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Dynamic regulation of the dopamine transporter

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Abstract

In the mammalian central nervous system the dopamine transporter (DAT) is the primary mechanism for clearance of dopamine from the extracellular space. Presynaptic receptors for dopamine and other neurotransmitters (auto-receptors and hetero-receptors) present on dopaminergic neurons are poised to regulate the activity of the dopamine transporter acutely through their actions on intracellular signaling systems. The mechanisms proposed for acute presynaptic regulation of dopamine transport include direct effects of phosphorylation on enzymatic rate, indirect effects through the alteration of the electrical and chemical gradients that drive transport and/or the modulation of transporter number through the trafficking of carriers to and from the cell surface. This review focuses on recent evidence for several distinct mechanisms which dynamically regulate dopamine transporter activity and thus have an important role in shaping the duration and amplitude of dopamine signals in the brain.

Keywords: Neurotransmitter transport; Dopamine transporter; Transporter regulation; Trafficking; Signaling mechanism; Phosphorylation; Cocaine; Amphetamine

1. Introduction

Dopamine, a major neurotransmitter in the mammalian central nervous system, is involved in the control of locomotor activity, and also in pathways regulating goal oriented behavior and reward (Schultz, 2002). The dopamine transporter clears neurotransmitter from the extracellular space and serves as an important regulator of signal amplitude and duration at dopaminergic synapses. The dopamine transporter also regulates the activation of extrasynaptic receptors and thus has a significant impact on volume neurotransmission, another established mode of dopamine signaling. The catalytic rate of the transporter itself is slow—less than one dopamine molecules/s in cultures of midbrain dopamine neurons—however, this low turnover rate may not be so surprising in that dopamine acts through G-protein coupled receptors that modulate intracellular events on a timescale of seconds (Prasad and Amara, 2001) The transporter is expressed selectively in dopaminergic neurons of the substantia nigra and the ventral tegmental area of the brain (Ciliax et al., 1995; Freed et al., 1995). These dopaminergic neurons project to the striatum, nucleus accumbens and the prefrontal cortex and the transporters are expressed throughout the cell on axons, dendrites and the soma, but have not been found in the active zones of synapses (Nirenberg et al., 1997).

Psychostimulants, such as methylphenidate, cocaine, and amphetamines, exert many of their effects by acting on the dopamine transporter, and thus provide a compelling clinical rationale for understanding the function and regulation of the carrier. These drugs either block transport of substrates, as established for cocaine or methylphenidate, or are carrier substrates, such as amphetamines, which both inhibit uptake of extracellular dopamine and stimulate efflux of intracellular dopamine. The result is an increase in extracellular dopamine that can activate the well-known motor and reward pathways of the midbrain and trigger the increased locomotor activity and euphoria associated with drug use. Dopamine transporter gene disruption experiments in mice have demonstrated the importance of the carrier in psychostimulant action; mice that completely lack a functional dopamine transporter display intense hyperactivity and a profound persistence of extracellular dopamine, mimicking the prominent actions of the drugs. However, the striking behavioral effects and changes in the rate of dopamine clearance observed in homozygous knock out animals are not further enhanced by cocaine or amphetamine administration, providing additional support for the

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importance of the dopamine transporter as an obligate target for these stimulant drugs (Giros et al., 1996). It is important to note that all the behavioral effects of cocaine cannot be attributed solely to dopamine transporter, as these mice still show conditioned place preference for cocaine (Rocha et al., 1998; Sora et al., 1998). Mice with a double disruption of both dopamine and serotonin transporter genes exhibit no conditioned place preference in response to cocaine, supporting the contribution of the serotonin transporter to cocaine reward and reinforcement (Sora et al., 2001). A mouse "knockdown" line which retains only 10% of wild type dopamine transport activity has provided a promising model for the counterintuitive calming effect of psychostimulants, such as methylphenidate, on patients with attention deficit hyperactivity disorder (ADHD) (Zhuang et al., 2001). These mice show the decreased dopamine clearance rates and hyperactivity characteristic of a hyperdopaminergic phenotype. Intriguingly, in this model, which retains some dopamine transporter function, psychostimulants also have a calming effect that resembles their therapeutic action in humans with ADHD.

