Regulation of the Dopamine Transporter Through Trafficking-Dependent and -Independent Mechanisms

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The dopamine transporter (DAT) is a vital protein involved in maintaining dopamine (DA) homeostasis in the brain by mediating reuptake of synaptic DA back into the presynaptic termi-nal. The regulation of this protein is critical for ensuring proper dopaminergic signaling, and its dysregulation can have dire consequences for a number of behaviors and neurological pro-cesses that are modulated by DA signaling. DAT activity can be regulated in both a positive and negative manner; the mechanisms of regulation involve both trafficking of the protein to and from the surface, as well as modulation of intrinsic transport activity independent of trafficking. Discussion of these trafficking-dependent and -independent modes of regulation will be the focus of this review.

Keywords: *Dopamine, transporter, presynaptic, trafficking, monoamines*

Introduction

The neurotransmitter dopamine (DA) is important for modulating many aspects of physiology and behavior, including motivation[1], movement[2], reward[3] and attention[4]. Importantly, perturbations to the central dopaminergic systems are associated with many disease states such as Parkinson's disease[2], attention deficit/hyperactivity disorder (ADHD)[5], schizophrenia[2] and addiction[6]. Therefore, understanding regulation of dopaminergic signaling is vital for understanding the pathophysiology of these diseases, as well as for gaining insights into the behaviors modulated by this neurotransmitter.

Presynaptic regulation of DA signaling is a dynamic process that can occur at many levels. These include regulation of DA synthesis by the enzyme tyrosine hydroxylase[7], excitability of the presynaptic neuron[8],and, most importantly for this review, regulation of the activity of the dopamine transporter (DAT), which acts to reuptake synaptic DA back into the presynaptic terminal to halt its signaling[9]. DAT is a twelve transmembrane domain protein that belongs to the sodium- and chloride-dependent (SLC6) family of transporters, of which the serotonin, norepinephrine, and GABA transporters are also members[10]. This family of transporters couple movement of their substrates across membranes to movement of sodium and chloride ions down their electrochemical gradients, allowing for energy-independent movement of substrate across the membrane[10]. DAT itself is the target for therapeutic agents such as the ADHD medications methylphenidate (Ritalin) and amphetamine formulations (e.g., Adderall)[11], and variants in this protein have been found to be associated with ADHD[12-15]. This, along with the fact that DAT is the target of addictive psychostimulants such as cocaine[9], amphetamine[11], and methamphetamine[16], implicates the importance of this protein for the pathophysiology of many of the disease states associated with altered DA signaling.

Importantly, DAT is not a static protein, and tight regulation of its activity has been observed experimentally[17-20]. Much research has demonstrated trafficking-dependent regulation of DAT through movement of the protein to and away from the surface by a number of interacting proteins and post-translational modifications, many of which will be discussed below. However, trafficking-independent regulation, where the intrinsic activity of DAT is modulated without trafficking to other compartments, has not been described as extensively. This review will focus on discussing these two broad classes of regulation that likely work in concert to achieve tight regulation of the activity of this vital protein.

