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## Palmitoylation mechanisms in dopamine transporter regulation

Danielle E. Rastedt<sup>1</sup>, Roxanne A. Vaughan, and James D. Foster\*

Department of Biomedical Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND 58202

### Abstract

The neurotransmitter dopamine (DA) plays a key role in several biological processes including reward, mood, motor activity and attention. Synaptic DA homeostasis is controlled by the dopamine transporter (DAT) which transports extracellular DA into the presynaptic neuron after release and regulates its availability to receptors. Many neurological disorders such as schizophrenia, bipolar disorder, Parkinson disease and attention-deficit hyperactivity disorder are associated with imbalances in DA homeostasis that may be related to DAT dysfunction. DAT is also a target of psychostimulant and therapeutic drugs that inhibit DA reuptake and lead to elevated dopaminergic neurotransmission. We have recently demonstrated the acute and chronic modulation of DA reuptake activity and DAT stability through *S*-palmitoylation, the linkage of a 16-carbon palmitate group to cysteine via a thioester bond. This review summarizes the properties and regulation of DAT palmitoylation and describes how it serves to affect various transporter functions. Better understanding of the role of palmitoylation in regulation of DAT function may lead to identification of therapeutic targets for modulation of DA homeostasis in the treatment of dopaminergic disorders.

### Keywords

posttranslational modification; palmitoyl acyl transferase; acyl protein thioesterase; phosphorylation; protein trafficking; 2 bromopalmitate

## 1. Introduction

Dopamine (DA) is a neurotransmitter that controls numerous functions including attention, mood, cognition, reward, and movement (Iversen and Iversen, 2007), and altered DA homeostasis is associated with many neurological diseases including depression, bipolar disorder (BD), schizophrenia, attention deficit hyperactivity disorder (ADHD), Parkinson disease and substance abuse, but the underlying mechanisms remain largely unknown

\*To whom correspondence should be addressed: James D. Foster, Ph.D., Department of Biomedical Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND 58202, Tel: 701-777-3193, Fax: 701-777-2382, james.d.foster@med.und.edu;

<sup>1</sup>Current address: Department of Obstetrics and Gynecology, University of Michigan Medical Center, Ann Arbor, MI, 48109

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(Bannon *et al.*, 1998; Kristensen *et al.*, 2011; Pramod *et al.*, 2013). Dopamine tone is modulated by the dopamine transporter (DAT), an integral membrane protein that removes neurotransmitter from the extracellular space and thus regulates signaling at pre- and postsynaptic DA receptors (Giros *et al.*, 1996). DAT is a target of numerous psychoactive drugs that suppress reuptake and induce elevated synaptic DA levels, including addictive compounds such as cocaine, amphetamine (AMPH), and methamphetamine (METH), and therapeutic drugs such as Adderall® (AMPH), Ritalin® (methylphenidate) and Wellbutrin® (bupropion) prescribed for dopamine related disorders including ADHD and depression.

DA reuptake activity undergoes acute and chronic regulation in response to signaling molecules and drug exposure, leading to impacts on DA tone that may contribute to DA imbalances in disease (Ramamoorthy *et al.*, 2011; Schmitt and Reith, 2010). These responses can occur by up- or down-regulation of transporter cell surface numbers or by kinetic modification of transport velocity. DAT is subject to several post-translational modifications that mediate these processes (Bermingham and Blakely, 2016; Kristensen *et al.*, 2011; Vaughan and Foster, 2013) and in this review we discuss the role of palmitoylation in control of reuptake and other DAT functions.

