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A Closer Look at Amphetamine-Induced Reverse Transport and Trafficking of the Dopamine and Norepinephrine Transporters

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Abstract Amphetamine (AMPH) and its derivatives are regularly used in the treatment of a wide array of disorders such as attention-deficit hyperactivity disorder (ADHD), obesity, traumatic brain injury, and narcolepsy (Prog Neurobiol 75:406–433, 2005; J Am Med Assoc 105:2051–2054, 1935; J Am Acad Child Adolesc Psychiatry 41:514–521, 2002; Neuron 43:261–269, 2004; Annu Rev Pharmacol Toxicol 47:681–698, 2007; Drugs Aging 21:67–79, 2004). Despite the important medicinal role for AMPH, it is more widely known for its psychostimulant and addictive properties as a drug of abuse. The primary molecular targets of AMPH are both the vesicular monoamine transporters (VMATs) and plasma membrane monoamine—dopamine (DA), norepinephrine (NE), and serotonin (5-HT)—transporters. The rewarding and addicting properties of AMPH rely on its ability to act as a substrate for these transporters and ultimately increase extracellular levels of monoamines. AMPH achieves this elevation in extracellular levels of neurotransmitter by inducing synaptic vesicle depletion, which increases intracellular monoamine levels, and also by promoting reverse transport (efflux) through plasma membrane monoamine transporters (J Biol Chem 237:2311–2317, 1962; Med Exp Int J Exp Med 6:47–53, 1962; Neuron 19:1271–1283, 1997; J Physiol 144:314–336, 1958; J Neurosci 18:1979–1986, 1998; Science 237:1219–1223, 1987; J Neurosc 15:4102–4108, 1995). This review will focus on two important aspects of AMPH-induced regulation of the plasma membrane monoamine transporters—transporter mediated monoamine efflux and transporter trafficking.

Keywords Amphetamine · AMPH · Norepinephrine transporter · NET · Dopamine transporter · DAT · Monoamine transporter · Neurotransmitter transporter · Monoamines · Efflux · Trafficking

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Monoamine Transporter Structure and Function

The monoamine transporters—dopamine, norepinephrine, and serotonin transporters (DAT, NET, and SERT, respectively)—belong to the SLC6 gene family of Na^+/Cl^--dependent transporters that are critical for regulating extracellular levels of neurotransmitters. These transporters rely mainly on the co-transport of Na^+ down its electrochemical gradient to facilitate the uptake of biogenic amines from the inter- and extrasynaptic space. This transporter mediated re-uptake controls both the duration and the intensity of monoamine signaling at the synapse and is hypothesized to occur via an alternating access mechanism [14–16]. This model of transporter function suggests that substrate and Na^+ binding trigger conformational changes that shift the transporter from an "outward-facing" conformation, in which the substrate is exposed extracellularly, to an "inward-facing" conformation where the substrate is exposed to the intracellular milieu [15–18]. This mechanism enables monoamine transporters to accumulate neurotransmitters back into the intracellular compartment after vesicular release in order to ensure both appropriate regulation and maintenance of synaptic signaling.

Topological predictions and experimental data to date indicate that the monoamine transporters have 12 transmembrane domains (TMD) with intracellular amino and carboxy termini, and these predictions have been confirmed by the crystal structure of the bacterial leucine transporter (LeuT), a close homolog of the neurotransmitter tansporters [19–23]. Numerous putative phosphorylation sites and binding domains have been identified within the intracellular domains of the various monoamine transporters. These domains are considered vital for transporter regulation, especially AMPH-induced reverse transport [24–26]. Another important region is the large extracellular domain, located between TMD3 and TMD4, which is posttranslationally modified in order to ensure appropriate targeting of the transporter to the surface [27].

Original Model for Transporter-Mediated Monoamine Efflux: Facilitated Exchange Diffusion

The molecular mechanism underlying AMPH action remained a mystery until the late 1950s when the work of Burn and Rand revealed that AMPH acts by "releasing a noradrenaline-like substance" [10]. Thus, the foundation of the field was established, and since then, numerous studies have focused intently on discovering the detailed mechanism behind AMPH's ability to induce monoamine release into the extracellular milieu. Investigations following the work of Burn and Rand implicated both vesicular and plasma membrane monoamine transporters as important conduits for monoamine release. This review, however, will focus on efforts surrounding the plasma membrane monoamine transporters. The reader is directed to our substantial review for a thorough discussion of vesicular monoamine transporter contribution to AMPH-mediated monoamine release [1].