Trafficking-dependent downregulation of DAT

The most robustly observed mechanism of DAT regulation,

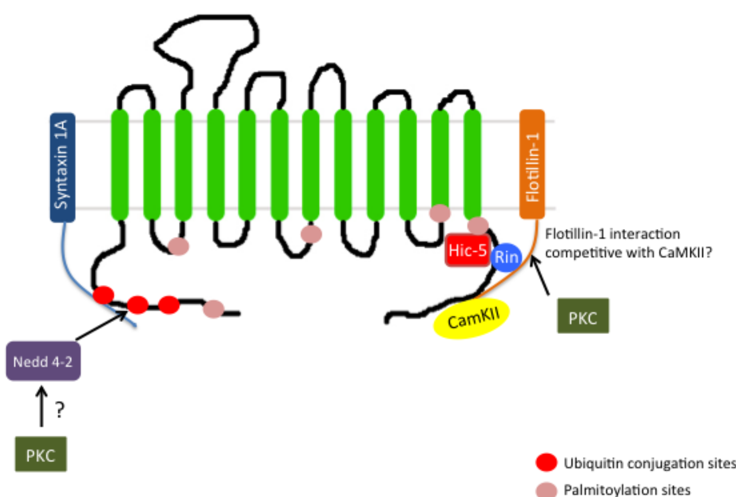

Figure 1: *Modulation of DAT activity.* DAT activity can be modulated by a number of interacting proteins, though which of these proteins may act together, or which may be exclusive and/or competitive is not entirely clear. Importantly, the site of interaction of Flotillin-1 has not been mapped, but has been placed based on evidence of potential competition with CaMKII. Post-translational modifications such as palmitoylation and ubiquitination also play important roles in regulating DAT activity and surface expression. Direct phosphorylation of the transporter has not been shown to regulate activity per se, but is important for regulating efflux-competent states of DAT.

downregulation of transport through trafficking of the transporter, is achieved through a large network of interacting proteins, the list of which is ever-expanding. Downregulation of DAT by Protein Kinase C (PKC) activation is among the most frequently studied mechanisms of DAT regulation, and is mediated by a number of proteins[21-25]. Activation of PKC by phorbol esters such as PMA[23, 24, 26] or by activation of G_q-coupled G-protein coupled receptors[27] induces a rapid reduction in surface DAT and a concomitant increase in intracellular DAT[28]. This redistribution of DAT is mediated by a clathrin-dependent mechanism, as knockdown of either clathrin heavy chain or dynamin II inhibits both constitutive as well as PKC-triggered endocytosis[26, 29]. However, direct phosphorylation of DAT by PKC is not required for internalization of the protein, suggesting that potentially another DAT-interacting protein is phosphorylated either by PKC or a downstream kinase to mediate this effect[30]. It was recently reported by Cremona et al. that the membrane raft-associated protein Flotillin-1 was required for internalization of DAT upon PKC activation, and that phosphorylation of this protein at Ser315 is also required for this effect[31]. These results suggest that Flotillin-1 may be either a direct or indirect target for phosphorylation by the PKC signaling cascade. It was also observed that Flotillin-1 was required for maintaining membrane raft-association of DAT, potentially pointing toward the importance of this localization for PKC-regulated endocytosis.

Further evidence of the importance of membrane microdomain targeting in PKC-regulated endocytosis has recently been reported. An ADHD-associated variant in the dopamine transporter (R615C) was shown to constitutively internalize and recycle back to the membrane at an accelerated rate, and was insensitive to PKC- and amphetamine (AMPH)-stimulated endocytosis[12]. Importantly, this variant showed reduced association with Flotillin-1 and reduced membrane raft localization, as measured by colocalization with an Alexa 647-conjugated cholera toxin B, which marks membrane raft-enriched GM1 Ganglioside. This reduction in association with Flotillin-1 and membrane rafts is contrasted by a significant increase in association with Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). It is possible that the reduced association between the 615C variant and Flotillin-1 may be due to the fact that CamKII and Flotillin-1 interactions with DAT are competitive due to overlapping sites of interaction. Therefore, an increased affinity for CaMKII might prevent Flotillin-1 interactions and raft association, though this is just speculation at this point since the site of interaction between Flotillin-1 and DAT has not been mapped (**Figure 1**). Regardless, these altered associations in this PKC- and AMPH-induced trafficking-insensitive DAT variant certainly provide further support for the importance of the Flotillin-1 interaction and raft localization for

Membrane rafts:
Cholesterol-rich microdomains that are thought to organize signaling molecules into discrete regions of the plasma membrane.

Clathrin:
A protein that mediates endocytosis of vesicles, a process that is important for many cellular physiological processes, including internalization of cell-surface proteins.

PKC and AMPH-triggered endocytosis of the transporter.

