Regulation of the Dopamine and Vesicular Monoamine Transporters: Pharmacological Targets and Implications for Disease

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Abstract——Dopamine (DA) plays a well recognized role in a variety of physiologic functions such as movement, cognition, mood, and reward. Consequently, many human disorders are due, in part, to dysfunctional dopaminergic systems, including Parkinson's disease, attention deficit hyperactivity disorder, and substance abuse. Drugs that modify the DA system are clinically effective in treating symptoms of these diseases or are involved in their manifestation, implicating DA in their etiology. DA signaling and distribution are primarily modulated by the DA transporter (DAT) and by vesicular monoamine transporter (VMAT)-2, which transport DA into presynaptic terminals and synaptic vesicles, respectively. These transporters are regulated by complex processes such as phosphorylation, protein–protein interactions, and changes in intracellular localization. This review provides an overview of 1) the current understanding of DAT and VMAT2 neurobiology, including discussion of studies ranging from those conducted in vitro to those involving human subjects; 2) the role of these transporters in disease and how these transporters are affected by disease; and 3) and how selected drugs alter the function and expression of these transporters. Understanding the regulatory processes and the pathologic consequences of DAT and VMAT2 dysfunction underlies the evolution of therapeutic development for the treatment of DA-related disorders.

I. Overview

Dopamine (DA) is a monoamine neurotransmitter first identified in 1957 by a team of researchers under the direction of Arvid Carlsson (for review of this historic development, see Hornykiewicz, 2006), which led to the Nobel Prize for Physiology or Medicine in 2000. DA signaling and distribution are dynamically regulated by several factors, including transport into the cytoplasm and synaptic vesicles through the dopamine transporter (DAT) and vesicular monoamine transporter (VMAT)-2, respectively. Perturbation of either DAT or VMAT2 function profoundly alters intracellular and extracellular DA concentrations. Dysregulation of dopaminergic neuronal function can, in turn, contribute to several disorders of the central nervous system (CNS), including Parkinson's disease (PD), attention deficit hyperactivity disorder (ADHD), and addictive disorders (Seeman and Niznik, 1990; Fiorino et al., 1993; Nestler and Carlezon, 2006; Swanson et al., 2007; Koob and Volkow, 2010). Understanding the function of these transporters provides insight into the mechanism of action of well known pharmacological agents and also provides opportunities for the development of new therapeutics. Given the vast scope of the field, only selected mechanisms and disorders are discussed herein. These are not all encompassing, and the reader is referred to additional recent reviews (Sulzer et al., 2005; Alter et al., 2013; Schmitt et al., 2013; Vaughan and Foster, 2013; Howell and Negus, 2014; Nickell et al., 2014).

II. Dopamine and Dopaminergic Terminals: A Brief Introduction

DA is a catecholamine neurotransmitter employed by several major CNS pathways, including the nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular systems. A canonical model of presynaptic DA terminal form and function is presented in Fig. 1. DA production, vesicular localization and release, and extracellular persistence are largely regulated by the coordinated activity of tyrosine hydroxylase (TH), VMAT2, and DAT, respectively.

TH is the rate-limiting enzyme in DA production (Levitt et al., 1965) that converts dietary tyrosine to L-dihydroxyphenylalanine (L-DOPA), which, in turn, is converted by aromatic amino acid decarboxylase (AADC) to DA. After synthesis, VMAT2 transports DA from the cytoplasmic space into synaptic vesicles within presynaptic terminals. VMAT2 activity largely dictates quantal size, affecting the scale of subsequent neurotransmitter release (Pothos et al., 2000; Omiatek et al., 2013).

Once released, DA can bind to and activate both presynpatic and postsynaptic DA receptors. As DA diffuses away from the synapse, it is taken back up into dopaminergic terminals via the perisynaptically localized DAT (Nirenberg et al., 1996b) and repackaged into synaptic vesicles or degraded. Extracellular reuptake, first characterized for norepinephrine, was initially identified as the primary mechanism for catecholamine synaptic clearance and signal cessation (Dengler et al., 1961; Glowinski et al., 1965; Snyder et al., 1965; Colburn et al., 1968). Although studies indicate that
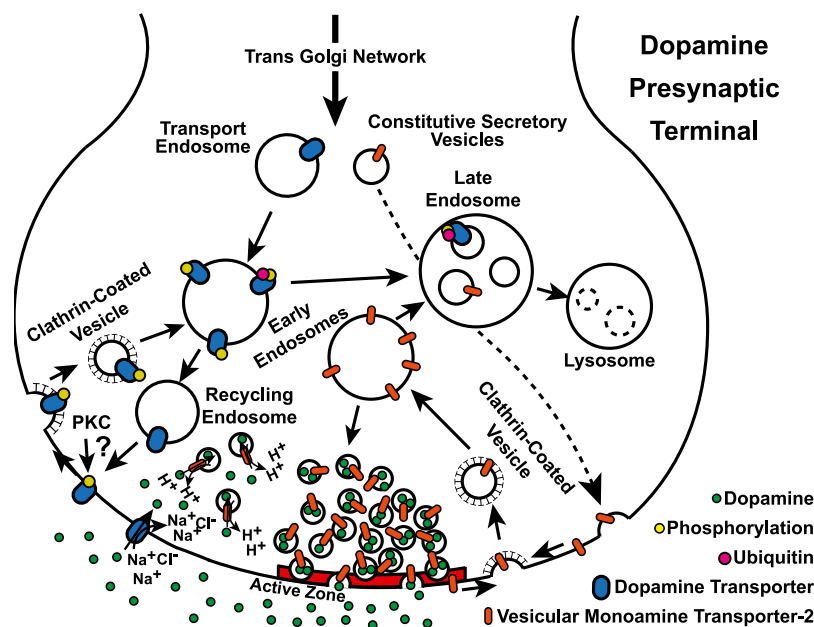
Fig. 1. Presynaptic dopaminergic terminal model.

DAT influences the duration and extent of presynaptic and postsynaptic DA receptor signaling (Jones et al., 1998), recent modeling data suggest that diffusion of DA away from the terminal and dilution within the extracellular milieu, rather than uptake through the DAT, may be the dominant factors governing the extent and duration of DA signaling (Cragg and Rice, 2004; Rice and Cragg, 2008). The perisynaptically localized DAT (Nirenberg et al., 1996b) may instead influence the kinetics and volume of extrasynaptic DA diffusion, rather than the DA content within individual synapses (Rice and Cragg, 2008).

III. Dopamine Transporter: Structure and Regulation

A. Dopamine Transporter Structure and Function

The DAT was first sequenced and cloned in 1991 (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991). It is a member of the solute carrier 6 (SLC6) family of solute transporters and is highly conserved among humans and other mammals, including rats and mice (92% and 93.4% homology, respectively; Giros et al., 1992). Crystallography of the closely related bacterial leucine transporter has been the primary source for DAT structure and function models, with more recent insight provided by crystallization of the *Drosophila melanogaster* DAT. These studies have identified several drug binding sites, including cocaine and antidepressants (Zhou et al., 2007; Beuming et al., 2008; Penmatsa et al., 2013). For extensive reviews of the protein structure and transport function of SLC6 transporters, see Kristensen et al. (2011) and Pramod et al. (2013).

In the rat, DAT is 619 amino acids long, with 12 transmembrane segments, a 68 amino acid–long cytoplasmic N-terminal regulatory section, and a 41 amino acid–long cytoplasmic C-terminal regulatory section. These N- and C-terminal regulatory domains are the primary regions for DAT post-translational modifications and protein interactions within the cytoplasmic space, and they are critical for regulating DAT function (see section III.B). In addition, a 75 amino acid–linking region between the third and fourth transmembrane segments serves as a regulatory substrate for post-translational modifications and protein interactions within the extracellular space. As demonstrated in *Drosophila*, the DAT has a 5–5 distribution of transmembrane domains, with domains 1–5 and 6–10 forming two groups of opposing domains that transport DA through a ligand binding core formed by transmembrane regions 1, 3, 6, and 8 (Kristensen et al., 2011; Penmatsa et al., 2013). These domains are bound by DA and its ionic carriers, Na^+ and Cl^-, to facilitate transport across the membrane, as well as by drugs such as cocaine and its analogs (Beuming et al., 2008).