Early evidence demonstrated that AMPH-like drugs act as substrates for monoamine transporters and that AMPH-induced monoamine release could be blocked by uptake inhibitors such as cocaine and nomifensine [11, 13, 28–32]. In tandem with these studies, Fischer and Cho proposed the facilitated exchange diffusion model as a model for AMPH-induced monoamine release via DAT [33, 34]. Fischer and Cho hypothesized that AMPH is transported as a substrate into the cell via DAT which subsequently results in the counter transport of DA extracellularly. Given that AMPH serves as a substrate for DAT, its transport into the cell increases the number of transporters in the inward facing conformation, and thus increases the probability that intracellular DA will bind to DAT and induce reverse transport. Evidence in support of this model of AMPH-induced efflux demonstrates that AMPH accumulation in rat synaptosomes is saturable, temperature-dependent, and ouabain-sensitive, implicating an active transport mechanism for AMPH [35]. Additional evidence for an active transport mechanism has been supported by several electrophysiology studies illustrating AMPH's ability to generate DA-like transporter-associated currents [36, 37].

Since the introduction of facilitated exchange diffusion in 1979, new experimental results have emerged that challenge this model. For example, direct intracellular injections of AMPH into the giant DA neuron of *Planorbis corneus*, the pond snail, induce reverse transport despite the fact that AMPH has not been taken up via the transporter [13]. Furthermore, AMPH increases intracellular Na^+ concentration, and an increase in intracellular Na^+ is sufficient to drive DA efflux even in the absence of extracellular AMPH [38]. In fact, numerous studies to date on AMPH-induced changes in uptake, charge transfer, and efflux all support the notion that the influx of extracellular sodium ions via the transporter triggers reverse transport [28, 37, 39–42]. Other experiments, utilizing concatemers of SERT (AMPH sensitive) and GAT (AMPH insensitive) reveal that AMPH-induced efflux also depends upon the oligomeric nature of the monoamine transporters [43]. Finally, additional studies have revealed that AMPH can cause DAT-mediated DA efflux by a process that results in rapid bursts of DA efflux through a channel-like mode of DAT, a process that is independent from the slow exchange-like mechanism [44]. This was demonstrated in outside-out patches from both heterologous cells stably expressing DAT and from dopaminergic neurons. Interestingly, this channel-like mode of DA release is approximately equivalent to a quantum of DA release from synaptic vesicle fusion. Therefore, this channel-like burst mode may actually influence the synaptic action and psychostimulant properties of AMPH. These studies suggest that while facilitated exchange diffusion may contribute to AMPH-mediated monoamine release, it cannot account for all experimental observations to date (Fig. 1).

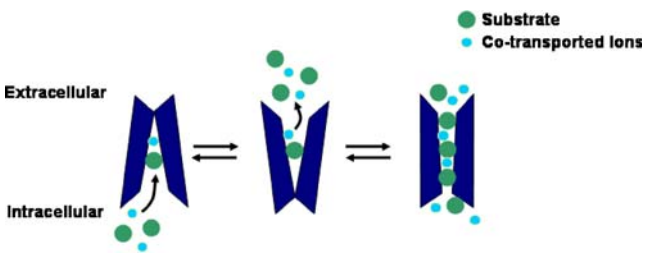

Fig. 1 Schematic representation of transporter mediated monoamine efflux. Transporters in the inward facing conformation are capable of mediating monoamine efflux by binding substrate and co-transported ions. Transporter reversal can be enhanced under certain conditions such as in the presence of AMPH or increased intracellular Na^+. In addition to the slow exchange-like mechanism of efflux, AMPH can also induce efflux through a channel-like mode of the transporter