Another recent study identified another DAT-interacting protein, the plasma membrane-associated GTPase, Rin, which is involved in mediating the effects of PKC on DAT[32]. Utilizing yeast two-hybrid, GST pull down, and FRET approaches, it was demonstrated that Rin directly associates with residues 587-596 of the DAT C-terminus, and that expression and activity of Rin is required for PKC-triggered endocytosis of the transporter. It was also shown that Rin and DAT interactions increase with PKC activation. Furthermore, Rin/DAT interactions were shown to occur preferentially in membrane rafts, again pointing toward the importance of this localization for PKC-regulated DAT trafficking. Unfortunately, attempts at demonstrating an interaction *in vivo* in dopaminergic neurons were unsuccessful due to technical issues, so the relevance of this interaction is unknown. Importantly, Rin is present in striatal tissue and a number of DAergic cell lines, supporting a potential role in endogenous DAT regulation. The role that Rin plays in DAT regulation is unclear, though it is possible that it helps regulate movement of DAT into or out of membrane raft microdomains, a process that has been suggested to be important for PKC regulation of DAT. However, more work is necessary to clarify just what role this interaction plays in DAT trafficking by PKC-dependent pathways.

It has been also been shown that PKC activation results in ubiquitination of DAT, and that three lysine residues in the N-terminus of DAT that act as ubiquitin conjugation sites are required for PKC-dependent endocytosis of the transporter[33]. Utilizing a large-scale RNAi screen for genes involved in PKC-dependent endocytosis, Sorkina et al. identified the E3 ubiquitin ligase, Nedd4-2, as being necessary for this process, as loss of this protein by siRNA-mediated knockdown abolished DAT ubiquitination[34, 35]. These data suggest that PKC activation induces ubiquitination of DAT by Nedd4-2, and that this process is necessary for PKC-regulated endocytosis of the transporter. It is possible that PKC itself regulates Nedd4-2 activity or recruitment to DAT, though this has not been shown experimentally. Also, the interplay between this Nedd4-2 dependent mechanism and the interaction with Flotillin-1 or Rin has yet to be investigated.

Another interesting DAT-interacting protein that appears to be involved in trafficking of the transporter is the focal adhesion protein Hic-5. This interaction was initially identified by the yeast two-hybrid system, and verified by co-immunoprecipitation from striatal extracts and immunostaining that showed presynaptic colocalization of these two proteins[36]. Co-expression of DAT and Hic-5 in HEK293 cells resulted in a decrease in transport and surface expression of DAT compared to expression of DAT alone. These results suggest that the association between DAT and Hic-5 may be important for surface trafficking of DAT. In further support of this, work done in platelets by Carneiro and Blakely has shown that Hic-5 also associates with the serotonin transporter (SERT)[37]. Interestingly, treatment with PMA, which also induces internalization of SERT, increases association between SERT and Hic-5 at times that correlate with decreases in SERT surface expression. If a similar mechanism occurs with Hic-5 and DAT, it may be the case that Hic-5 is involved in the network of proteins mediating PKC-regulated DAT endocytosis, though direct evidence for this has yet to be presented. Also worth noting is the fact that this interaction between Hic-5 and DAT occurs in the membrane-proximal region of the C-terminus DAT, very close to the interaction site of Rin. It is possible that these proteins function together to regulate DAT trafficking, or may be competitive for binding to DAT and may mediate different forms of DAT regulation, though these possibilities have yet to be investigated.