The result of transporter knock outs have underscored how important short term and chronic regulation of the dopamine transporter are during normal dopaminergic neurotransmission, and in a variety of neurological and neuropsychiatric disorders, including ADHD, schizophrenia, psychostimulant addiction and Parkinson's disease. Both the chronic regulatory changes and the acute regulation of transport activity have been the subject of an excellent, comprehensive review on the biogenic amine transporters (Zahniser and Doolen, 2001). Activation of intracellular signaling systems has been proposed to acutely modulate transport activity directly by altering the phosphorylation state of carriers, or indirectly by changing the electrical and chemical gradients that drive transport or by regulating the trafficking of carriers to and from the cell surface. A summary of these processes is illustrated in Fig. 1. With an emphasis on the dopamine

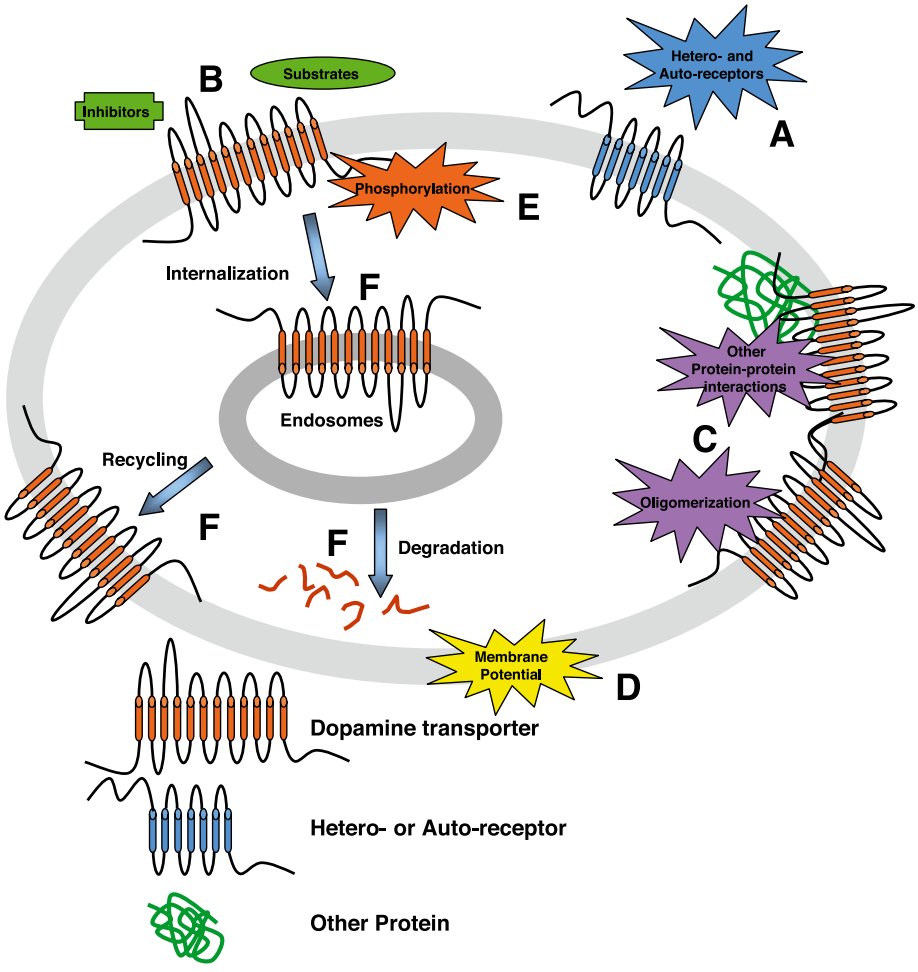

Fig. 1. Illustration of potential processes involved in regulating dopamine transporter activity this review will cover. (A) Extracellular signals can be relayed through either dopamine auto-receptors or hetero-receptors for other neurotransmitters. These receptors would activate intracellular signaling pathways and thus modulate transporter activity. (B) Smaller molecules including substrates of the dopamine transporter, inhibitors, and arachidonic acid may interact directly with and regulate the transporter. (C) Formation of either homo-multimers of the dopamine transporter or hetero-multimers with other proteins could modulate transporter activity. (D) Because dopamine transport is electrogenic the process could be modulated by membrane potential. (E) Posttranslational modifications such as phosphorylation could regulate the intrinsic catalytic activity of the transporter. (F) Internalization and recycling and ultimately degradation dictate the number of available transporters at the surface, thus regulating transport activity.

transporter, our review will consider on recent developments in our understanding of the dynamic cellular events that regulate short-term changes in transport activity.