## 2. Transport Mechanism

DAT belongs to the SLC6 family of Na<sup>+</sup>-Cl<sup>-</sup> dependent transporters and is composed of 12 transmembrane spanning domains (TMDs) arranged in two antiparallel aligned pentahelical bundles with cytoplasmically oriented N- and C-termini (Kristensen *et al.*, 2011; Pramod *et al.*, 2013) (Fig. 1). DAT structural and transport mechanism details have been elucidated by homology to the crystallized bacterial leucine transporter (LeuT) and the *Drosophila* (d)DAT, which facilitated the identification of the helix packing arrangement, substrate active site and translocation pathway, and extracellular and intracellular gating networks that dictate substrate movement (Beuming *et al.*, 2006; Dahal *et al.*, 2014; Wang *et al.*, 2015). The inner core that forms the permeation pathway consists primarily of TMD1, TMD3, TMD6, and TMD8 (Fig. 1) with the DA binding site generated by critical residues contained within unwound segments in the middle of TMDs 1 and 6 (Fig. 1). Substrate translocation occurs through a series of conformational changes in which substrate is bound and released on opposite sides of the membrane (Forrest *et al.*, 2008; Kristensen *et al.*, 2011), a process known as the alternating access mechanism. Substrate binds to the outward facing conformation the transporter adopts when the extracellular gate is open and the intracellular gate is closed, sealing off the cytoplasmic side of the pathway from the extracellular environment (Fig. 2, *Outward Facing*). Once the substrate is bound, the transporter transitions into an occluded state in which both the extracellular and intracellular gates are closed (Fig. 2, *Occluded*), and then transitions into the inward facing conformation where the intracellular gate opens, releasing substrate to the cell interior (Fig. 2, *Inward Facing*). After substrate has been released the intracellular gate is reestablished and the empty transporter returns to the outward facing conformation, allowing the next molecule of extracellular substrate to bind. The overall kinetic capacity of the protein is thus dictated by the rate of these transitions.

In addition, mammalian DATs have large N- and C-termini that extend into the cytoplasm (Fig. 1). These domains receive regulatory input via post-translational modifications and interactions with binding partners, and transmit this information to the functional core. The N-terminal domain is a site for input from protein kinases including PKC and ERK (reviewed in Foster and Vaughan, 2016 this issue), and we have recently determined that modification by S-palmitoylation occurs near the C-terminal domain (Foster and Vaughan, 2011) (Fig. 1). This review will describe the characterization of palmitoylation and its linkage to changes in transport activity and other functions.

### 3. Protein S-palmitoylation

S-palmitoylation of proteins is the thioesterification of palmitate, a 16-carbon fatty acid, to a cysteine residue. For integral membrane proteins this modification regulates a variety of properties including functional activity, trafficking, turnover, membrane raft targeting, and cholesterol binding (el-Husseini Ael and Brecht, 2002; Fang *et al.*, 2006; Greaves and Chamberlain, 2011; Huang and El-Husseini, 2005; Huber *et al.*, 2006; Lai and Linder, 2013). The thioester bond between the fatty acid and the protein is labile and reversible, allowing for dynamically regulated cycles of palmitoylation and depalmitoylation to occur throughout the protein's lifetime (Linder and Deschenes, 2007) in a manner analogous to phosphorylation and dephosphorylation (Fukata and Fukata, 2010; Smotryst and Linder, 2004).

Catalysis of palmitoylation is driven by palmitoyl acyltransferases (PATs) (Fig. 3). The human genome contains 23 PAT enzymes (Fang *et al.*, 2006; Fukata *et al.*, 2004; Fukata *et al.*, 2006; Roth *et al.*, 2002), that are also known as DHHC enzymes in reference to the conserved amino acid sequence Asp-His-His-Cys in the active site (Roth *et al.*, 2002). The enzymes have varied tissue distributions, including several that are highly expressed in the brain, and intracellular localizations, with most being localized to the Golgi apparatus, endoplasmic reticulum, or plasma membrane (Korycka *et al.*, 2012; Ohno *et al.*, 2006). PATs are polytopic membrane proteins that typically contain 4 TMDs with the catalytic domain present in the intracellular loop between TMDs 2 and 3 (Fig. 3) (Fukata *et al.*, 2004; Mitchell *et al.*, 2006), suggesting that palmitoylation reactions with integral proteins generally occur near the cytosolmembrane interface (Salaun *et al.*, 2010). Little is known regarding mechanisms of substrate specificity or enzyme regulation, but the large number of PATs and their specific subcellular localization suggests that palmitoylation machinery is tightly regulated. Currently there are few specific pharmacological PAT activators or inhibitors, limiting the experimental approaches available for examining functional properties. However, functional consequences of modification can be probed by reducing palmitoylation with the compound 2-bromopalmitate (2BP) a nonspecific irreversible PAT inhibitor (Jennings *et al.*, 2009), or by increasing palmitoylation by overexpression of PAT enzymes (Fukata *et al.*, 2004; Moritz *et al.*, 2015).