Importantly, it should be noted that the timing and cellular contexts of these various PKC-related mechanisms of DAT endocytosis remain unclear. For instance, it is unknown if these proteins work in tandem in DAergic terminals through a single PKC-related mechanism of DAT endocytosis, or if there is exclusivity between these different interactors depending on factors such as the active signaling pathways or the membrane microdomain localization of DAT. Foster et al. demonstrated that treatment with β–PMA causes PKC-α to be recruited to membrane rafts and may preferentially regulate endocytosis of DAT in these fractions through some of the mechanisms mentioned above. However, it is possible that activation of other PKC isoforms by other signaling pathways may lead to modulation of DAT endocytosis by entirely separate mechanisms. Sakrikar et al. have proposed a model based on work with the R615C DAT variant that postulates that DAT endocytosis occurs in both regulated and constitutive manners that depend upon microdomain localization. In this model, PKC regulates endocytosis from membrane rafts, whereas DAT localized outside of these rafts endocytoses in a constitutive manner. This is consistent with the finding that PKC-α moves into raft fractions after PMA treatment, but Foster et al. also showed that PKC activation does not

reduce surface DAT in raft fractions. These inconsistencies may reflect the different cell types used or the methods employed, or it may be the case that PKC-α mediates a trafficking-independent mode of DAT regulation, and other isoforms, such as PKC-β underlie mechanisms of trafficking-dependent regulation of DAT. Clearly, the picture of PKC regulation of DAT is quite complicated at this point, and this complication is made worse by the inconsistency in cell lines and techniques employed by various groups. Regardless, it certainly seems as if PKC regulation of DAT surface expression may actually occur through a number of mechanisms; future work on the interaction of these various PKC-related mechanisms, as well as the PKC subtypes mediating these effects, may clarify where and when these different pathways may modulate DAT surface expression *in vivo*.

The neuronal SNARE protein Syntaxin 1A is another DAT regulatory partner whose interaction appears to have a number of functional consequences for DAT activity. Tissue treatment with Botulinum Neurotoxin C (BoNT/C) results in degradation of Syntaxin 1A, and treatment of rat striatal tissue with this toxin causes an increase in DA uptake and DAT surface levels[38]. Conversely, heterologous co-expression of these two proteins results in decreased DA uptake and DAT surface levels compared to DAT expression alone. Together, these results suggest that the interaction between these two proteins promotes suppression of DA uptake through reduced DAT surface levels. Importantly, PMA-induced endocytosis of DAT was intact even with BoNT/C treatment, suggesting that the Syntaxin 1A/DAT interaction is not required for PKC-triggered endocytosis of the transporter.. This interaction is interesting because it may have relevance for the localization of DAT to sites of release, as Syntaxin 1A is an important member of the SNARE complex that mediates vesicular fusion and neurotransmitter release. What the relevance of this interaction is remains unclear, but it is important to note that Syntaxin 1A is also essential for facilitating an efflux-competent state of DAT and decreases DAT channel currents, which, combined with the apparent effects on trafficking, likely have important functional consequences for how DAT behaves endogenously.

Trafficking-dependent upregulation of DAT

There are a number of signaling pathways that have been shown to promote surface expression of DAT through trafficking-dependent mechanisms. For instance, insulin increases dopamine uptake by increasing surface expression of DAT, and this increase can be blocked by inhibition of Phosphatidylinositide 3-kinase (PI3K)[39]. Importantly, PI3K inhibition also reduces basal uptake and surface expression in a dynamin-dependent manner, suggesting that this regulation is vital for opposing endocytosis of the protein by other pathways such as PKC, etc[39]. Also vital for this process is the kinase Akt, whose level of phosphorylation increases upon insulin stimulation[40]. Constitutively active Akt prevents AMPH-induced endocytosis of DAT, and inhibition of Akt reduces DAT uptake and surface expression, supporting its role in the insulin/PI3K pathway that promotes surface expression of DAT.