2. The dopamine transporter

2.1. Na^+- and Cl^--dependent transporter family

The dopamine transporter belongs to the family of Na^+/Cl^- dependent neurotransmitter transporters. The cloning of two transporters by different groups initially facilitated the identification of this family. Amino acid sequence information from a γ-hydroxybutyric acid (GABA) transporter purified from rat brain was the basis for cloning the first GABA transporter, GAT1 (Guastella et al., 1990). A norepinephrine transporter was cloned using a functional expression assay based on its ability to transport a radio-iodinated substrate analog when expressed in green monkey fibroblast COS cells (Pacholczyk et al., 1991). The sequence similarity shared by the two carriers was the basis for identifying many other structurally and functionally related carriers. Currently in mammals, the family consists of the serotonin transporter (SERT), the dopamine transporter (DAT), glycine transporters (GLYTs 1a, 1b, 1c, and 2), the norepinephrine transporter (NET), GABA transporters (GATs 1–4) the proline transporter (PROT), and the taurine transporter (TAUT), reviewed in (Masson et al., 1999). Interestingly, in mammals there still exist orphan transporters in this family for which substrates have not yet been identified.

2.2. Substrate and inhibitor selectivity of the dopamine transporter

The dopamine transporter has been cloned and characterized from several species (Gallant et al., 2003; Giros et al., 1991, 1992; Jayanthi et al., 1998; Kilty et al., 1991; Miller et al., 2001; Porzgen et al., 2001; Shimada et al., 1991; Usdin et al., 1991). It has a well-characterized and unique pharmacological profile. Transport is inhibited by non-selective psychostimulants such as cocaine, mazindol, and methylphenidate, and a few selective compounds that include the GBR compounds, 12909 and 12935, the non-classical antidepressant bupropion, and the muscarinic antagonist benztropine. On the other hand most clinically used antidepressants classified as reuptake inhibitors, including fluoxetine, citalopram venlafaxine and paroxetine, are more potent at inhibiting the serotonin and/or norepinephrine transporters. In addition to clearing dopamine, the dopamine transporter can also transport other substrates such as amphetamines, which block transport, but also cause dopamine efflux, and the neurotoxin 1-methyl-4-phenylpyridinium ion (MPP^+), which kills dopamine neurons, and triggers a Parkinson's disease-like syndrome. Interestingly, the *Drosophila* dopamine transporter has been proposed to be a primordial catecholamine transporter because its substrate selectivity parallels that of the mammalian dopamine carrier, but its rank order of potency for inhibitors is more similar to that of a norepinephrine transporter (Porzgen et al., 2001).

2.3. Thermodynamic coupling of ions

Like most members of Na^+/Cl^- dependent carrier family, dopamine transporters are expressed at the plasma membrane, where they catalyze the accumulation of their respective substrates within the cell. In order to move substrate against a concentration gradient, dopamine transporters co-transport sodium and chloride ions along with substrate to provide the thermodynamic driving force for inward flux. Thus, transport requires sodium and chloride and, depending on the net charge of substrates and ions, the process is potentially electrogenic. Estimates of the ionic stoichiometry of monoamine carriers have come predominantly from experiments measuring the ionic dependence of initial rates of substrate transport. In the cloned carrier, dopamine and other substrates appears to be transported with two sodium ions and one chloride ion (Gu et al., 1994). However, electrophysiological analyses of the currents associated with transporters have provided additional insights into the electrical properties and the pathways of ion permeation of the carrier proteins. Although one might assume that the currents associated with sodium-coupled co-transporters should reflect the net charge of substrates and ions transported, an electrophysiological investigation of dopamine transport demonstrates more complexity than would be expected from a simple model of co-transport of Na^+, Cl^-, and substrate (Sonders et al., 1997).