A well accepted model of DAT function proposes that DA is transported across the plasma membrane by DAT shifting between outward- and inward-facing conformations, which is driven by the symport of Na^+ and Cl^- along their concentration gradients (Shan et al., 2011) (Fig. 2A). This process is initiated by one DA molecule binding the pocket formed by transmembrane regions 1, 3, 6, and 8 of outward-facing DAT (Fig. 2A). Binding of two Na^+ ions and one Cl^- ion to this pocket causes a DAT conformational change from outward facing to inward facing (Fig. 2A), releasing DA, Na^+, and Cl^- into

A Dopamine Transporter

B Vesicular Monoamine Transporter-2
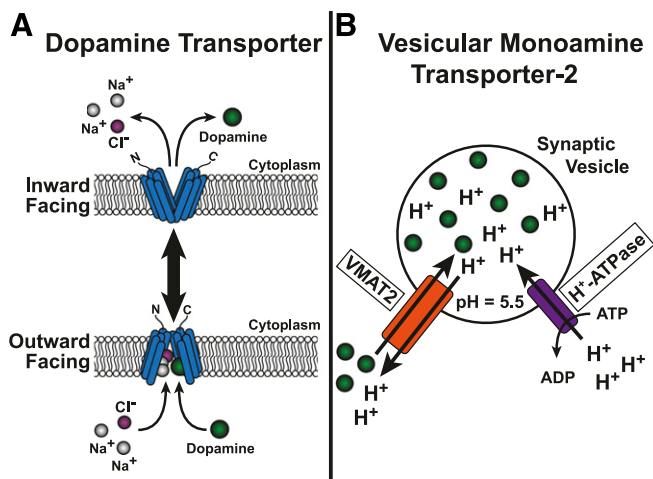
Fig. 2. Models of DAT and VMAT2 function. (A) The movement of DA from the extracellular to cytoplasmic space through transition of DAT from an outward-facing to inward-facing state. (B) The transport of DA into synaptic vesicles from the cytoplasmic space through VMAT2, facilitated by H^+-ATPase function.

the cytoplasm. Substrate release shifts the DAT from its inward-facing conformation to the outward-facing conformation. In this model, DA is stoichiometrically transported across the membrane and is highly dependent upon the concentration of extracellular Na^+ (Wheeler et al., 1993). Inward and outward conformations also expose different DAT residues to the extracellular space, which affects the binding properties of specific pharmacologic agents (e.g., cocaine), as discussed in section VI.A.2.

DAT creates a complex electrogenic current associated with substrate transport as Cl^- and Na^+ move into the cell along their concentration gradients (Sonders et al., 1997). In addition to the stoichiometric current associated with substrate transport, an uncoupled anionic current is created when substrates are transported, resulting in a larger than expected charge movement (Sonders et al., 1997). Underlying substrate transport, DAT has a small tonic leak current that nonselectively transports cations and is blocked by both substrates and uptake inhibitors (e.g., cocaine) (Sonders et al., 1997). These DA-coupled and DA-uncoupled currents directly alter membrane excitability and may affect DA release (Sonders et al., 1997; Ingram et al., 2002). Current-like behavior is also hypothesized to play a role in stimulant-induced DA efflux through DAT (Kahlig et al., 2005). Enhancing or impairing DAT channel-like properties may provide a unique means to manipulate dopaminergic membrane potential, although this is unexplored.

B. Dopamine Transporter Regulation

DAT is primarily regulated by three mechanisms: post-translational modification, protein–protein interactions, and intracellular localization. A high degree of overlap exists between these mechanisms. Post-translational

modifications are often the result of protein–protein interactions, and the localization of DAT on the plasma membrane versus within the intracellular space is often dictated by post-translational modifications and interactions with trafficking and regulatory proteins. Of note, these mechanisms have primarily been identified in in vitro systems that may not fully model the in vivo state (e.g., cell culture expression systems) or in systems intended to model diseased and/or dysfunctional states (e.g., in animals treated with psychostimulants or animal models of PD; see section VI.A.1). Consequently, it is unclear to what extent many of these mechanisms contribute to the regulation of basal DAT function in vivo.

1. Post-Translational Modification. DAT undergoes at least four forms of post-translational modification: phosphorylation, ubiquitination, glycosylation, and palmitoylation. Two additional processes, oxidation and nitrosylation, may also regulate DAT function. These modifications facilitate interactions between DAT and other proteins, alter DAT transport kinetics (e.g., V_{max}), and/or change the distribution of DAT within plasma membrane lipid domains or between the cell surface and intracellular space. The vast majority of DAT post-translational modification identified to date occurs within the N-terminal cytoplasmic domain, the C-terminal cytoplasmic domain, and the extracellular segments between the third and fourth transmembrane domains. Pharmacologically manipulating these post-translational modifications may provide unique therapeutic routes to regulate DAT expression and function.

Phosphorylation is the most well characterized but functionally opaque DAT post-translational modification. The DAT N terminus contains several threonine and serine residues that may be phosphorylated by protein kinase C (PKC), protein kinase A (PKA), protein kinase G, and Ca^{2+}/calmodulin protein kinase II (Gorentla et al., 2009). Proline-directed phosphorylation of DAT threonine 53 may be involved in maintaining DAT transport (point mutations decrease DAT V_{max}) and may also be involved in amphetamine (AMPH)–induced reverse transport (Gorentla et al., 2009; Foster et al., 2012). Acute, in vitro activation of PKC by phorbol esters has been associated with a downregulation of DAT activity, which coincides with DAT phosphorylation and reduced plasma membrane expression (Copeland et al., 1996; Vaughan et al., 1997; Zhu et al., 1997; Daniels and Amara, 1999; Melikian and Buckley, 1999). Taken together, these observations led to the hypothesis that PKC-mediated downregulation of DAT activity is a consequence of phosphorylation-induced DAT trafficking, likely internalization. The identification of several DAT N-terminal serines that are phosphorylated in vitro by PKC led to the further proposal that these residues are involved in regulating DAT plasma membrane expression (Foster et al., 2002). However, limited evidence has come to support this hypothesis. Mutating or deleting serines on the DAT N-terminal

and intracellular domains prevents PKC-mediated phosphorylation, but it does not alter DAT localization (Chang et al., 2001; Granas et al., 2003). The role of DAT N-terminal serine phosphorylation remains unclear, but it may be involved in facilitating protein–protein interactions, regulating constitutive DAT turnover, and/or altering substrate transport during stimulant exposure (see section VI.A.1). Instead, DAT internalization may be regulated by PKC interaction at the DAT C terminus. A unique motif identified on the C terminus of DAT and other SLC6 family transporters drives constitutive clathrin-mediated endocytosis (Holton et al., 2005). PKC has been proposed to regulate the degree of DAT internalization by removing a braking mechanism that physically masks the C-terminal DAT internalization motif, allowing this exposed motif to enhance basal internalization (Boudanova et al., 2008).

Ubiquitination of the DAT N terminus determines whether DAT internalization is temporary and DAT is recycled back to the membrane, or it is more permanent and leads to DAT degradation. Ubiquitinated DAT is sorted away from the constitutive recycling pathway and into a late endocytic pathway, resulting in lysosomal degradation (Daniels and Amara, 1999; Miranda et al., 2005, 2007). To date, two ubiquitin ligases that ubiquitinate and sort misfolded DAT into the lysosomal degradation pathway (Fig. 1) have been identified: neural precursor cell expressed developmentally downregulated protein 4-2 (Sorkina et al., 2006) and Parkin (Jiang et al., 2004).

The extracellular regulatory domain between transmembrane domains 3 and 4 contains asparagines that are targets for glycosylation (Vaughan and Kuhar, 1996). DAT glycosylation progressively increases throughout development and appears to both stabilize DAT localization to the plasma membrane and increase the DA transport rate (V_{max}) (Patel et al., 1994; Li et al., 2004). Glycosylation is not necessary for plasma membrane expression, but partially glycosylated and nonglycosylated DAT is preferentially endocytosed, is more predominant in endocytic structures, and inefficiently transports DA compared with fully glycosylated DAT (Li et al., 2004). Glycosylation plays an important role in the susceptibility of DAT to the effects of drugs and disease. Cocaine has a greater inhibitory effect on nonglycosylated DAT (Li et al., 2004), and reduced DAT glycosylation within the human striatum and midbrain strongly correlates with greater PD susceptibility (Afonso-Oramas et al., 2009). Maintaining or enhancing DAT glycosylation, perhaps via pharmacological manipulation, may stabilize DAT plasma membrane localization and uptake and may aid in the preservation of DAT function.

On the C terminus, DAT function is regulated through palmitoylation, the addition of a saturated fatty acid via thioester bonding. Palmitoylation regulates DAT function and structure by maintaining V_{max} and reducing PKC-mediated plasma membrane turnover (Foster and Vaughan, 2011). Although much of this process requires elucidation, palmitoylation is important in activity-driven changes in synapse morphology and function (Kang et al., 2008) and may serve as a future target for pharmacological manipulation.

Finally, nitric oxide–mediated nitrosylation can regulate DAT activity (Volz and Schenk, 2004). In addition, exposure to reactive oxygen species decreases DAT function (Berman et al., 1996; Fleckenstein et al., 1997a) and likely contributes to the formation of high molecular weight DAT complexes (Baucum et al., 2004), both of which are components of methamphetamine (METH)–induced dopaminergic deficits (see section VI).

