *et al.*, that the ability of AMPH to rapidly increase surface DAT reflects an increase in membrane targeting of DAT, not a slowing of internalization [39]. Taken together, these data suggest that PKC β is intrinsically important for rapid transport of DAT to the surface and, thus, the degree of responsiveness of the transporter to the presence of substrate.

Importantly, it appears that PKC β and CaMKII have opposite effects on DAT trafficking, such that PKC β promotes rapid AMPH-evoked DAT trafficking to the plasma membrane [32,69] and CaMKII appears to be involved in DAT trafficking away from the plasma membrane [64]. It is of interest to know whether continuous DAT trafficking is regulated sequentially by PKC β and CaMKII. If the evidence bears out that CaMKII is required for AMPH-stimulated DA efflux in the naive, untreated rat, then CaMKII could, similar to PKC, exhibit biphasic effects on surface DAT. This is suggested by the fact that CaMKII is required for AMPH to couple to syntaxin 1A at the cell surface [78]. Furthermore, DAT is a substrate for protein phosphatase 1 *in vitro* and in rat striatal tissue, but not for PP2A [79], although PP2A forms a complex with DAT [80]. Whether PKC β -promoted rapid DAT trafficking is dampened by these DAT-associated phosphatases warrants future investigation.

SNARE protein dependence

Although the demonstration of real-time imaging of fusion of an individual DAT-containing vesicle on the membrane upon short-term substrate exposure has not yet been achieved, the rapid increase of surface DAT expression is dependent on soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins [69]. SNARE proteins, such as syntaxin 1A and the vesicle-associated membrane protein (VAMP)/synaptobrevin, participate in the process of membrane fusion and exocytosis. Multiple neurotransmitter transporters, including DAT, NET and SERT, form a complex with the SNARE protein, syntaxin 1A, through direct physical interaction [81–83]. The DA-stimulated ultra-rapid increase of surface DAT in GFP-rDAT-N2A cells is blocked by overexpression of the light chain of botulinum neurotoxin C (BoNT C) or tetanus neurotoxin (TeNT), both of which inhibit vesicle fusion [69]. BoNT C proteolyzes the plasmalemmal membrane SNARE protein, syntaxin 1A, while TeNT proteolyzes VAMP/synaptobrevin. Presumably, the VAMP/synaptobrevin is located on the DAT-containing vesicle. Although DAT has not been directly demonstrated to be present in vesicles, the homolog GAT1 has been localized in small vesicles with a diameter of approximately 50 nm [84]. Overexpression of syntaxin 1A increases AMPH-stimulated DA

efflux in hDAT-HEK293 cells, and treatment with 10 μ M AMPH for 5 min increased a syntaxin 1A–DAT interaction [78]. Syntaxin 1A is crucial for docking of vesicles to the plasmalemmal membrane [85] and may function similarly for DAT-containing vesicles. These data suggest that the substrate-induced rapid increase in surface DAT is an exocytotic event that is regulated by SNARE proteins. The effect of syntaxin 1A on surface DAT has not yet been reported, but our laboratory has found that cleavage of syntaxin 1A in rat brain synaptosomes by BoNT C reduces basal DAT expression [KIM *ET AL.*, UNPUBLISHED OBSERVATIONS]. It has been reported that cleavage of syntaxin 1A in mouse brain synaptosomes by BoNT C reduces basal NET surface expression [82]. Furthermore, NET and syntaxin 1A interaction mediates NET sequestration in response to AMPH [86]. Although syntaxin 1A facilitates AMPH-induced DA efflux [78], it remains unknown whether the association of syntaxin 1A and DAT modulates basal and AMPH-stimulated DAT internalization or recycling.