Protein depalmitoylation is catalyzed by acyl protein thioesterases (APTs), enzymes that belong to a superfamily of serine hydrolases. There are multiple forms of these proteins with various subcellular distribution and potential functions. Enzymes named APT1, APT2, and APT1-like proteins remove palmitate from cytosolically accessible sites of membrane

proteins (Linder and Deschenes, 2007), and are thought to function in regulated turnover of palmitate. Palmitoyl protein thioesterase 1 and 2 (PPT1, PPT2) are associated with lysosomes and are linked with control of protein stability by catalyzing protein depalmitoylation prior to lysosomal degradation (Fukata and Fukata, 2010; Vesa *et al.*, 1995). Recently members of a subfamily of  $\alpha/\beta$ -hydrolase domain-containing serine hydrolases not belonging to either of these families were found to depalmitoylate the neuronal protein PSD-95 (Yokoi *et al.*, 2016), suggesting that additional proteins involved in depalmitoylation remain to be discovered.

#### 4. DAT palmitoylation characteristics

Our labs have demonstrated the modification of rat (r), mouse (m), and human (h) DATs by S-palmitoylation in both rodent brain tissue and heterologous cell systems using metabolic labeling of DAT with [<sup>3</sup>H]palmitic acid in living cells or tissue, and acyl biotinyl exchange (ABE), an *in vitro* assay for post-hoc detection of endogenous palmitoylation (Foster *et al.*, 2016; Foster and Vaughan, 2011). Both methods have yielded comparable results in numerous analyses, indicating their interchangeability. Metabolic labeling of DAT with [<sup>3</sup>H]palmitic acid is easily detected within 1h, indicative of a rapid rate of palmitate turnover, and ABE analysis also indicates the presence of robust tonic palmitoylation levels. Palmitoylation of DAT is inhibited by 2BP, consistent with catalysis driven by PATs, and is reversible *in vitro* with hydroxylamine, which cleaves thioester bonds, confirming that the modification occurs via a thioester linkage.

Rodent and human DATs contain five conserved cysteine residues, Cys6, Cys135, Cys341/342, Cys522/523, and Cys580/581, that are predicted to be exposed to the cytoplasm and thus represent potential S-palmitoylation sites (Fig. 1). To identify the site(s) modified, we mutated each of these residues individually to alanine (A) and assessed the proteins for [<sup>3</sup>H]palmitic acid labeling (Foster and Vaughan, 2011). Analysis of rDAT Cys→Ala mutants showed that incorporation was reduced by ~60% in C580A DAT, indicating this residue as a major palmitoylation site (Fig. 1), with a similar reduction of labeling obtained for hDAT C581A (Shetty and Foster, unpublished results). This cysteine is located at the membrane-cytoplasm interface of TMD12, the most C-terminal membrane spanning helix, a common palmitoylation site for membrane proteins (Salaun *et al.*, 2010). However, a substantial portion of [<sup>3</sup>H]palmitate labeling still remains on C580/581A DATs, demonstrating that palmitoylation is occurring on one or more additional site(s). None of the other Cys→Ala mutants in these studies displayed decreased labeling, suggesting that an unexamined site is modified, that multiple sites are modified at low stoichiometries that are difficult to detect by this method, or that the mutations induced compensatory palmitoylation on another site, possibly Cys580. Our studies to date have focused on properties of Cys580/581, but identification of the additional site(s) is an important issue as each modification site may confer distinct regulatory effects (Hayashi *et al.*, 2005).

To examine the functional ramifications of palmitoylation in the WT protein we utilized the PAT inhibitor 2BP (Foster and Vaughan, 2011). In rat striatal synaptosomes, significant inhibition of DAT palmitoylation was detectable within 30 min of 2BP treatment, with further decreases occurring between 45 and 60 min. In contrast, inhibition of DAT

[<sup>3</sup>H]palmitate labeling in LLC<sub>PK</sub><sub>1</sub> cells was less pronounced and required treatment times of 6–18h indicating major differences in palmitoylation kinetics between these systems. As a consequence, we have developed rDAT-expressing N2a neuroblastoma cells and immortalized rat dopaminergic midbrain neurons (N27), as neuronal cell lines for palmitoylation analyses. In these cells DAT palmitoylation is considerably more sensitive to 2BP (Rastedt, Stanislawski, Vaughan and Foster, unpublished results), indicating a greater similarity of this property to brain tissue.