Regulation of Efflux by Second Messenger Systems

While the model of AMPH-induced efflux evolved to include not only facilitated exchange diffusion but also channel-like modes of the transporter, its development is incomplete without considering regulation by second messenger systems. As mentioned previously, numerous putative phosphorylation sites for various protein kinases have been identified within the intracellular regions of the monoamine transporters. In fact, several studies have demonstrated that DAT function is heavily regulated by a plethora of protein kinases [24, 45–47]. As evidence emerged that AMPH is capable of increasing protein kinase C (PKC) activity in vivo, the possibility that kinase regulation may impact AMPH's ability to induce transporter-mediated monoamine efflux became enticing [48]. Soon thereafter, strong evidence for the involvement of PKC in AMPH-induced DAT-mediated DA efflux appeared in experiments that utilized specific PKC inhibitors to prevent AMPH-stimulated DA release altogether [49, 50]. A similar role for PKC was also established for AMPH regulation of NET efflux in undifferentiated PC12 cells [51]. Not surprisingly, these experiments also revealed a requirement for intracellular Ca^{2+} in AMPH-induced NET efflux, along with the necessity for PKC activity. In addition to a role for intracellular Ca^{2+}, the effects of repeated AMPH exposure on NET were shown to depend on N-type and L-type Ca^{2+} channel activity [52]. These studies were further extended by research that demonstrated the ability of intracellular Ca^{2+} to regulate both AMPH-induced DAT currents and efflux [53]. Finally, recent studies investigating PKC's involvement in AMPH-mediated DA release have provided evidence for the importance of a physical association of $PKC\beta_{II}$ with DAT in the rat striatum [54].

Considering the wealth of data supporting a role for PKC in AMPH-stimulated neurotransmitter release, the finding that PKC activation leads to N-terminal phosphorylation of DAT in the rat striatum is not surprising [55]. Nevertheless, a DAT mutant lacking the first 22 amino acids of the N-terminus, which eliminates ^{32}P incorporation in response to PKC, shows no significant deficits in uptake, inhibitor binding, internalization, or oligomerization [24]. Given the substantial evidence for PKC involvement in AMPH-induced transporter efflux, Galli and coworkers chose to investigate the role of the N terminus in regulating DAT-mediated DA efflux by using the 22 amino acid deletion mutant. The study revealed an 80% reduction in AMPH-induced DA efflux in the mutant DAT [25]. Additionally, they demonstrated that mutation of five N-terminal serine residues to alanine residues (S/A) produced an identical phenotype to that of the 22 amino acid deletion DAT mutant. Furthermore, mutation of the same five residues to aspartate (S/D) restored AMPH-induced DA efflux to normal levels. From this study, the authors proposed a novel model for AMPH-induced regulation of DAT efflux that implies a role for N-terminal phosphorylation in shifting DAT from a "reluctant" to a more "willing" state for efflux.

While an abundance of evidence insinuates a role for phosphorylation via PKC in AMPH-induced DAT efflux, additional data implicates the involvement of other kinases in this complex process [56, 57]. In fact, recent data indicates that Ca^{2+}/calmodulin-dependent protein kinase α (CaMKIIα) is a key component of AMPH-induced regulation of DAT efflux [26]. In this study, the researchers clearly demonstrated that inactivation or inhibition of CaMKIIα dramatically reduced AMPH-induced DA efflux. Furthermore, a physical association between CaMKIIα and the DAT C-terminus was observed, and subsequent disruption of this association was sufficient to diminish AMPH-stimulated DA release. Thus, research clearly indicates a role for both PKC and CaMKIIα signaling in AMPH-induced DAT-mediated DA efflux. Whether or not these two signaling pathways contribute to efflux in parallel or sequentially, however, has yet to be determined. Interestingly, recent research, focused on the role of CaMKII in AMPH-mediated DA efflux, has identified syntaxin1A (SYN1A) as an important link between CaMKII signaling and transporter reversal [58]. SYN1A is a SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) that is critical for synaptic vesicle release. In addition to its role in vesicular fusion, SYN1A also interacts with and regulates numerous transmembrane proteins including ion channels and importantly, neurotransmitter transporters. In fact, DAT/SYN1A and NET/SYN1A associations have been shown to increase in response to AMPH, and evidence now indicates that this enhancement in SYN1A association is vital for AMPH-mediated DA efflux [58, 59]. Interestingly, SYN1A interaction with NET and DAT has also been shown to influence transporter channel-like activity, a mode of the transporter that is important for AMPH-induced efflux [60, 61]. By utilizing pharmacological and peptide inhibitors of CaMKII, experiments have clearly established that the increase in SYN1A/DAT association in response to AMPH requires CaMKII activity. From these findings, a model for AMPH-induced DA efflux can be formulated, whereby the binding of CaMKIIα to the C-terminus of DAT facilitates the phosphorylation of the N-terminus and the binding of SYN1A, promoting the shift of DAT toward a "willing" state for AMPH-induced DA efflux (Fig. 2).