2. Dopamine Transporter–Protein Interactions. DAT interacts directly and indirectly with numerous proteins (in addition to the kinases and ubiquitinases already discussed) that are important in regulating localization and function (for review, see Eriksen et al., 2010b). DAT localization to intracellular locales or plasma membrane domains requires interaction with numerous synaptic scaffolding proteins. Binding of the PDZ domain protein interacting with C-kinase 1 (PICK1) to the DAT C-terminal PDZ domain has been demonstrated both in vitro and in vivo and is thought to be involved in regulating plasma membrane expression, although opposing functions have been demonstrated (Torres et al., 2001; Bjerggaard et al., 2004). Recent work indicates that rather than facilitating plasma membrane expression, PICK1 may function as an endocytic compartment–specific anchor, holding DAT within Rab11-positive endocytic compartments and preventing recycling back to the plasma membrane (Madsen et al., 2012).

Despite the unclear role of PICK1 binding, DAT C-terminal PDZ interactions are incredibly important in transporter localization. DAT knock-in mice with disrupted PDZ domains experience significant loss of transporter expression within the striatum (Rickhag et al., 2013a). PDZ domain interactions appear necessary for stabilizing DAT at the plasma membrane, and their absence enhances constitutive internalization (Rickhag et al., 2013a).

DAT–protein interactions also facilitate the formation of complexes between proteins involved in regulating the DA system. DAT may directly bind DA D_2 receptors, which promotes the recruitment of DAT to the plasma membrane (Lee et al., 2007). Disrupting this interaction in vivo leads to hyperlocomotion in mice, suggesting impaired DA uptake and/or D_2 receptor signaling (Lee et al., 2007). The DAT may be indirectly influenced by VMAT2 through interaction with the synaptic vesicle protein synaptogyrin-3 (Egaña et al., 2009). Increasing synaptogyrin-3 expression in vitro increases DAT function, which is blocked by treatment with the VMAT2 inhibitor reserpine (Egaña et al., 2009). This leads to the hypothesis that synaptogyrin-3

tethers VMAT2 synaptic vesicles to DAT, facilitating the immediate reuptake of DA into synaptic vesicles after transport through DAT.

Localization and stabilization of DAT at the plasma membrane is regulated through interactions with proteins such as flotillin-1 and α-synuclein. Flotillin-1 is a membrane scaffolding protein that was recently identified as necessary for localizing DAT to plasma membrane lipid rafts, thereby dictating the binding partners to which DAT may be physically exposed (Cremona et al., 2011). In addition, the binding of α-synuclein to the DAT C terminus increases DAT membrane expression and DA uptake, stabilizing the transporter at the plasma membrane under normal conditions. However, α-synuclein overexpression may be an important factor driving PD dopaminergic toxicity, as discussed in section V.B (Fountaine and Wade-Martins, 2007; Moszczynska et al., 2007). Thus, pharmacologically targeting interactions between DAT and its protein-binding partners, such as flotillin-1 or α-synuclein, may prevent the progression of disorders such as PD.

In addition to binding other proteins, the DAT has been observed in vitro to form oligomeric complexes, often tetramers, which are involved in plasma membrane expression and function (Milner et al., 1994; Hastrup et al., 2001; Torres et al., 2003). DAT oligomeric complexes are formed within the endoplasmic reticulum and maintained throughout constitutive cycling between the plasma membrane and endocytic structures (Sorkina et al., 2003). Whether oligomeric complexes are the predominant state of DAT orientation in vivo is unknown, but some evidence supports the existence of DAT tetramers within striatal membranes (Milner et al., 1994). However, the potential significance of oligomers has been demonstrated by recent work showing that, under certain conditions, DAT protomers within an oligomeric complex affect the conformational state of one another, altering DA transport activity and sensitivity to stimulants historically classified as DAT reuptake inhibitors such as cocaine (Zhen et al., 2015). The size, composition, and distribution of DAT oligomeric complexes may therefore influence the sensitivity of particular dopaminergic neurons to stimulants and any subsequent toxicity. Of note, the formation of large molecular weight DAT complexes is observed after multiple high-dose administrations of METH in vivo and is associated with excessive DA signaling, dysfunctional VMAT2, and hyperthermia (Baucum et al., 2004; Hadlock et al., 2010). How METH-induced DAT complexes may be related to or involved with unimpaired DAT oligomers, as well as the role of these complexes in a METH-stressed striatum, is unknown.

3. Transporter Localization. DAT can only transport DA when expressed at the plasma membrane. Under normal conditions, DAT is perisynaptically expressed and constitutively internalized. After clathrin-mediated endocytosis, DAT is primarily sorted down the degradation pathway of the late endosome and lysosome, but it may also be sorted into recycling endosomes and then reexpressed on the plasma membrane (Loder and Melikian, 2003; Sorkina et al., 2005; Eriksen et al., 2010a; Rao et al., 2011). Two aspects of localization are important when considering DAT function: the localization of DAT within the plasma membrane and the distribution of DAT between the plasma membrane and intracellular endosomal structures. The localization of DAT within plasma membrane lipid rafts dictates many of its protein interactions and relative mobility on the membrane. Under basal conditions, DAT is evenly distributed between raft and nonraft domains (Adkins et al., 2007; Foster et al., 2008) and is highly mobile within the plasma membrane (Eriksen et al., 2009); however, changes to this distribution may play a role in DA-related disorders. For example, one case study revealed a rare coding variant in the human DAT C terminus of a patient with ADHD (R615C); this variant impairs flotillin 1 interactions with DAT and reduces DAT lipid raft localization, which, in turn, may have altered DA signaling in this individual (Sakrikar et al., 2012).

Given the importance of DAT plasma membrane expression to its function, facilitated removal of DAT from the plasma membrane by endocytosis has been proposed as a primary mechanism for the reduction in DAT uptake observed in many DA-related disorders (for review, see Miller et al., 1999). Indeed, DAT internalization is hypothesized as a primary mechanism underlying AMPH-induced DAT downregulation (see section VI.A.1). The degree to which facilitated removal of DAT from the membrane is responsible for reduced DA uptake during DA-related disorders is unclear.

IV. Vesicular Monoamine Transporter-2: Structure and Regulation

A. Vesicular Monoamine Transporter-2 Structure and Function

The VMAT was first identified and cloned in rats as the primary mediator of monoamine uptake into intracellular vesicles in 1992 (Erickson et al., 1992; Liu and Edwards, 1997), followed by identification of both isoforms (VMAT1 and VMAT2) in 1996 (Erickson et al., 1996). Neuroendocrine cells primarily express VMAT1, whereas neurons of the central, peripheral, and enteric nervous systems exclusively express VMAT2 (Erickson et al., 1996). VMAT2 is well conserved across mammals, with 96% sequence homology between rats and mice and 92% sequence homology between rats and humans (Takahashi and Uhl, 1997).

No VMAT2 crystal structure exists to date, so the structure–function relationship of VMAT2 has been primarily determined through mutagenesis and photoaffinity labeling studies. In the rat, VMAT2 is 515

amino acids long, with 12 transmembrane domains and a cytoplasmic N terminus and C terminus. The primary VMAT2 regulatory domains exist on the N terminus and C terminus, along with an intravesicular-linking region between the first and second transmembrane domains and the cytoplasmic linking region between the sixth and seventh transmembrane domains. The terminal domains are targets for phosphorylation, whereas the linking region between the first and second transmembrane domains is glycosylated.

Within the CNS, the VMAT2 is predominantly localized to small synaptic vesicles, with only a small portion localized to dense-core vesicles or structures of the endocytic pathway (Nirenberg et al., 1997b). As discussed in section VI.B, VMAT2 synaptic vesicle localization within presynaptic terminals can play an important role in monoamine uptake; for example, stimulant-induced relocalization strongly correlates with increases or decreases in VMAT2-mediated DA uptake and synaptic vesicle DA content (Brown et al., 2001b, 2002; Sandoval et al., 2002, 2003; Volz et al., 2007).

Once present on synaptic vesicles, VMAT2 functions as a stoichiometric antiporter, transporting two H^+ ions out of a vesicle to transport one monoamine molecule into a vesicle (Johnson et al., 1981; Knoth et al., 1981) (Fig. 2B). H^+-ATPase activity establishes the high intravesicular H^+ concentration upon which VMAT2 transport depends, and reducing H^+-ATPase function or increasing vesicular pH impairs VMAT2 transport activity (for review, see Sulzer et al., 2005; Fleckenstein et al., 2007). This can affect VMAT2 function: AMPH and its analogs may function as weak bases to disrupt the synaptic vesicle pH and contribute to VMAT2

dysfunction (Sulzer et al., 1993; Omiatek et al., 2013; for review, see Fleckenstein et al., 2007) (Fig. 3B).