Using 2BP to examine functional ramifications of DAT palmitoylation we have identified multiple effects that can be broadly categorized as rapid or slow changes. In synaptosomes (Foster and Vaughan, 2011) and in rDAT-N2a and rDAT-N27 cell lines (Rastedt, Stanislawski, Vaughan, and Foster, unpublished results), low dose and short-term 2BP treatments cause up to ~50% reductions in DA transport  $V_{max}$ , with no detectable change in total or surface transporter levels. The acute decreases in the kinetic efficiency of transport with reduced palmitoylation indicates that palmitoylation functions to enhance the kinetic capacity of the transporter. Consistent with these findings transport  $V_{max}$  of palmitoylation-deficient C580A DAT is strongly reduced relative to the WT protein (Moritz *et al.*, 2015). PMA-induced downregulation is also increased in 2BP-treated and C580A DATs, indicating that the enhanced activity derived from palmitoylation functions to oppose PKC-induced down-regulation (Foster and Vaughan, 2011). These findings thus identify a novel mechanism for control of DAT transport activity and implicate Cys580 palmitoylation in enhancement of the kinetic capacity of the transporter.

Longer-term 2BP treatments in both rDAT-LLC<sub>PK</sub><sub>1</sub> cells and synaptosomes were also associated with further losses of transport that were accompanied by loss of DAT protein and appearance of low  $M_r$  transporter fragments. This indicates that prolonged suppression of palmitoylation drives DAT into the degradation pathway (Foster and Vaughan, 2011). Consistent with this finding, C580A DAT displays increased turnover assessed via [<sup>35</sup>S] methionine pulse-chase analysis (Moritz, Vaughan, and Foster, unpublished results), indicating that palmitoylation supports the metabolic stability of DAT and functions as a mechanism for long-term control of total transporter levels.

More recently we have been able to elevate DAT palmitoylation by overexpressing DHHC enzymes in rDAT cell systems (Moritz *et al.*, 2015). Our findings indicate that DAT palmitoylation can be catalyzed by a subset of neuronally-expressed PATs, although many PATs were without effect, indicative of substrate specificity. Increased DAT palmitoylation driven by DHHC enzyme co-expression led to increased DA uptake  $V_{max}$  without changes in transporter surface expression or  $K_M$ , further supporting the contention that DAT palmitoylation increases the rate of transport (Rastedt *et al.*, 2014). Together these findings are consistent with the mechanism shown in Fig. 3 in which conditions that enhance palmitoylation lead to acute increases in transport  $V_{max}$  driven by a kinetic mechanism, while sustained suppression of palmitoylation leads to targeting of DAT for degradation.

## 5. Reciprocal regulation of DAT palmitoylation and phosphorylation

The enhancement of transport by palmitoylation is thus opposite to the reduction of transport imparted by phosphorylation, prompting us to examine the relationship of these modifications in greater detail. DAT undergoes protein kinase C (PKC)-dependent phosphorylation in a serine cluster on the distal end of the N-terminus (Fig. 1), with phosphorylation of Ser7 within this domain a primary determinant in PKC-induced transport down-regulation (reviewed in Foster and Vaughan, 2016 this issue). We have now shown that not only do Cys580 palmitoylation and Ser7 phosphorylation exert opposing effects on transport activity, but that they are mechanistically linked in a reciprocal manner (Moritz *et al.*, 2015). Palmitoylation status affects phosphorylation, as C580A mutation and 2BP treatment, conditions that reduce DAT palmitoylation, lead to enhanced basal and PKC-stimulated DAT phosphorylation, which in some cases was localized to Ser7, and elevation of palmitoylation with DHHC 2 overexpression leads to reduced transporter  $^{32}\text{PO}_4$  labeling (Moritz *et al.*, 2015). Conversely, phosphorylation conditions affect palmitoylation, as activation of PKC, which elevates Ser7 phosphorylation, reduces DAT palmitoylation, and conditions that reduce Ser7 phosphorylation (S7A mutation, PKC inhibitor) enhance DAT palmitoylation. Similar reciprocity of palmitoylation and phosphorylation have been shown for other proteins (Charych *et al.*, 2010; Dorfleutner and Ruf, 2003; Lin *et al.*, 2009; Moffett *et al.*, 1996), although in most cases the modified sites are close in primary sequence and the reciprocal relationship of the modifications has been attributed to steric hindrance. As the modified sites in DAT are far apart in primary sequence, the underlying mechanism for the reciprocal regulation remains unknown. Importantly, transporter activity follows these modifications, as decreased transport  $V_{\max}$  is driven by increased phosphorylation/decreased palmitoylation and increased transport  $V_{\max}$  is driven by decreased phosphorylation/increased palmitoylation supporting the concerted regulation of transport by the balance between these modifications (Fig 3).