AMPH-Induced Trafficking of the Dopamine Transporter

Unlike AMPH-induced neurotransmitter efflux, a phenomenon recognized in the late 1950s, another aspect of AMPH regulation of monoamine transporters did not emerge from the literature until the late 1990s. Fleckenstein and

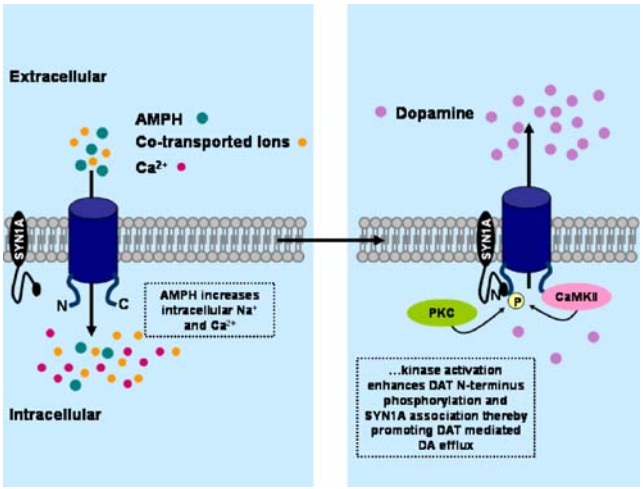

Fig. 2 AMPH-induced DAT efflux is regulated by second messengers. A model of AMPH-induced regulation of DAT mediated DA efflux via intracellular signaling pathways. AMPH transport via DAT into the intracellular milieu results in an increase in both intracellular Na^+ and Ca^{2+} levels. As a consequence, PKC and CaMKII activation initiates both phosphorylation of the N terminus of DAT and enhancement of DAT/SYN1A association, shifting the transporter from a "reluctant" to a "willing" state for efflux

coworkers hypothesized that AMPH not only induces transporter efflux, but also may regulate transporter surface expression levels based on their observation that, in rats, a single high dose injection of AMPH results in a decrease in DAT function 1 h later [62]. Since then, AMPH-induced regulation of DAT trafficking has been confirmed by numerous studies, and the investigation into the mechanisms underlying this phenomenon has become an area of intense research [63–66].

The first comprehensive demonstration of AMPH-induced trafficking of DAT in heterologous systems surfaced a few years after Fleckenstein's original proposal. In this study, acute treatment with the DAT substrates AMPH and DA not only reduced [^{3}H]DA uptake and AMPH-induced currents, but also clearly decreased DAT cell surface expression [63]. By utilizing a dominant negative mutant of dynamin I (K44A) to prevent substrate-induced trafficking, the authors also provided evidence to suggest that AMPH-stimulated DAT endocytosis occurs via a dynamin-dependent pathway. Following these experiments, a series of investigations verified these results in other heterologous systems (*Xenopus* oocytes), rat synaptosomal preparations, and finally, indirectly, in vivo via high speed chronoamperometry [65, 67–70]. Interestingly, application of DAT inhibitors such as cocaine, mazindol, and nomifensine is sufficient to prevent the AMPH-induced DAT trafficking, implying that transport of AMPH into the cell may be an important component of this regulation. To address this hypothesis, a mutant DAT (Y335A) capable of substrate binding but impaired in substrate transport was exposed to AMPH and analyzed for redistribution from the cell surface to the cytosol. Interestingly, extracellular AMPH application did not induce internalization of the uptake-impaired DAT, but when applied directly into the intracellular milieu, AMPH was sufficient for inducing trafficking of the mutant [66]. From this, the researchers concluded that while the DAT transport cycle is unnecessary for AMPH-induced DAT trafficking, an increase in intracellular AMPH is an essential component of this regulation. An important caveat to consider in these studies is the timing of transporter cell surface redistribution in response to AMPH application. Indeed, recent studies point out that AMPH-induced trafficking of DAT is dependent upon the time of AMPH exposure. For example, a rapid enhancement of DAT surface expression occurs within seconds of AMPH application and diminishes by 2.5 min implying that AMPH's regulation of transporter trafficking differs with respect to acute versus long-lasting effects [71].