Presynaptic dopamine D₂/D₃-receptor dependence

DAT function and trafficking is regulated by D₂ DA receptors, presumably the short form of the D₂ DA receptor (D2SR), which is probably the autoreceptor [87]. Both D₂ and D₃ DA receptors are found on DA neurons and both regulate presynaptic function [88]. D₂/D₃ DA-receptor agonists increase the V_{max} value of DAT substrates and surface DAT expression [72–74,89]. Quinpirole, a D₂/D₃ DA-receptor agonist, increases surface DAT expression within the first 5 min of exposure in cells overexpressing either D₂ or D₃ DA receptors [73, 89].

Modulation of DAT trafficking by D2SR is G_i/G_o-dependent and requires ERK1/2 but not PI3K activation [73]. However, D₂ receptor-independent DAT trafficking has also been reported in N2A cells stably expressing hDAT [69], suggesting that DA can modulate DAT trafficking both as a substrate for DAT and as an agonist for D₂/D₃ DA receptors. DAT is regulated by D2SR through a direct physical interaction involving the DAT N-terminus and the third intracellular loop of D2SR [74], which probably facilitates intracellular DAT trafficking to the surface. Disruption of D2SR–DAT interaction in the mouse brain *in vivo* results in decreased striatal synaptosomal DA uptake and increased locomotor activity, mimicking *DAT*-knockout mice [74]. The strong relationship between D₂ DA receptors and DAT is also expressed at the genetic level. There is significant D₂ receptor/DAT interaction in prefrontal cortex and striatum during both working memory and encoding of recognition memory as assessed by patients undergoing blood oxygenation level-dependent functional magnetic resonance imaging [90].

Insulin-stimulated trafficking

Insulin signaling has been demonstrated to increase surface DAT in heterologous cells [36]. Furthermore, the ability of AMPH to elicit behaviors and DA efflux is reduced in hypoinsulinemic rats [91,92]. Insulin can also reverse AMPH-induced DAT internalization through the action of PI3K, suggesting a hormonal regulation of DA homeostasis [64]. Activation of Akt via insulin signaling or overexpression of a dominant-negative Akt mutant counteracts AMPH-evoked DAT internalization, while Akt inhibition, similar to AMPH treatment, induces DAT trafficking away from the plasma membrane [52]. The earliest time point at which insulin significantly increased [³H]DA uptake in FLAG–hDAT-EM4 cells was 5 min, although there was a trend to an increase at 2 min, suggesting that the effect of insulin on surface DAT is relatively rapid [26]. Similarly, the reduction of [³H]DA uptake following treatment of hDAT overexpressed cells in response to 100 μ M of the Akt inhibitor ML9 was significant within 5 min. The latter experiment, which was performed in the absence of insulin, suggests that Akt is important for constitutive surface DAT, similar to the effect of PKC β . A time course of the effect of insulin on surface DAT would clarify whether insulin elicits ultra-rapid trafficking of DAT to the surface.

Future perspective

DAT is one of the most important regulators of dopaminergic homeostasis, and is potentially involved in several neurodegenerative and psychiatric diseases. Since DAT is active when situated at the plasmalemmal membrane, the concentration of DAT at the plasmalemmal membrane is crucial to proper dopaminergic function. Research in the past few years has led to an explosion of knowledge concerning the modulation of DAT trafficking by substrates, protein kinases, second messengers and interacting proteins. Continuous exposure to DAT substrates elicits a downregulation of DAT at the plasmalemmal membrane, which could serve as a protection mechanism against a build-up of toxic substrates in the terminal. However, recent studies have demonstrated that substrates also elicit, within seconds, trafficking of DAT to the plasmalemmal membrane. This ultra-rapid trafficking takes place during the physiological time frame of DA uptake in response to exocytotically-released DA and DA efflux in response to the psychostimulant AMPH. One can imagine that blockade of the ultra-rapid response would reduce the response to AMPH but permit sufficient uptake to maintain normal function. Thus, blockade of the rapid AMPH-induced trafficking of DAT to the surface, especially by locally targeting PKC β , could be a worthwhile approach to treat drug abuse.