## 6. Possible palmitoylation mechanisms in transport kinetics

In the studies described above altered transport levels were not associated with changes in DAT plasma membrane levels detected by surface biotinylation indicating that regulation of uptake occurred via a kinetic mechanism. Kinetic regulation of transport by palmitoylation implies that the modification is affecting the rate at which the protein transitions through the transport cycle. As Cys580 lies outside the core TM bundles that function in substrate binding and permeation, it is likely that palmitoylation affects transport capacity indirectly through effects on gating networks, permeation pathway conformational changes or binding partner interactions.

Palmitoylation can affect integral membrane proteins in numerous ways. At the level of protein - lipid bilayer interaction, transmembrane domains that are longer than the width of the membrane bilayer create a situation of hydrophobic mismatch that is energetically unfavorable (van Duyl *et al.*, 2002) and can affect protein function. Palmitoylation may assist in reducing mismatch by promoting changes in helix orientation or tilting (Strandberg and Killian, 2003), a possibility for DAT that is consistent with the site of palmitoylation at the base of TMD12. Partitioning of proteins into membrane raft domains, which possess

greater membrane thickness than non-raft regions may also reduce mismatch (Andersen and Koeppe, 2007) or promote interactions with cholesterol or raft binding partners that are impacted by palmitoylation. DAT is associated with membrane raft microdomains (Cremona *et al.*, 2011; Foster *et al.*, 2008; Gabriel *et al.*, 2013; Hong and Amara, 2006; Navaroli *et al.*, 2011; Sakrikar *et al.*, 2012) and phosphorylation of DAT is enriched at these sites (Foster *et al.*, 2008), suggesting this as a possible mechanism for the concerted regulation of phosphorylation and palmitoylation. It is also possible that palmitoylation of DAT at Cys580 may affect its ability to form a dimer, as the LeuT crystal structure showed a dimer interface at TMD12 (Yamashita *et al.*, 2005). Dimer and monomer forms of DAT at the cell surface may exhibit unique transport characteristic or subcellular localization (Foster *et al.*, 2008; Hong and Amara, 2006; Hong and Amara, 2010; Jones *et al.*, 2012; Seidel *et al.*, 2005; Zhen *et al.*, 2015) that affect DAT transport kinetics.

A novel attribute revealed by the dDAT crystal structure is the presence of a motif formed by a hairpin loop between the base of TMD12 and an adjacent helix formed by residues 586–595. This helix lies parallel to the plane of the membrane and interacts with intracellular loop 3 (IL3) to stabilize the intracellular gate and indirectly impact the ability of TMD1 to undergo conformational changes needed for transport (Penmatsa *et al.*, 2015). The interaction between the helix and IL3 is stabilized by hydrogen bonding between Thr582 and Arg589 which are conserved in rDAT as Ser581 and Arg587 (Fig. 1), strongly suggesting that structural impacts on TMD12 by palmitoylation at Cys580 could affect the ability of Ser581 to form this H-bond, providing a potential mechanistic explanation for its ability to modulate transport activity. This helix was originally identified as an endocytosis motif that mediates PKC-dependent endocytosis (Holton *et al.*, 2005; Navaroli *et al.*, 2011). Current surface biotinylation findings indicate that palmitoylation-mediated regulation of transport occurs independently of plasma membrane changes, but further examination of the effect of these modifications on trafficking may be warranted due to the proximity of the palmitoylation site to the helix.

## 7. Palmitoylation in disease

Many proteins with essential functions in neurotransmission and synaptic plasticity are palmitoylated (Duncan and Gilman, 1998; el-Husseini Ael and Brecht, 2002; Fang *et al.*, 2006; Fukata *et al.*, 2004; Hayashi *et al.*, 2005; Keller *et al.*, 2004), and dysregulation of palmitoylation is associated with multiple neurological disorders, including intellectual disability, Huntington disease, Alzheimer disease, schizophrenia, and the lysosomal storage disease infantile neuronal ceroid lipofuscinosis (reviewed in Cho and Park, 2016). In many cases these diseases are caused or associated with dysregulation of the acyl transferases or thioesterases that cause inappropriate palmitoylation of target proteins, suggesting that similar dysregulation of palmitoylation inputs into DAT could have strong impacts on its associated functions. In particular, reduced palmitoylation of glial glutamate transporter-1 results in decreased glutamate uptake, that has been suggested as a cause of excitotoxicity (Huang *et al.*, 2010).