B. Vesicular Monoamine Transporter-2 Regulation

Similar to DAT, VMAT2-mediated monoamine transport is regulated by post-translational modification, protein interactions, and presynaptic localization, although less is known about these processes. VMAT2 has been assumed to follow stages of the synaptic vesicle cycle in a manner similar to other neurotransmitter systems. Vesicles are trafficked to the plasma membrane, neurotransmitter is released into the synapse, vesicles are reformed through internalization, and neurotransmitter is transported from the cytoplasm back into these vesicles through membrane-integrated transporters. However, recent evidence indicates that VMAT2-associated synaptic vesicles are processed differently within the synaptic vesicle cycle than other neurotransmitter systems, indicating alternate pathways and mechanisms of VMAT2 vesicle cycling may exist (Onoa et al., 2010). In the midbrain, VMAT2 is internalized and vesicles are reformed faster, segregate to different terminal and axonal locations, and release at a lower rate than vesicles associated with the vesicular glutamate transporter (Onoa et al., 2010). The post-translational modifications, protein interactions, and sorting patterns of VMAT2 likely underlie the unique way in which these vesicles engage the synaptic vesicle cycle, as well as their response to different pharmacologic agents.

1. Post-Translational Modification. Phosphorylation and glycosylation are the primary VMAT2 post-translational modifications reported to date. VMAT2 is constitutively phosphorylated on both the N-terminal and
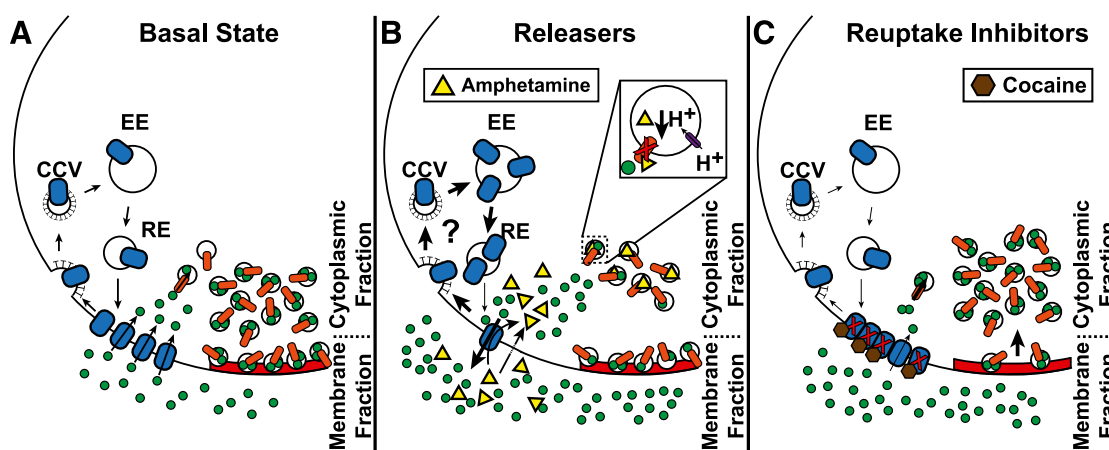
Fig. 3. The effects of stimulants on dopaminergic terminal function. (A–C) Exposure of dopaminergic terminals (A) to releasers (B) and reuptake inhibitors (C) alters the distribution and function of DAT and VMAT2. (B) Releasers function as DAT substrates and diffuse through the cytoplasmic membrane, leading to reversal of DA transport through the DAT. PKC-mediated DAT internalization may occur but has not been demonstrated in vivo. Releasers also reduce DA uptake into VMAT2 synaptic vesicles, by potentially functioning as a weak base to decrease vesicular pH or by binding VMAT2 directly, presumably increasing cytoplasmic DA. Finally, releasers reduce VMAT2 synaptic vesicles within the cytoplasmic fraction. (C) Reuptake inhibitors prevent DA transport via the DAT while also increasing DAT expression at the plasma membrane, increasing extracellular DA. In addition, reuptake inhibitors increase VMAT2 synaptic vesicles within the cytoplasmic fraction while reducing the number of vesicles in the membrane fraction, increasing DA content within striatal dopaminergic terminals. DAT, VMAT2, and DA are depicted as in Fig. 1. CCV, clathrin-coated vesicle; EE, early endosome; RE, recycling endosome.

C-terminal cytoplasmic regions. Although the precise role of phosphorylation is unclear, N-terminal phosphorylation is necessary for the maintenance of VMAT2 monoamine uptake, and perhaps for stimulant-induced monoamine efflux from synaptic vesicles (Krantz et al., 1997; Torres and Ruoho, 2014). The protein kinases involved in N-terminal phosphorylation have yet to be identified, but PKA and PKC are probable candidates, given the VMAT2 N-terminal structure. VMAT2 C-terminal phosphorylation by casein kinases I and II is important in retaining VMAT2 in large dense-core vesicles (Waites et al., 2001; Fei et al., 2008). Removal of C-terminal region phosphorylation by casein kinases results in VMAT2 trafficking into small synaptic vesicles instead of large dense-core granules (Waites et al., 2001). Thus, C-terminal modification may play an important in vivo role in localizing VMAT2 into monoaminergic synaptic vesicles. In addition to the terminal regions, several amino acids with kinase binding motifs exist on the cytoplasmic linking region between the sixth and seventh transmembrane domains, although no studies to date have evaluated their involvement in VMAT2 regulation.

N-linked glycosylation of the C terminus and linking region between the first and second transmembrane domains is responsible for VMAT2 vesicular targeting. VMAT2 is synthesized in the endoplasmic reticulum, glycosylated in the trans-Golgi network, and sorted into constitutive secretory vesicles or large dense-core vesicles in a glycosylation-dependent manner (Nirenberg et al., 1995, 1996a, 1997a). Glycosylation of both transmembrane regions is necessary to traffic VMAT2 into dense-core vesicles—the expression of each region alone is insufficient (Yao and Hersh, 2007). In the absence of C-terminal and linking domain glycosylation, VMAT2 is trafficked to small vesicles (Yao and Hersh, 2007). The majority of VMAT2 identified in DA synaptic terminals is present in small synaptic vesicles (Nirenberg et al., 1997a), indicating that only a small fraction of VMAT2 is glycosylated within the Golgi body and sorted into large dense-core vesicles within dopaminergic cell bodies. These large dense-core vesicles either remain within the cell body or are trafficked down axons to presynaptic terminals. Nonglycosylated VMAT2 is sorted into constitutive secretory vesicles within dopaminergic cell bodies. These secretory vesicles are then trafficked down axons and integrate into the plasma membrane, upon which internalization drives the formation of VMAT2 synaptic vesicles (Fig. 1). For further review of synaptic vesicle biogenesis and transporter trafficking, see Hannah et al. (1999) and Fei et al. (2008).

Of note, nitrosylation of the VMAT2 is reported to occur shortly after METH exposure and results in reduced vesicular DA uptake (Eyerman and Yamamoto, 2007). Inhibiting neuronal nitric oxide synthase activity prior to METH exposure prevents VMAT2 nitrosylation and subsequent loss of activity (Eyerman and Yamamoto, 2007), suggesting that reactive species modification of VMAT2 may alter function under at least some pathophysiological conditions.

2. Vesicular Monoamine Transporter-2–Protein Interactions. Relatively few direct VMAT2–protein interactions have been identified. Among these, complex formation between VMAT2, TH, and AADC has been described. TH and AADC, the rate-limiting and final enzymes in DA synthesis, respectively, directly interact with VMAT2 on synaptic vesicles isolated from dopaminergic brain regions (Cartier et al., 2010). The existence of these complexes suggests that DA is spatially restricted within the presynaptic terminal, because DA uptake into synaptic vesicles will occur through VMAT2 immediately after production. Since DA can be rapidly converted to reactive species within the oxidizing environment of the cytoplasm (see section VI.B), restricting cytoplasmic DA localization with these complexes may be important in preventing DA from damaging presynaptic terminals. Loss or disruption of these VMAT2-TH-AADC complexes may drive or enhance presynaptic terminal dysfunction. Trafficking and sorting may play an important role in forming VMAT2-TH-AADC complexes, which appear to reside only on a subset of vesicles.

VMAT2 also directly interacts with heat shock chaperone 70 (Hsc70), a heat-shock protein involved in the removal of clathrin-coats from vesicles that allows subsequent trafficking into the endocytic pathway (Requena et al., 2009). The exact role of Hsc70 in vesicle trafficking is unclear, but Hsc70 binding to VMAT2 synaptic vesicles leads to a significant reduction in monoamine uptake in both cell culture and synaptosome models. This suggests that VMAT2 function may be directly inhibited at specific stages of internalization and synaptic vesicle reformation or until particular vesicle components are in place (e.g., H^+-ATPase). Improper VMAT2 synaptic vesicle maturation or sorting, which occurs after exposure to stimulants (see section VI.B.3), may therefore impair transporter function.

3. Presynaptic Localization. The distribution of VMAT2 within different synaptic vesicle populations, and perhaps within discrete regions of the presynaptic terminal, is closely related to transporter function. As further discussed in section VI.B.3, psychostimulants differentially redistribute VMAT2 between membrane-associated and cytoplasmic crude synaptic vesicle fractions, which differentially alters VMAT2 uptake and vesicular DA content. Whether VMAT2 redistribution is the cause or a consequence of reduced VMAT2 uptake is unclear, but presynaptic localization plays a critical role in regulating the capacity of VMAT2 to sequester monoamines.