As AMPH-induced DAT trafficking became well established, researchers shifted their focus toward identifying underlying key components of the phenomenon. Considering the wealth of evidence supporting a role for AMPH-induced PKC activation in transporter efflux, the idea that PKC activity may also be involved with transporter trafficking seemed plausible. In fact, numerous studies had already demonstrated that PKC activation leads to the rapid redistribution of DAT from the cell surface in both heterologous and neuronal systems [24, 47, 72–74]. However, recent results show that while AMPH application induces N-terminal phosphorylation of DAT via PKC, prevention of this phosphorylation by mutation does not deter AMPH-induced DAT trafficking [24, 75]. Furthermore, more recent studies have demonstrated that residues required for PKC-induced internalization are not critical for AMPH-triggered DAT sequestration, and PKC inhibition also failed to inhibit AMPH-induced DAT redistribution [76, 77]. Therefore, while experiments to date clearly implicate a role for PKC in AMPH-induced regulation of DAT efflux, it does not appear to be involved in AMPH-induced transporter trafficking. Interestingly, new work from Vaughan and co-workers indicates that PKC induced DAT regulation may differ depending on the membrane localization of the transporter [78]. In this study, the authors demonstrate that PKC-stimulated phosphorylation of DAT occurs to a significantly higher level in lipid raft populations of DAT compared to non-lipid raft populations. In fact, the PKC-triggered DAT internalization happens primarily from non-raft population. Thus, PKC regulation of DAT depends heavily upon the discrete membrane localization of the transporter. Given AMPH's dual effect on transporters, one can imagine a scenario where non-raft populations are primarily responsible for AMPH-induced DAT internalization, whereas raft populations may primarily constitute transporter-mediating monoamine efflux.

Whether membrane localization is important or not for AMPH-induced efflux and trafficking has yet to be determined, but it will be an interesting question for future studies. In addition to PKC, other kinases have also been implicated in AMPH-induced DAT trafficking. For example, AMPH application results in a time-dependent increase in CaMKII activity which is required for DAT trafficking [79]. Importantly, this CaMKII activation inhibits an important kinase in the insulin signaling pathway, Akt. This study suggests that insulin signaling is involved in DAT trafficking and, as a consequence, may impact DA homeostasis. Thus, the current model of AMPH-induced DAT internalization proposes an intersection of AMPH-stimulated signaling and the insulin signaling pathway.

The notion that insulin signaling may be involved in the regulation of DA clearance began with studies that revealed striking alterations in the dopaminergic system of rodents rendered diabetic through streptozotocin (STZ) treatment, which results in necrosis of insulin-producing pancreatic β cells [80–82]. Additionally, tyrosine kinase inhibitors, which block the receptors activated by insulin and insulin-like growth factor were shown to reduce DA clearance due to a decrease in the surface expression of DAT [83]. Concurrently, other investigators expanded these results by illustrating that inhibition of downstream components of the insulin signaling pathway, such as phosphatidylinositol 3-kinase (PI3K) and Akt, also dramatically reduced DA clearance and surface expression of DAT [46, 84]. Thus, the idea that basal insulin signaling is critical for the appropriate maintenance of DA clearance via DAT became firmly established. Given the importance of insulin signaling for maintaining DAT at the plasma membrane, researchers quickly realized the ability of insulin signaling to impact AMPH action in the brain. For example, the reinforcing properties of AMPH are tremendously diminished in the STZ model of diabetes, as demonstrated by the AMPH self-administration paradigm [85]. Additionally, selective inhibition of PI3K via LY294002 results in a dramatic reduction in AMPH's ability to elicit DAT-mediated DA efflux in heterologous cells, dopaminergic neurons, and in vivo within the striatum of rats as measured by both in vivo voltammetry and functional magnetic resonance imaging [70, 86]. These data suggest that kinases linked to both glucose homeostasis and food intake regulation are also capable of regulating the reward pathways in the brain that are targeted by psychostimulants such as AMPH.