Several DAT SNPs associated with disease states have now been identified, including A559V, identified from ADHD and BD patients (Mazei-Robison *et al.*, 2005). This residue

is found at the extracellular end of TMD12, and induces significant effects on transport functions including reverse transport (Mazei-Robison *et al.*, 2008), which is driven by DAT hyperphosphorylation (Khoshbouei *et al.*, 2004; Wang *et al.*, 2016). This mutant displays reduced palmitoylation, which could mechanistically link it to altered phosphorylation and efflux, resulting in hyperdopaminergia that is associated with ADHD and BD (Shetty *et al.*, 2015), and supporting the concept that DA imbalances could follow from dysregulated palmitoylation.

## 8. Summary

The findings described here highlight the importance of palmitoylation in the functionality and regulation of DAT. Changes in transporter functionality driven by palmitoylation thus suggest this modification as a potential therapeutic target for modulation of DA reuptake in disease states through modulation of the palmitoyl transferases or thioesterases that regulate the modification. Mutations in the palmitoyl transferases and APT/PPT enzymes have also resulted in disease states, indicating that the palmitoylation status of a protein is relevant to disease processes. Understanding the mechanisms of these posttranslational modifications and the effect they have on DAT, along with elucidation of the N- and C-terminal domain structures, is critical in understanding the regulatory processes that occur in disease states and potential treatment via these specific therapeutic targets.