DAT activity can also be modulated by interactions with the D2 dopamine receptor (D2R). D2Rs are expressed both postsynaptically and presynaptically, and can be alternatively spliced into short and long isoforms (D2S and D2L), with the D2S isoform as the predominant presynaptic isoform. It has been observed that D2-deficient animals have reduced DAT function[41], and heterologous expression of D2 and DAT suggests that this D2 effect on DAT occurs cell-autonomously[42]. By co-expressing D2 and DAT, it has been shown that these proteins directly interact[43, 44], and increases uptake and DAT surface expression in heterologous systems compared to singly transfected DAT[43]. In addition to this direct interaction, there also appears to be a functional interaction between these two proteins. When DAT and D2S were co-expressed in cells, the D2 agonist quinpirole significantly increased dopamine

SNARE:
A family of proteins that mediate docking and fusion of vesicles to the plasma membrane, which allows for the release of neurotransmitter.

uptake and DAT surface expression, and a specific ERK1/2 antagonist could block this increase[44]. This suggests that D2 modulation of DAT activity likely occurs via at least two mechanisms: a direct interaction of the proteins promoting increased surface expression of the transporter, as well as a functional interaction that involves D2-dependent ERK1/2 signaling cascades.

An important thing to keep in mind when considering how these signaling pathways influence DAT regulation is that these pathways overlap and interact in many ways. For instance, in addition to activating PI 3-kinase, insulin can also activate PKC signaling pathways via PLC gamma, which would presumably oppose PI 3-kinase upregulation of DAT. It is likely that compartmentalization of DAT with its regulatory partners helps organize these regulatory mechanisms in manners that aren't well understood, and it should always be kept in mind that much of the work on DAT regulation is done in heterologous expression systems that may not accurately reflect the environment in which DAT is endogenously expressed. Because of this, care must be taken in how the results of such studies are interpreted until they are repeated in endogenous DAT-expressing systems.

Trafficking-independent downregulation of DAT

In recent years, it has become clear that trafficking of DAT alone cannot explain regulation of its activity, and that there are trafficking-independent modes of regulation of this protein. Foster et al. initially reported findings that suggest that reduction in DAT activity by PMA-induced PKC activation could only partially be explained by internalization of the transporter[45]. They demonstrated this by inhibiting clathrin-mediated endocytosis using either the chemical inhibitor Concanavalin A (Con A) or a dominant-negative dynamin. This inhibition was sufficient to prevent internalization of the protein, but only partially prevented PKC-induced downregulation of transport activity. Also, using a cholesterol depletor, methyl-β–cyclodextrin (MβC), it was demonstrated that PKC-induced downregulation was also partially inhibited by loss of cholesterol, but DAT internalization was about equivalent to PMA treatment alone, suggesting a loss of PKC-induced downregulation that was independent of trafficking. In further support of this, another study showed that PKC-induced downregulation of DAT in synaptosomes occurs even in the presence of high sucrose, which blocks endocytosis[46]. These lines of evidence suggest that PKC causes a decrease in DAT activity via a mechanism that does not require internalization of the protein.

The association of DAT with cholesterol-rich membrane rafts has been well characterized, and this association can be decreased by MβC treatment and increased by treatment with water-soluble cholesterol (wsChol). By augmenting membrane cholesterol of DAT-transfected HEK cells. as well as striatal synaptosomes with wsChol, Hong and Amara demonstrated that binding of cocaine-analogs [^{125}I] RTI-55 and [^{3}H]WIN35428 was significantly increased compared to untreated controls[47]. They also showed that binding of Maleimide-PEO$_2$-biotin to DAT, which specifically recognizes sulfhydryl (–SH) moieties on surface-accessible cysteine residues, is increased with no change in total surface DAT. Using site-directed mutagenesis, the site of increased reactivity was found to be cysteine 306, and its increased availability to the –SH-specific biotin was attributed to an increase in outward conformation of the DAT protein. This cholesterol-dependent change in DAT conformation may underlie some of the modulation of DAT activity by altered membrane cholesterol content, and may represent a mechanism through which altered cholesterol-rich membrane raft association of DAT can regulate transport, independent of trafficking to and away from the cell surface.