AMPH-induced Trafficking of the Norepinephrine Transporter

While evidence for AMPH-induced internalization of DAT has accumulated since the 1990s, experiments supporting a similar phenomenon with respect to NET did not appear until the 2000s [59, 87]. The first studies, from Ordway and colleagues, which investigated the effects of chronic AMPH exposure on NET revealed that long-term AMPH exposure reduces NET expression in both a time- and concentration-dependent manner [87]. Furthermore, a study from 2007 demonstrated that, like DAT, acute AMPH application stimulated a slow, significant reduction in surface levels of NET in a catecholaminergic cell line [59]. While these results come as no surprise, the study also revealed novel aspects of AMPH-induced regulation of the transporter. First, the authors clearly demonstrated that this process is Ca^{2+} dependent by utilizing BAPTA-AM and Cd^{2+} to diminish intracellular Ca^{2+} levels and prevent AMPH-stimulated NET internalization. Furthermore, inhibition of CaMKII via KN93 also prevented AMPH-induced NET trafficking, implying that this process for NET is both Ca^{2+} and CaMKII dependent just as it is for DAT. Finally, the study of an N-terminal deletion mutant (hNET$\Delta_{28–47}$) also indicated that AMPH-stimulated regulation of NET internalization may be mediated through the N terminus [59]. Interestingly, whether a similar region is critical for AMPH-induced DAT internalization has yet to be determined. In comparison to the wealth of research dedicated to determining how AMPH regulates both trafficking and efflux of DAT, knowledge related to AMPH-induced regulation of NET is rather limited. Despite this gap in the field, much research has been done on β-PMA induced downregulation of NET, and evidence supports a role for lipid rafts in NET internalization [88, 89]. Perhaps, similar mechanisms will be elucidated for AMPH-mediated NET trafficking which would suggest a divergence in the regulation of NET compared to DAT given the clathrin dependence of AMPH-induced DAT trafficking. Thus, much research is still needed in the field of AMPH-induced NET internalization and efflux. Many important questions remain unanswered such as which compartment does the transporter travel through during AMPH-stimulated trafficking, and what intracellular signals and modifications of NET are required for this trafficking phenomenon? Furthermore, does the insulin signaling pathway play any role in the ability of AMPH to induce NET-mediated NE efflux and NET trafficking?

Concluding Remarks

Since the first clues behind AMPH's mechanism of action began to emerge as early as the late 1950s, this field of research has seen an exponential amount of growth. After almost 50 years of investigation, the original model for AMPH-induced monoamine efflux has evolved from its simplest form of facilitated exchange diffusion to a

multifaceted mechanism that requires not only exchange diffusion and channel-like modes of release but also regulation by second messenger systems. Perhaps, even more remarkable than the transformation of the efflux field is the discovery of a second mechanism of action for AMPH altogether—transporter trafficking. Despite the advancements over the last few years, both the broad implications and the intricate details of AMPH's actions continue to elude us. For instance, how does AMPH's ability to dynamically regulate monoamine transporter membrane expression contribute to its psychostimulant and addictive properties? Which intracellular signaling pathways are critical for AMPH-induced regulation of transporter efflux and trafficking, and how do they differ? Research aimed at addressing these types of questions promises to bring us one step closer to comprehending the basis of not only AMPH abuse and addiction but also its role as a treatment for various pathological conditions. As our understanding of AMPH action continues to progress, so too will our comprehension of monoaminergic regulation, in general. For example, recent work involving a DAT-coding variant associated with ADHD that effluxes DA as if it were exposed continuously to AMPH has been shown to have aberrant regulation under normal conditions that closely parallels regulation of wild-type DAT by AMPH [90]. Thus, uncovering the secrets of AMPH-mediated monoamine transporter regulation promises to enhance our capacity to generate novel therapeutic strategies for treating drug abuse as well as disorders associated with monoaminergic dysfunction such as depression and ADHD.

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