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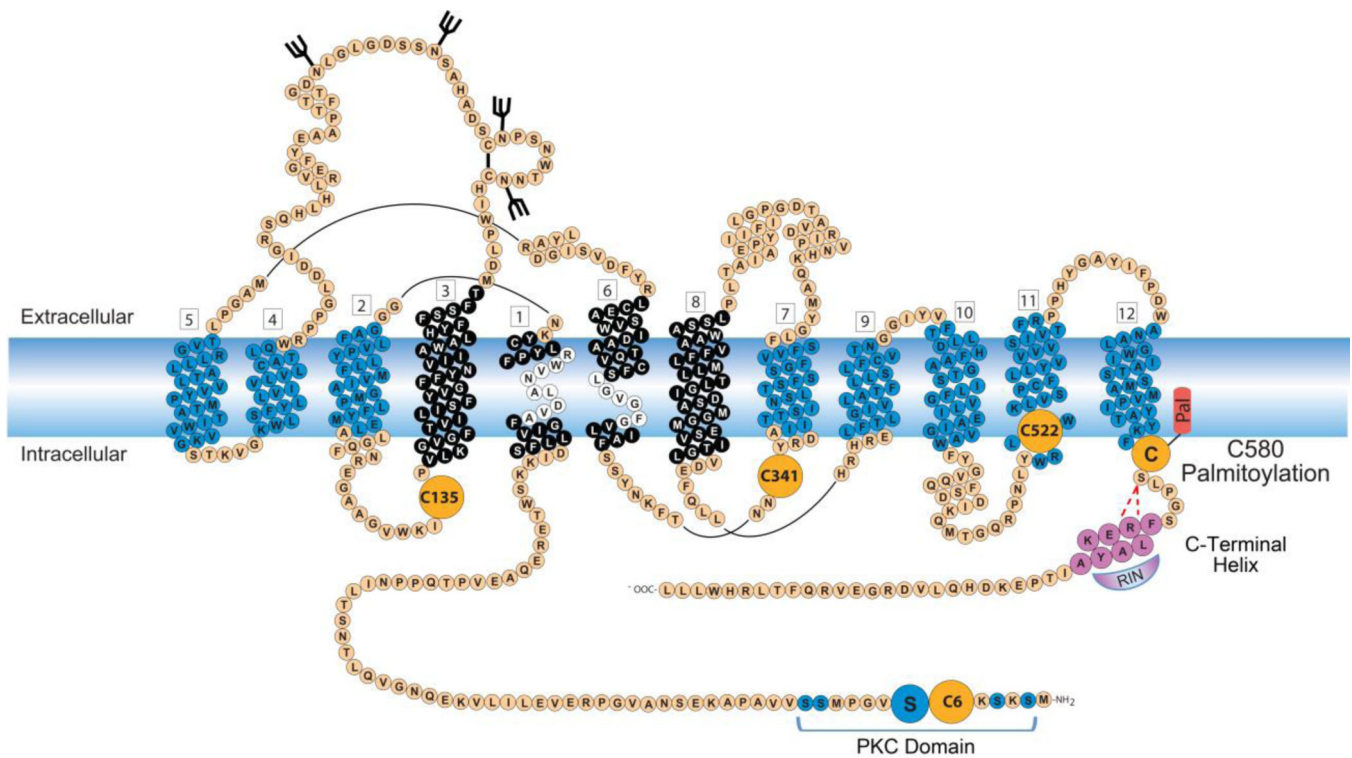
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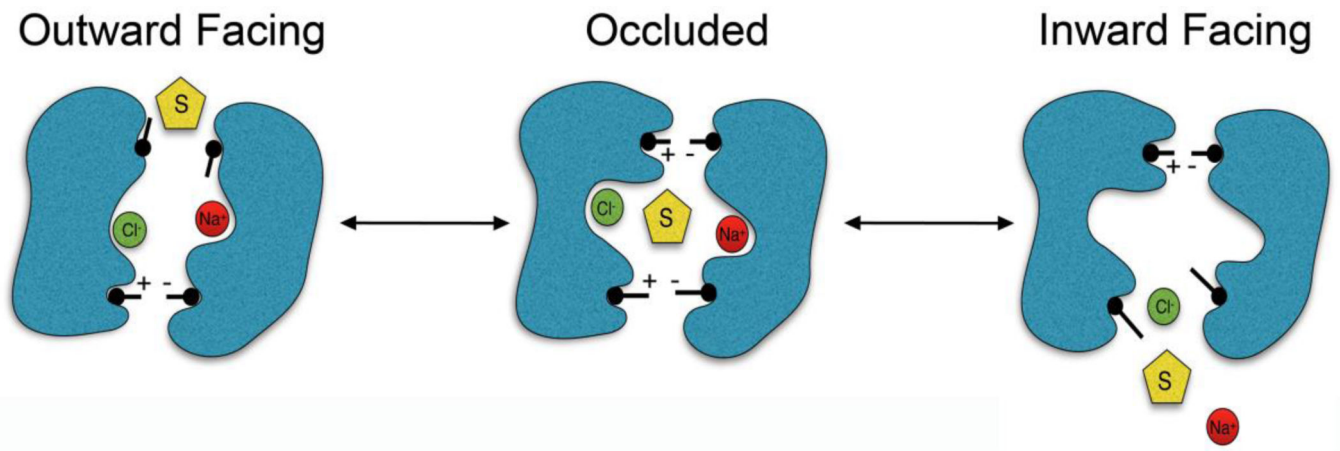
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### Highlights

- DAT is reversibly modified with a 16-carbon palmitate group through a thioester bond.
- Enhanced DAT palmitoylation leads to acute increases in transport  $V_{\max}$  driven by a kinetic mechanism.
- Sustained suppression of palmitoylation leads to targeting of DAT for degradation.
- DAT is regulated in a reciprocal manner by palmitoylation and phosphorylation.