Trafficking-independent upregulation of DAT

Though very little direct evidence for trafficking-independent upregulation of DAT activity has been observed, there are a few findings that indicate that this may occur. Foster and Vaughn have shown that DAT is palmitoylated, and that this modification has functional consequences for transport[46]. By inhibiting palmitoyl acyltransferase using 2-bromopalmitate (2BP), which prevents protein palmitoylation, they showed that blocking palmitoylation of DAT induces a rapid reduction in transport with no changes in DAT protein or surface levels at early time points. It is important to note that 2BP inhibited palmitoylation of DAT by about 40% within 45 minutes, suggesting that palmitate turnover is quite rapid, and that this modification may be used to acutely regulate DAT activity. If this is the case, then palmitoylation and depalmitoylation may represent mechanisms by which DAT kinetics can be rapidly up- or downregulated, independent of trafficking of the protein. Additionally, since palmitoyl groups can mediate interactions between proteins and membrane lipids, the palmitoylation status of DAT may impact its membrane microdomain localization and, therefore, its regulation, providing a potential mechanism through which activity

and surface expression may be altered by this modification.

Further evidence from the related transporters SERT, as well as the norepinephrine transporter (NET), demonstrates that trafficking-independent upregulation of transporters does occur, and it is tempting to think that this mode of regulation may generalize to other family members such as DAT. For instance, activation of adenosine A3 receptors on serotonergic neurons increases SERT activity by a mechanism that is partially independent of trafficking to the surface[48-54]. The signaling pathways involved have been worked out, and it appears that activation of PKG and p38 MAP kinase underlies this upregulation[50-53]. In the case of NET, it has been demonstrated that insulin stimulation also activates p38 MAP kinase, and this activation induces upregulation of transport without any significant increase in surface expression[55, 56]. This raises the question of whether mechanisms that upregulate DAT activity may be doing so via similar mechanisms that may be due in part to trafficking-independent activation. It seems reasonable to think that perhaps trafficking-independent upregulation of DAT has not been reported because of a lack of temporal resolution in monitoring DAT surface expression. For example, many studies that showed increased uptake and a concomitant increase in DAT surface levels only looked at DAT surface levels at later time points, and may have missed an earlier increase in DAT activity prior to trafficking of DAT to the surface. Hopefully, future work will begin to clarify whether this mode of regulation is indeed employed to regulate DAT, as has been shown for related transporters.

Conclusions

Understanding how DA homeostasis is maintained and dynamically regulated is essential for gaining insights into how DA mediates its effects on behavior, and how dysfunction of this system can lead to diseases such as ADHD, schizophrenia, and addiction. At the center of DA signaling regulation is DAT, whose activity is vital for controlling the proper amount of synaptic DA during neurotransmission. Because DAT dysfunction has been shown to be associated with a subset of individuals with diseases such as ADHD, it is imperative that there be a focus on studying how this protein is regulated in order to understand its role in these disease states, as well as in normal cognition and behavior. As this review has shown, there are many different modes of DAT regulation involving trafficking and modulation of intrinsic transport activity, though when and where these pathways may regulate DAT *in vivo* and how they may interact with one another remains poorly understood. As these regulatory networks of proteins are worked out further, it will hopefully expand out understanding of DA signaling in the brain and open up avenues for treating individuals with DA-related brain disorders.

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    This paper is important because it demonstrates how the signaling pathways and regulation of a ADHD-associated DAT variant are altered, and potentially brings the field closer to understanding how alterations in DA signaling regulation can contribute to this disease.

It also furthers our understanding of the importance of membrane microdomain localization for DAT function.

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This is a landmark paper because it is one of the first to really demonstrate the importance of membrane microdomain localization of DAT for its regulation and function, and identifies Flotillin-1 as a potential target for PKC that may play a role in mediating endocytosis of DAT by this pathway.

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D2 autoreceptor regulation of DAT mediates an important feedback loop for regulating DA signaling, and this paper expands our understanding of the functional interaction between these proteins, and uncovers the signaling pathways involved in activity-dependent regulation of DAT by this receptor.

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