Finally, it has been suggested that synaptic vesicles are regulated in a similar manner across neurotransmitter systems. Recent evidence, however, indicates

significant differences between the vesicle cycles of VMAT2 compared with the vesicular glutamate transporter (Onoa et al., 2010). The susceptibility of different neurotransmitter systems to various drugs and disorders may be due, in part, to variance in synaptic vesicle trafficking, exocytosis, endocytosis, and sorting that indirectly affects neurotransmitter reuptake and release. Thus, this is an important area for future investigation.

V. Dopamine Transporter, Vesicular Monoamine Transporter-2, and Disorders of the Central Nervous System

Dysregulation of monoamine systems plays a critical role in a variety of disease states (for reviews, see Seeman and Niznik, 1990; Fiorino et al., 1993; Nestler and Carlezon, 2006; Swanson et al., 2007; Koob and Volkow, 2010). The following sections include a description of some commonly investigated diseases and the putative role of DAT and VMAT (for examples of other diseases such as autism and bipolar disorder, see Grünhage et al., 2000; Nakamura et al., 2010; Bowton et al., 2014; Nguyen et al., 2014).

A. Attention Deficit Hyperactivity Disorder. ADHD is a common neuropsychiatric disorder characterized by symptoms of inattention, hyperactivity, and impulsivity that may contribute to cognitive and behavioral impairment. Approximately 9% of American children aged 13–18 years (Merikangas et al., 2010) and 4.1% of American adults aged ≥18 years (Kessler et al., 2005) are affected by ADHD. Although the etiology of the disorder is not well understood, several lines of evidence implicate dysregulation of DA systems in ADHD (for reviews, see Swanson et al., 2007; Volkow et al., 2007a,b, 2009). For instance, the impact of DAT genetic variation on behavioral changes underlying mental illness stems from pioneering studies of the DAT knockout mouse, which demonstrated that genetic elimination of DAT expression reduces presynaptic DA stores, elevates extracellular DA, and produces hyperactivity in a novel environment (Giros et al., 1996). However, humans that are homozygous for loss-of-function DAT (SLC6A3) alleles present with a complex disorder that resembles PD, rather than ADHD (see section V.B; Kurian et al., 2011; Ng et al., 2014). Instead, clinical studies that genetically screen individuals with ADHD, as well as preclinical studies using cell lines and genetic mouse models, have suggested that variations in the genes encoding for DAT, rather than simple loss of function, and/or DA receptors are associated with risk for ADHD (Gill et al., 1997; Bobb et al., 2005; Mazei-Robison et al., 2005; Sakrikar et al., 2012; Mergy et al., 2014). Importantly, only a few human case studies of DAT variants have been implicated in ADHD to date. Nonetheless, a further link between DAT function and ADHD is suggested from the therapeutic utility of drugs with actions at DAT, such as methylphenidate (MPD) and AMPH, which are clinically effective in treating children, adolescents, and adults with ADHD. Finally, evidence from brain imaging studies, albeit mixed, indicates that DAT levels and DA signaling may be altered in patients with ADHD (Dougherty et al., 1999; Krause et al., 2000; Volkow et al., 2007a,b, 2009), although other transmitter systems are also likely to play an important role.

Although it is unclear how drugs that work in part by targeting DAT treat symptoms of ADHD, these drugs may help normalize brain function within the limbic and frontal cortex circuit (Rubia et al., 2009). In untreated children with ADHD, MPD increased fronto-striato-parieto-cerebellar activation as well as decreased activation in the orbitofrontal and superior temporal regions during a rewarded continuous performance task (Rubia et al., 2009). Compared with medication-naïve subjects with ADHD, patients with ADHD who had received long-term stimulant treatment also had increased activation of the right caudate during an attentional control task and had similar activation to that of control subjects (Hart et al., 2013). To the extent that altered brain circuitry contributes to ADHD, these findings suggest that stimulant medication may help normalize corticostriatal function.

Finally, stimulant medications have been a first-line treatment of ADHD for over 50 years (Wigal et al., 2010) and are clinically effective in treating symptoms. However, there is concern that these medications have high potential for misuse and abuse among certain populations (e.g., college students; for review, see Weyandt et al., 2014). Thus, emerging research is investigating how other novel drugs that target DAT might increase the therapeutic effects and decrease unwanted side effects (i.e., abuse), such as atypical DAT ligands (see section VI.A.2; for review, see Reith et al., 2015). For example, modafinil, an atypical DAT inhibitor, is suggested to have relatively little potential for abuse because it interacts with DAT in a different manner compared with drugs such as MPD (i.e., modafinil stabilizes the closed conformation; see Schmitt and Reith, 2011). Furthermore, modafinil has been demonstrated to be clinically effective and a viable alternative compared with traditional stimulant medication (e.g., Adderall; Shire, Lexington, MA) for the treatment of ADHD (Taylor and Russo, 2000; Turner, 2006). These studies highlight the notion that understanding atypical ligands and their interactions with DAT might lead to improved medications for other disorders linked to perturbations in dopaminergic systems, such as substance abuse (Tanda et al., 2009b; Loland et al., 2012).

B. Parkinson's Disease. PD is a neurodegenerative disorder affecting 3% of people aged ≥65 years (Gillies and McArthur, 2010). This disorder is characterized by a progressive loss of nigrostriatal dopaminergic neurons that results in bradykinesia, rigidity, resting tremor,

and postural instability (Halliday and McCann, 2010). PD is treated by administration of L-DOPA or dopaminergic agonists. Postmortem studies suggest that 30%–50% of dopaminergic cell bodies are lost in the substantia nigra at the time motor symptoms appear (Fearnley and Lees, 1991; Ishibashi et al., 2014) and imaging studies of human patients with PD have shown decreased DAT levels beginning in the posterior putamen (Ishibashi et al., 2014). Furthermore, the disorder is also associated with the accumulation of Lewy bodies containing α-synuclein within the substantia nigra pars compacta (Braak et al., 2003).

Multiple genes have been implicated in the heritable form of PD, including a mutation of the α-synuclein (SNCA) gene (Spatola and Wider, 2014), which is thought to be involved in the production of α-synuclein, a major protein constituent of PD Lewy bodies. This heritable form of PD is associated with earlier onset and aggressive progression (Polymeropoulos et al., 1997). Although the role of α-synuclein in regulating DAT function is poorly defined, α-synuclein appears to recruit and stabilize DAT at the plasma membrane and to enhance V_{max} under normal conditions (Fountaine and Wade-Martins, 2007). How the interaction between α-synuclein and DAT may contribute to dopaminergic neuronal disorders in PD is less clear, but α-synuclein overexpression has been proposed to cluster and locally accelerate DA uptake, leading to oxidative stress and terminal damage (Lee et al., 2001). In cell models, the ubiquitin ligase Parkin disrupts this DAT–α-synuclein interaction, protecting against α-synuclein–induced dopaminergic toxicity (Moszczynska et al., 2007). In addition, loss-of-function mutations of the human SLC6A3 gene that encodes DAT have been linked to infantile parkinsonism-dystonia (also known as DAT deficiency syndrome; Kurian et al., 2009, 2011), which is a severe neurologic syndrome that usually presents in early infancy with hypokinetic parkinsonism (Assmann et al., 2004). Of note, recent studies have shown evidence that DAT missense mutations can also lead to later-onset DAT deficiency syndrome and parkinsonism in adolescents and adults (Hansen et al., 2014; Ng et al., 2014). Decreases in VMAT2 within the striatal brain regions as assessed by [^{11}C]dihydrotetrabenazine (DHTBZ) binding have also been reported in patients with PD (de la Fuente-Fernández et al., 2011). Postmortem studies have shown that VMAT2 function is decreased in humans with PD, even after correcting for DA nerve terminal loss (Pifl et al., 2014). This is in contrast with preclinical models of PD induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in which the decrease in vesicular DA uptake was attributable to a loss of terminals (Pifl et al., 2014). Finally, mutations in human SLC18A3, the gene that encodes VMAT2, have been associated with infantile parkinsonism-dystonia, further suggesting a role for this transporter in complex movement disorders (Rilstone et al., 2013).

As reviewed by Bernstein et al. (2014), several classes of environmental toxicants, including pesticides, polychlorinated biphenyls, and brominated flame retardants, have been associated with PD. These compounds induce damage by disrupting vesicular and synaptosomal DA uptake and thus promoting reactive species formation. As discussed in section VI.B.3, pharmacological manipulation of DAT and VMAT2 function (e.g., MPD) may be critical for the development of therapies to slow the progression of PD.