Executive summary

Background

- The dopamine transporter (DAT) is a primary determinant of the concentration of dopamine in the synapse.
- DAT is involved in neurological diseases, including Parkinson's disease, and psychiatric diseases, such as psychosis, attention-deficit/hyperactivity disorder and substance abuse.

Role of DAT

- Synaptic dopamine released through exocytosis is taken up through the DAT back into the nerve terminal.
- Ligands for DAT include dopamine, amphetamine, methamphetamine (substrates), and cocaine and methylphenidate (blockers).

Substrate-regulated trafficking of DAT

- Substrates initially stimulate trafficking toward the plasmalemmal membrane, increasing reuptake.
- When the substrate is amphetamine, the rapid trafficking also increases the reverse transport of dopamine.
- The continuous presence of substrate results in downregulation of the transporter activity, followed by internalization of the transporter.

Strategies for altering DAT trafficking

- Ultra-rapid substrate-induced trafficking of the transporter to the surface is mediated, at least partially, by PKC β .
- Blockade of PKC β could prove useful to reduce the effectiveness of methamphetamine, yet permit enough normal uptake to keep the neuron functional.
- The endocytic pathway could be modulated to keep more DAT on the surface, thus reducing synaptic dopamine, which would limit reinforcement to rewarding

stimuli, or to reduce surface transporter, to protect the neuron from having too much intracellular dopamine.

Future perspective

- Delineation of the molecular mechanisms involved in the exocytic/endocytic pathway of DAT will provide therapeutic opportunities for regulating synaptic and neuronal dopamine that will be useful in treating neurologic and psychiatric diseases.
- Since the pathways are probably regulated separately by various signal transduction mechanisms, drugs altering the pathway can be tailored to the specific pathology.

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Figure 1. Transporters regulate dopamine reuptake and storage in the nerve terminal

The plasmalemmal transporter, DAT, is a Na^+ , Cl^- -dependent transporter that is normally outward-facing on the plasmalemmal membrane. Na^+ that is transported into the terminal is transported back to the extracellular milieu by Na^+ , K^+ -ATPase. Following exocytotic release, the substrate DA diffuses to the transporter, binds to DAT and is transported into the cytosol. DA in the cytosol is transported into synaptic vesicles through the H^+ -ATPase-coupled VMAT2. AMPH (and methamphetamine), as a substrate, binds to DAT, is transported inside the terminal along with Na^+ and Cl^- , and depletes DA from the vesicle. However, upon dissociation of AMPH, cytosolic DA can rebind to the now inward-facing transporter; the transporter then reverses such that DA is transported back into the synapse. AMPH also inhibits the ability of DA to be transported by VMAT2 into the synaptic vesicle. The increased concentration of DA is then transported into the synapse, increasing the maximal amount of DA that can be effluxed in response to AMPH.

AMPH: Amphetamine; DA: Dopamine; DAT: DA transporter; SV: Synaptic vesicles; VMAT2: Vesicular monoamine transporter 2.

Figure 2. Dopamine transporter undergoes constitutive and substrate-induced trafficking to the surface and endocytosis

DAT constitutively internalizes to and recycles from the recycling endosome and early endosome). The basal surface DAT endocytic rate is 3–5%/min. The maintenance of the basal surface DAT is PKC β - and Rab11-dependent. DAT also undergoes time-dependent biphasic trafficking upon substrate exposure. Short-term exposure of substrates (DA or AMPH) stimulate an ultra-rapid recycling of DAT that is PKC β dependent. Upon continuous substrate administration, DAT invaginates into membrane pits (probably when it is in the inward-facing form) and internalizes. CaMKII contributes to substrate-induced internalization. AMPH: Amphetamine; DA: Dopamine; DAT: DA transporter; CaMKII: Ca²⁺/calmodulin-dependent protein kinase II; EE: Early endosome; RE: Recycling endosome. VMAT2: Vesicular monoamine transporter 2.