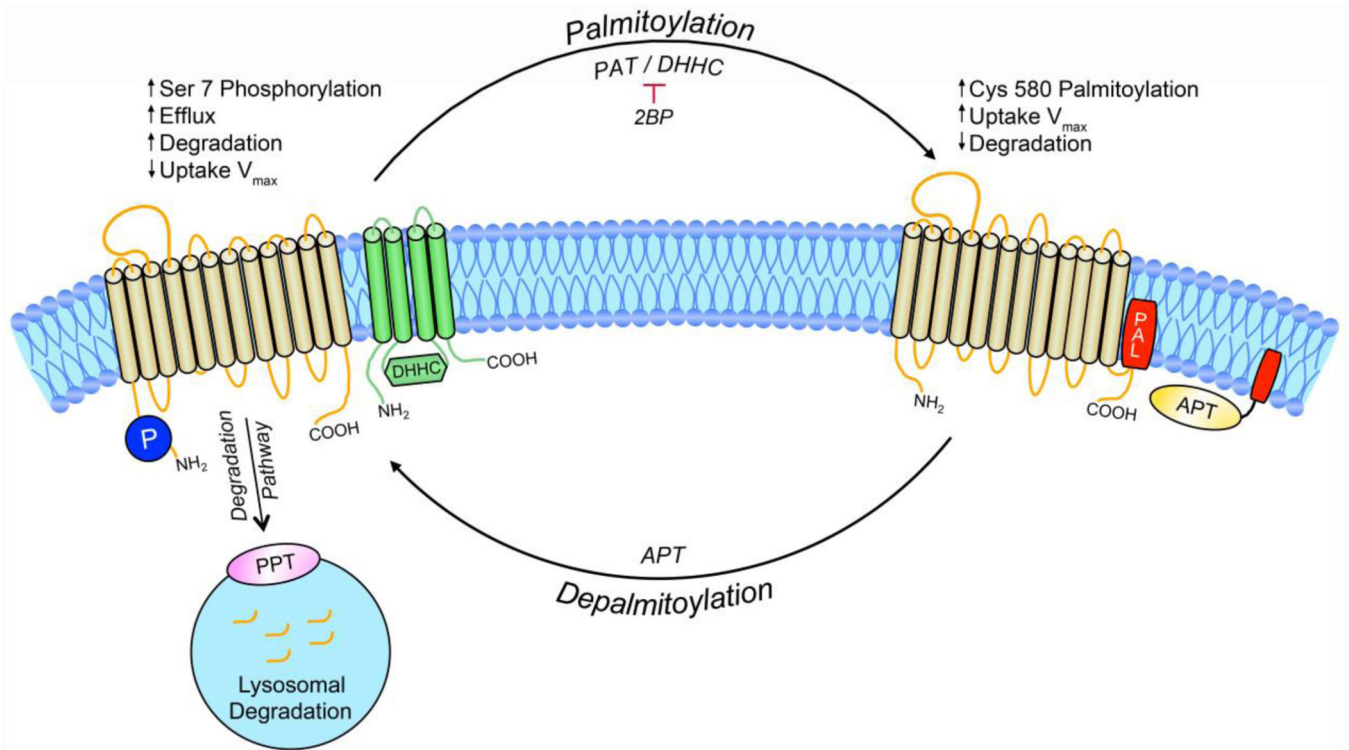


**Fig. 1.** Schematic diagram of the expanded membrane topology of DAT with 12 TMDs and intracellularly facing N- and C-termini. Vertical stacks of amino acids (blue, black and white) represent  $\alpha$ -helical membrane spanning domains arranged in two antiparallel aligned pentahelical bundles (TMDs 1–5 and 6–10) with TMDs 11 and 12 located peripheral to these bundles. The DA binding site is positioned within the inner core (black) with critical residues contained within unwound segments found in the middle of TMD1 and TMD6 (white). The known sites of glycosylation (black branched sticks), phosphorylation within the PKC-domain containing Ser7 (blue circles) and palmitoylation (orange circle with red appendage) are shown. Potential additional sites of palmitoylation are shown as large orange circles. The C-terminal helix (mauve, FREKLAYA) is also shown with Ras-like GTPase Rin 1 bound (Rin, mauve) and red dashed lines depicting hydrogen bonding between Arg 587 and Ser 581.



**Fig. 2.**

Illustration of the alternating access mechanism of DA transport. Substrate (S) and ions (Na<sup>+</sup> and Cl<sup>-</sup>) access the binding site of the outward facing transporter via the open outer gate, the outer gate closes yielding the occluded state, which then transitions to the inward facing state releasing substrate and ions to the cytosol. The transporter then rectifies to the outward facing structure. Black sticks represent the gating network of amino acid side chains.



**Fig. 3.** Functional consequences of DAT palmitoylation. The cycle of DAT palmitoylation and depalmitoylation by palmitoyl acyltransferases (green, *PAT/DHHC*) and acyl protein thioesterases (yellow, *APT*), respectively, is shown. Identified palmitoylation site Cys580 (red, PAL) and the reciprocally regulated Ser 7 phosphorylation site (dark blue, P) are also shown. DAT destined for degradation is directed toward the lysosome via an unknown sorting mechanism which could include depalmitoylation by lysosomal localized palmitoyl-protein thioesterase (PPT, pink).