C. Stimulant Abuse. Stimulant drug abuse is the second most common form of substance abuse according to the World Health Organization (http://www.who.int/substance_abuse/facts/psychoactives/en/), and it is a major public health problem. Loss and/or dysfunction of the DAT is well identified in humans with a history of stimulant abuse. Individuals that abuse METH display a 15%–25% decrease in caudate DAT densities compared with control subjects, as assessed via positron emission tomography imaging (Volkow et al., 2001; Johanson et al., 2006; Chang et al., 2007), and these decreases persist for years after abstinence (McCann et al., 1998). Furthermore, epidemiologic studies suggest that individuals who abuse METH/AMPH may be more likely to develop PD (Callaghan et al., 2012; Curtin et al., 2015). Reports of changes in VMAT2 expression are mixed and are likely attributable to differences in drug use or time abstinent across studies. A postmortem study of human METH abusers reported no significant change in VMAT2 levels (Wilson et al., 1996). However, more current imaging studies in recently abstinent METH-abusing subjects demonstrated increased VMAT2 levels in the caudate, putamen, and ventral striatum compared with control subjects (Boileau et al., 2008), and VMAT2 levels were decreased in METH-abusing subjects that had been abstinent for at least 3 months (Johanson et al., 2006).

Clinical data investigating the effects of cocaine on DAT and VMAT2 have also yielded mixed results. Some reports demonstrate reduced DAT and VMAT2 protein levels, but normal DAT binding (Wilson et al., 1996), in cocaine-abusing subjects postmortem (Wilson et al., 1996; Little et al., 2003). Recently abstinent cocaine users also have reductions in dihydrotetrabenazine binding of VMAT2 in vivo (Narendran et al., 2012). Interestingly, recent data indicate that cocaine abuse, in contrast with METH/AMPH abuse, does not lead to PD (Curtin et al., 2015). Furthermore, other studies report increased DAT levels in striatal regions of recently abstinent cocaine users or postmortem cocaine-abusing subjects, compared with control subjects (Mash et al., 2002; Crits-Christoph et al., 2008). In addition, DAT function as assessed by [^{3}H]DA uptake is elevated postmortem in cocaine-abusing subjects (Mash et al., 2002). Overall, these findings highlight the need for further work to determine the effects of cocaine abuse and abstinence on DAT and VMAT2 expression and function.

To date, there is no U.S. Food and Drug Administration–approved pharmacological treatment for stimulant addiction (Yahyavi-Firouz-Abadi and See, 2009; Kufahl and Olive, 2011). However, targets for the DAT and VMAT2 may hold potential therapeutic value. Numerous clinical trials have investigated the ability of DA reuptake inhibitors and releasers to treat cocaine AMPH dependencies. At least some studies demonstrate that DAT inhibitors are more effective than DA releasers for reducing use of DA releasers (Grabowski et al., 1997; Tiihonen et al., 2007), whereas DA releasers are more effective than DAT inhibitors for reducing use of DA inhibitors (Grabowski et al., 2001; Mooney et al., 2009; Longo et al., 2010; Galloway et al., 2011). For example, the DAT inhibitor MPD has been tested as a putative agonist replacement therapy for cocaine and METH/AMPH dependence. Although clinical trials testing MPD substitution as a treatment for cocaine dependence are generally negative (see Grabowski et al., 1997; Schubiner et al., 2002), MPD may be effective for managing AMPH use disorders (Tiihonen et al., 2007; Ling et al., 2014). Abstinence was not achieved in many of these participants, however, suggesting that DAT inhibitors may hold some therapeutic potential but other DAT and VMAT2 pharmacological manipulations, as discussed below, should be explored for stimulant abuse treatment (e.g., atypical compounds; see section VI.A.2). Finally, drugs that mimic the neuropeptide neurotensin, a modulator of DA system activity, reduce animal self-administration of stimulants such as AMPHs and cocaine and are potential agents for the treatment of stimulant dependence (Richelson et al., 2003; Hanson et al., 2013). Although these neurotensin receptor agonists have been shown to block stimulant-induced increases in extracellular DA, their effects on DAT and VMAT2 function have not been reported.

VI. Pharmacological Manipulation of the Dopamine Transporter and Vesicular Monoamine Transporter-2

The structure, function, and mechanisms regulating DAT and VMAT2 are exciting and expanding fields; however, much remains to be learned regarding how these mechanisms regulate transporter function in vivo. Investigating the role of drugs in affecting DAT and VMAT2 function and expression in vivo is important for understanding variances in response to therapeutic use, abuse-related effects, and the development of novel, more effective compounds. The following section provides an overview of the current knowledge regarding interactions between various pharmacological agents and DAT and VMAT2 processes, with suggestions for opportunities for future study.

A. Pharmacological Regulators of the Dopamine Transporter

1. Releasers and Reuptake Inhibitors. Psychostimulants that affect DAT, such as AMPH and cocaine analogs, have been historically classified based on their mechanism of action as either reuptake inhibitors or substrate-type (e.g., DA) releasers. Reuptake inhibitors (e.g., MPD and cocaine) bind to DAT and prevent the reuptake of DA into neurons, thereby increasing extracellular DA concentration (Heikkila et al., 1975; Ritz et al., 1987; Nicolaysen and Justice, 1988). By contrast, releasers (e.g., AMPH analogs) elevate extracellular DA levels by reversing the process of transporter-mediated exchange, thereby enhancing DA efflux (Liang and Rutledge, 1982; Jones et al., 1999; for review, also see Sulzer et al., 2005; Fleckenstein et al., 2007).

Altering the post-translational modifications and protein interactions that occur on the DAT N-terminal and C-terminal domains is a primary target for drug-induced regulation of DAT function. N-terminal DAT phosphorylation is suggested to be involved in AMPH-induced DA efflux (Khoshbouei et al., 2004) through an interaction with phosphatidylinositol (4,5)-bisphosphate (Hamilton et al., 2014). Interaction of calmodulin protein kinase II with the DAT C terminus has also been demonstrated, both in vitro and in vivo, to be involved in AMPH-induced DA efflux (Fog et al., 2006; Rickhag et al., 2013b). Novel therapeutic agents that can selectively target and enhance or prevent N-terminal or C-terminal DAT modifications or interactions may modulate the effects of releasers and reuptake inhibitors.

In vitro findings indicate that DAT inhibitors and releasers differentially affect DAT cell surface expression. Reuptake inhibitors, such as cocaine, rapidly increase DAT cell surface expression (approximately 10-minute incubation; Saunders et al., 2000; Daws et al., 2002) (Fig. 3C). By contrast, releasers, such as AMPH, biphasically alter DAT surface expression. AMPH increases DAT cell surface expression within the first minutes of exposure (1–3 minutes), followed by DAT internalization and accumulation within endocytic structures (15–60 minutes; Saunders et al., 2000; Johnson et al., 2005; Furman et al., 2009; Richards and Zahniser, 2009) (Fig. 3B). PKC plays a well established role in basal DAT trafficking and function (Vaughan et al., 1997); however, the role of PKC in drug-induced DAT relocalization is an area of current investigation. In vitro, PKC inhibitors block METH-induced downregulation of DAT expression and function (Sandoval et al., 2001; Cervinski et al., 2005) and attenuate AMPH-stimulated DA release (Kantor and Gnegy, 1998). At a behavioral level, PKC knockout mice are less sensitive to the locomotor effects of AMPH, compared with wild-type mice. Taken together, these data suggest that PKC may be an

important regulator of stimulant-induced alterations in DAT function (Fig. 3B). Whether this is due to DAT internalization or modification of DAT on the membrane is unclear.

DAT inhibitors and releasers also differentially affect in vivo DAT function. In synaptosomes prepared from drug-treated rats (noting that this effect is not due to residual drug introduced by the original subcutaneous injection), in vivo METH administration rapidly (within 1 hour) decreases DAT function, a property shared by numerous other releasers such as AMPH and methylenedioxymethamphetamine (Fleckenstein et al., 1997b, 1999). By contrast, in vivo administration of reuptake inhibitors has no effect on (e.g., MPD), or slightly increases (e.g., cocaine), DAT function (Fleckenstein et al., 1997b) as assessed in this preparation. The differences among psychostimulants in altering in vivo DAT function, along with other dopaminergic markers, plays an important role in the development of neurotoxicity (for review, see Fleckenstein et al., 2000).

Limited in vivo evidence supports stimulant-induced DAT internalization. On the contrary, one recent study failed to demonstrate a change in DAT plasma membrane expression after exposure to a broad range of in vivo AMPH or METH treatment paradigms (German et al., 2012). Likewise, neither Richards and Zahniser (2009) nor Moszczynska et al. (2007) observed DAT internalization after in vivo AMPH/METH administrations. Nonetheless, AMPH analogs rapidly decrease in vivo DAT function, as assessed in synaptosomes prepared from treated rats (Fleckenstein et al., 1997b). Taken together, these data suggest that AMPH/METH-induced internalization may not be responsible for reduced DAT function in vivo. Instead, DAT modification at the plasma membrane, perhaps through PKC or oxidative inactivation as described in section III.B.1, may underlie the AMPH analog-induced loss of DAT function observed in vivo. How the underlying mechanisms regulating DAT (e.g., post-translational modifications and protein interactions) are differentially affected by releasers and inhibitors, and whether these correspond to differences in DAT function and expression associated with these drugs, is an expansive area for future research.

Finally, recent voltammetry studies suggest additional differences between the effects of AMPH and cocaine. In particular, both cause phasic DA activation through augmented vesicular DA release and increased burst firing. However, these agents differ in their effect on tonic activation. For example, cocaine, but not AMPH, requires action potential-dependent mechanisms (for discussion, see Covey et al., 2013).

2. Atypical Dopamine Transporter Reuptake Inhibitors and Partial Substrate Releasers. Because DAT inhibitors and releasers both increase extracellular DA concentration, it was originally assumed that all drugs interacting with DAT would induce behavioral effects identical to a prototypical DAT inhibitor such as cocaine (e.g., readily self-administered, locomotor-stimulating properties, and shared discriminative properties). Although a multitude of DAT inhibitors that have cocaine-like properties exist (e.g., RTI55 [methyl-(1*R*,2*S*,3*S*)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1] octane-2-carboxylate] and WIN35428 [methyl-(1*R*,2*S*,3*S*,5*S*)-3-(4-fluorophenyl)-8-methyl- 8-azabicyclo [3.2.1]octane-2-carboxylate]), atypical DAT inhibitors (e.g., analogs of benztropine, modafinil) exhibit different behavioral properties, especially in humans (Søgaard et al., 1990; Carroll et al., 2009; Vosburg et al., 2010). One problem with using traditional DAT inhibitors for stimulant abuse treatment is that these inhibitors also have abuse liability and the potential to enhance the effects of abused drugs (see Barrett et al., 2004; Hiranita et al., 2011). Thus, atypical compounds that may not have these effects are novel therapeutics of interest. For example, several potent atypical DAT inhibitors, such as JHW007 [(3-endo)-3-[bis(4-fluorophenyl)methoxy]-8-butyl-8-azabicyclo[3.2.1]octane hydrochloride] and JHW013 [n-cyclopropyl methyl-3α-[bis(4′-fluorophenyl)methoxy]-tropane], are not readily self-administered in animal models (Desai et al., 2005b; Li et al., 2011), demonstrating that the reinforcing effects of a drug are not solely a property of actions at DAT or that these inhibitors have a different manner of interaction with DAT (see below). Furthermore, several atypical DAT inhibitors, including JHW007, antagonize various effects of cocaine and METH/AMPH, including locomotor activity (Desai et al., 2005b; Velázquez-Sánchez et al., 2013), increases in extracellular DA concentrations (Tanda et al., 2009a), and self-administration (Hiranita et al., 2009, 2014).

Several hypotheses have been suggested for mechanisms underlying the effects of atypical compounds and include actions on non-DA systems (Hiranita et al., 2011), rate of DAT occupancy (Desai et al., 2005a,b), and the conformational state of DAT (Loland et al., 2008). For instance, whereas cocaine and its analogs bind and induce an outward-facing DAT conformation, atypical DAT inhibitors promote an inward-facing DAT conformation (Reith et al., 2001; Loland et al., 2008; Hong and Amara, 2010). These studies underscore the importance of understanding how compounds interact with DAT and that atypical DAT compounds might serve as exciting leads for potential medications for psychostimulant addiction (Dutta et al., 2003; Carroll et al., 2006).

Recent investigation of substrate-type releasers has identified compounds with unique partial substrate properties (Rothman et al., 2012). Similar to the prototypical DAT releasers (e.g., AMPH, METH), these partial substrate releasers (e.g., PAL-1045 [(*S*)-*N*-ethyl-1-(2-naphthyl)propan-2-amine], PAL-193 [XXXX], PAL-738 [XXXX]) are transported by DAT but are significantly less effective at inducing reverse transport

(i.e., DAT-mediated substrate efflux). For example, PAL-1045 was unable to fully elicit DAT-mediated efflux of the labeled substrate [^{3}H]1-methyl-4-phenylpyridinium (MPP+) (E_{max} of approximately 65%) compared with a full substrate releaser (Rothman et al., 2012). These authors speculate that partial releasers are less effective at promoting the conformational changes in the DAT protein required for the overall process of alternating exchange (Rothman et al., 2012).

The identification of novel ligands as atypical inhibitors and partial substrates has challenged the notion that all ligands acting at DAT are functionally homogeneous and that perhaps both the behavioral and neurochemical effects of a particular ligand are contingent on how it interacts with the transporter (for review, see Schmitt et al., 2013; Reith et al., 2015). Although the molecular mechanisms underlying these compounds are poorly understood, DAT ligands with different chemical structures exert unique conformational effects on the transporter, stabilizing the protein in different structural states (Reith et al., 2001; Loland et al., 2008; Schmitt and Reith, 2011) that might influence the effects of each compound. An important new avenue of research involves whether these novel atypical ligands affect modifications, protein interactions, and relocalization events that affect DAT function differently than typical ligands. Further study of how these novel compounds interact with transporters might lead to improved medications for disorders linked to perturbations in dopaminergic systems, such as substance abuse and PD.

3. Dopamine Receptors. DA interacts with five G protein–coupled receptors that are divided into two families. Receptors of the D_1-like family (D_1 and D_5 receptors) are predominantly excitatory and postsynaptic (Huang et al., 1992; Cameron and Williams, 1993; Levey et al., 1993). Receptors of the D_2-like family (D_2, D_3, and D_4 receptors) are predominantly inhibitory and are localized both presynaptically and postsynaptically, and D_2-like autoreceptors regulate presynaptic DA activity.

DA receptors are important regulators of DAT function. For instance, quinpirole, a nonselective D_2/D_3 agonist, reduces extracellular DA concentration via acute upregulation of DAT function (Meiergerd et al., 1993). This effect is reversed by a nonselective D_2/D_3 receptor antagonist, further indicating that quinpirole-induced upregulation of DAT function is mediated by D_2/D_3 receptors. Similarly, the more selective D_3 receptor ligand, PD128907 [(4aR,10bR)-3,4a,4,10b-tetrahydro-4-propyl-2H,5H-[1]benzopyrano-[4,3-b]-1,4-oxazin-9-ol hydrochloride, a D_3-preferring agonist] increases electrochemically measured DA clearance (Zapata and Shippenberg, 2002). Taken together, in combination with binding studies (Mayfield and Zahniser, 2001), these data suggest that activation of D_2/D_3 receptors triggers rapid trafficking of intracellular DAT

to the plasma membrane. Moreover, D_2/D_3 receptor agonist–induced upregulation of DAT function is blocked by pertussis toxin, indicating the involvement of G_i/G_o protein–coupled signaling (Mayfield and Zahniser, 2001).

In addition to evidence that DA receptors directly regulate DAT and thus the amount of extracellular DA, DA receptors mediate the early decreases in DAT function caused by METH. Pretreatment with either a D_1 or a D_2 antagonist attenuates the acute decrease in DAT activity caused by METH (Metzger et al., 2000). D_2 antagonist pretreatment attenuates the formation of reactive oxygen species–associated high molecular weight DAT complexes as well (Hadlock et al., 2010). Further findings that METH causes reactive oxygen species formation (Giovanni et al., 1995; Fleckenstein et al., 1997a) and that DAT is vulnerable to oxidative modification (Berman et al., 1996; Fleckenstein et al., 1997a; Haughey et al., 1999) suggest an interaction among these factors that may serve as a target for pharmacological intervention (for further review, see Fleckenstein et al., 2000, 2007).

4. Insulin. Insulin provides hormonal inputs to homeostatic metabolic systems (Figlewicz, 2003); however, emerging data indicate that it also directly affects DA systems (for reviews, see Daws et al., 2011; Niswender et al., 2011; Baladi et al., 2012). Insulin receptors are expressed in brain regions that are rich in DA neurons, receptors, and transporters (Figlewicz et al., 2003), and insulin, via the phosphoinositide 3-kinase/Akt signaling pathway (Williams et al., 2007), regulates the expression and activity of DAT. Acute administration of insulin enhances DAT activity and expression, whereas rats made hypoinsulinemic (e.g., via pharmacological agents or feeding conditions) have decreased DAT activity and expression (Figlewicz et al., 1994; Patterson et al., 1998; Carvelli et al., 2002; Owens et al., 2005; Sevak et al., 2008). Moreover, manipulating insulin levels (pharmacologically or via feeding conditions) can decrease AMPH-induced DA efflux through DAT (Williams et al., 2007) and can dramatically alter the behavioral effects of drugs acting at DAT, such as cocaine and METH (McGuire et al., 2011; Baladi et al., 2012, 2015).

The importance of understanding interactions between insulin, the DA systems, and drugs is highlighted by comorbidities such as eating disorders and drug abuse (Wiederman and Pryor, 1996; Franko et al., 2008), diabetes and schizophrenia (Mukherjee et al., 1996; Dixon et al., 2000), diabetes and tobacco use (Facchini et al., 1992; Cho et al., 2009), and obesity and depression (Faith et al., 2002; McElroy et al., 2004; Simon et al., 2006). Understanding the relationship between insulin and drug response could provide important keys to understanding the individual differences in response to therapeutic drugs and in vulnerability to drug abuse.

5. Estrogen. A multitude of preclinical and clinical studies demonstrate that the effects of drugs, including those acting at DAT, differ between females and males (for reviews, see Lynch et al., 2002; Anker and Carroll, 2011). Although numerous mechanisms may account for differences in the effects of drugs between females and males, hormones such as estrogen likely play a role. Estrogens are well known to act through intracellular receptors and regulate gene transcription. Interestingly, emerging data suggest that estrogens can affect proteins at the plasma membrane that can be dynamically regulated over short time frames (Watson et al., 2006). For example, estrogen can regulate DAT function and expression via rapid, transcription-independent mechanisms (Bossé et al., 1997; Disshon et al., 1998; Watson et al., 2006; Chavez et al., 2010). Estrogen may also activate protein kinases (including PKC) via transcription-independent pathways (Watson and Gametchu, 1999), which may also affect DAT trafficking and function. Understanding the mechanistic role for estrogens in modulating DAT might have implications for the etiology and treatment of disorders involving the dysregulation of DA systems in female patients.

B. Pharmacological Manipulation of Vesicular Monoamine Transporter-2

The development of novel VMAT2 ligands has been hindered by the lack of high-throughput screens (Bernstein et al., 2014). Hence, some laboratories are developing whole-cell fluorescent assays for VMAT transport function that have important advantages including the use of an intact cell (versus isolated vesicles that may not recapitulate the cell signaling). This technology holds significant promise for identifying ligands that may not directly interact with the VMAT2/tetrabenazine (TBZ) binding site but may still alter VMAT2 function. The following section describes the current compounds that directly interact with VMAT2 as well as other compounds that have indirect actions at the transporter (e.g., METH, MPD).

1. Reserpine and Tetrabenazine. Reserpine and TBZ are "classical" VMAT2 inhibitors (Pletscher, 1977) that prevent the transport of amines into synaptic vesicles. Although these agents are the most widely studied, their clinical utility is relatively limited. Reserpine binds irreversibly and thus can have effects lasting days or even weeks. Furthermore, reserpine and TBZ deplete both DA- and serotonin-containing vesicles, leading to symptoms including gastrointestinal distress, depression, and fatigue. Radiolabeled TBZ and related derivatives with high affinity for VMAT2 are used as imaging tools, especially in relation to diagnosis and understanding of several disease states, such as PD, Huntington's disease, bipolar disorder, and schizophrenia (Zubieta et al., 2000, 2001; Frey et al., 2001; Suzuki et al., 2001; Bohnen et al., 2006). TBZ is approved by the U.S. Food and Drug Administration for symptomatic treatment of Huntington's disease, which is characterized, in part, by dysregulation of dopaminergic neurotransmission (Frank and Jankovic, 2010).

2. Lobeline and Analogs. Lobeline, the major alkaloid in the plant genus *Lobelia*, is a potent inhibitor of the VMAT2. Lobeline has received considerable attention, owing to preclinical demonstrations that this agent inhibits both METH-induced striatal DA release and rat self-administration. This effect may be due, in part, to the ability of lobeline to decrease the availability of presynaptic DA for reverse transport through DAT (Harrod et al., 2001). Interestingly, and despite findings that pretreatment with VMAT2 inhibitors such as reserpine worsens METH-induced dopaminergic deficits, lobeline protects against such deficits (Eyerman and Yamamoto, 2005) presumably by causing a redistribution of VMAT2 resembling that afforded by MPD (see discussion below).

The clinical development of lobeline as a treatment for METH abuse was hindered, in part, by its significant affinity for nicotinic acetylcholine receptors. Its analog, lobelane, also inhibits the neurochemical and behavioral effects of METH; however, development of this agent as a therapy is confounded by the development of tolerance to its ability to decrease METH self-administration. More recent attention has focused on the lobelane analog, GZ-793A [(R)-3-[2,6-*cis*-di(4-methoxyphenethyl)piperidin-1-yl]propane-1,2-diol hydrochloride], which inhibits both the behavioral and neurochemical effects of METH, without exhibiting tolerance. These and other studies by Dwoskin, Crooks, and coworkers suggest that selective VMAT2 inhibitors provide the requisite preclinical behavioral profile for evaluation as pharmacotherapeutics for METH abuse, particularly utilizing agents that are selective for VMAT2 relative to DAT (for review, see Nickell et al., 2014).

3. Methamphetamine, Methylphenidate, and Dopaminergic Agonists. AMPH and related amphetamines disrupt VMAT2 transport of DA into synaptic vesicles through at least two proposed mechanisms. First, AMPH-related drugs are membrane-permeable weak bases that disrupt intravesicular pH, diminishing the proton gradient on which VMAT2 depends for antiport function and reducing DA uptake into synaptic vesicles (for extensive discussion of the weak base hypothesis, see Sulzer, 2011). Second, amphetamines bind the transporter directly (Peter et al., 1994), preventing substrate interaction with the transporter or potentially functioning as substrates themselves. The involvement of transporter binding in AMPH-induced VMAT2 dysfunction is supported by two observations. First, AMPH reduces VMAT2 function to a greater degree than proton gradient disruption would predict (Reith and Coffey, 1994). Second, treatment of synaptic vesicles with bafilomycin, a non-VMAT substrate proton

pump inhibitor, decreases vesicular pH to a greater degree than AMPH but only causes one-half of the DA release observed with AMPH (Floor and Meng, 1996).

Intracellular localization may also play an important role in regulating VMAT2 function, which is differentially altered by the releaser and reuptake inhibitor stimulants. Brown et al. (2000) reported that repeated high-dose administrations of METH rapidly (within 1 hour) decrease VMAT2 function, as assessed in nonplasmalemmal membrane–associated (presumably cytoplasmic) vesicles prepared from treated rats (Fig. 3B). This effect was not due to residual METH introduced by the parenteral administration. Rather, this effect was associated with a redistribution of VMAT2 protein, and presumably associated vesicles, from the cytoplasmic fraction to a subcellular compartment not retained by the differential centrifugation used to prepare these vesicles (Riddle et al., 2002). Importantly, VMAT2 immunoreactivity in the whole homogenate is not decreased 1 hour after binge METH treatment (Fleckenstein et al., unpublished observations), indicating that protein degradation is not responsible for the fractional loss of immunoreactivity in the whole synaptosomal and vesicle-enriched fractions at this time point (see also Hogan et al., 2000). This decrease in VMAT2 function corresponds with a decrease in VMAT2 sequestration capacity (Chu et al., 2010). It is hypothesized that the resulting decrease in DA sequestration capacity causes aberrant cytoplasmic DA accumulation and reactive oxygen species formation, which, in turn, contributes to a persistent loss of dopaminergic nerve terminals.

By contrast, the DA reuptake inhibitor MPD increases VMAT2 immunoreactivity and function in this same vesicle fraction via D_1 and D_2 receptor–mediated mechanisms (Sandoval et al., 2002). Furthermore, MPD post-treatment attenuates the persistent dopaminergic deficits caused by METH, presumably by compensating for the METH-induced loss of DA sequestration capacity (Sandoval et al., 2003). Post-treatment with the DAT reuptake inhibitor amfonelic acid protects against METH-induced dopaminergic deficits as well (Marek et al., 1990). Of related interest are findings that administration of nonselective D_2/D_3 receptor agonists increases VMAT2-mediated uptake, DHTBZ binding, and VMAT2 immunoreactivity (Brown et al., 2001a; Truong et al., 2003, 2004). Given the presumed role for decreased VMAT2 function in the dopaminergic deficits associated with PD (see discussion above), it has been suggested that treatment with reuptake inhibitors such as MPD or D_2/D_3 agonists may slow the neurodegenerative processes associated with the disorder.

These findings, as well as differences in the effect of reuptake inhibitors and releasers on VMAT2 function, likely contribute mechanistically to preclinical findings that releasing agents generally cause persistent dopaminergic deficits, whereas reuptake inhibitors are largely devoid of this property (for review, see Riddle et al., 2005).

4. Pituitary Adenylyl Cyclase Activating Polypeptide 38. Pituitary adenylyl cyclase activating polypeptide 38 (PACAP38) is a brain-gut peptide that is neuroprotective in several models of neurodegeneration. PACAP38 has several biologic functions, including increasing VMAT2 expression and function. Importantly, pretreatment with PACAP38 attenuates the persistent dopaminergic deficits caused by METH (Guillot et al., 2008) and those in several models of PD, although multiple mechanisms of action likely underlie this neuroprotective effect (for review, see Lee and Seo, 2014).

VII. Summary

In vitro studies have provided important insights into subcellular mechanisms whereby the structure and function of DAT and VMAT2 can be modified. Furthermore, emerging data concerning binding pockets, conformational alterations, and critical residues important for the functions of these transporters have led to a variety of novel targets for pharmacological manipulation. In vivo studies have and will continue to provide important extensions of this work as the translational relevance of potential therapeutics is evaluated. Pharmacological approaches ranging from atypical ligands to post-translational modifiers to agents that regulate vesicular trafficking hold significant promise for the development of novel therapeutics.

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