2.4. Transporter-mediated conductances

Many studies have now shown that members of several transporter families can mediate macroscopic ionic currents, which are not stoichiometrically linked to substrate movement. The results of this work support an emerging concept that transporters share more common properties with ionic channels than was previously thought. Our laboratory has successfully used voltage clamp analysis in *Xenopus* oocytes to analyze currents generated by the human, rat and *Drosophila* dopamine transporters (Sonders et al., 1997). Several currents appear to be associated with the carrier and these currents can be differentially influenced by the application of a variety of substrates and transport inhibitors. When the human dopamine transporter is expressed in oocytes, two steady-state ionic conductances that display a common pharmacological sensitivity to dopamine transporter ligands are observed. An inward steady-state current—the transport current—is elicited by substrates such as dopamine and amphetamine, and can be blocked by non-substrate blockers. A second conductance—a tonic leak conductance, generates a steady-state current which is not coupled to substrate transport but which is blocked by cocaine-like drugs and by substrates.

It has now been possible to characterize dopamine transport-associated currents in cultures of rat dopaminergic neurons (Ingram et al., 2002). In these neurons, whole-cell and perforated patch clamp recordings were used to demonstrate that low concentrations of substrates elicit inward currents that are sodium-dependent, blocked by cocaine, and are primarily constituted by chloride flux, a previously uncharacterized property of monoamine transporters. Unlike receptor-mediated anion conductances that are generally inhibitory, the biophysical properties of the dopamine transporter-associated anion conductance result in depolarization which causes an increase in neuronal firing rate that occurs even when dopamine receptors are blocked. This transporter mediated anion conductance implicates a role for dopamine transporter in modulating neurotransmitter release in addition to its established function in dopamine clearance.

3. Regulation by ion gradients and membrane potential

3.1. Voltage dependence of transport in cloned and endogenous carriers

As transport involves the electrogenic movement of charged substrates and ions, the amount of transport could vary with changes in membrane potential. *Xenopus* oocytes provided a unique system in which to address this issue because uptake can be measured while simultaneously maintaining a constant membrane potential. This approach was used to show that dopamine uptake by the human dopamine transporter is a modestly voltage-dependent process and that transport increases with hyperpolarization and decreases with depolarization (Sonders et al., 1997). The observation that dopamine transport is regulated by membrane potential suggests one possible way in which dopamine autoreceptor activation and consequent hyperpolarization could act to enhance presynaptic dopamine uptake. However, an examination of the voltage-dependence of transport under more physiological conditions in cultured dopamine neurons argues against this hypothesis. Current clamp recordings of dopamine neurons have shown that activation of endogenous G-protein coupled receptors by dopamine, baclofen and orphanin-FQ (OFQ) cause different degrees of hyperpolarization, but did not alter or increase uptake as might be predicted from studies with the cloned carrier (Prasad and Amara, 2001). Furthermore, when dopamine transport activity in neurons actively firing action potentials (2–3 Hz) was compared to the activity in neurons that had been silenced with tetrodotoxin no differences were observed.

3.2. Effects of dopamine D2 receptor activation on dopamine clearance

The involvement of receptor systems in the regulation of the dopamine transporter has also been investigated. Pre-synaptic auto-receptors are known to be activated by released dopamine and serve as an important feedback loop. When activated they inhibit both the release and the synthesis of neurotransmitter. The dopamine D2 receptor is the pre-synaptic auto-receptor in dopaminergic neurons, but like many other receptor subtypes that serve as auto-receptors, they can also be found post-synaptically, an observation that can make some pharmacological studies difficult to interpret. The dopamine D2 receptor couples pre-synaptically to the activation of an inward rectifying potassium channel, which causes the cell to become hyperpolarized. Antagonists of these receptors have been shown to modestly decrease the clearance of dopamine in brain (Cass and Gerhardt, 1994), and tissue preparations (Meiergerd et al., 1993) but the mechanism of this has not been clarified as both hyperpolarization and intracellular signaling mechanisms have been implicated (Mayfield and Zahniser, 2001a). Mice in which the dopamine D2 receptor has been knocked out have slower dopamine clearance than wild type mice, again linking this receptor to the regulation of the transporter (Dickinson et al., 1999). These results are not entirely consistent with the work noted above, which showed that dopamine D2 receptor agonists and other hyperpolarizing stimuli had no effect on transport in cultured dopamine neurons (Prasad and Amara, 2001). These inconsistencies could easily arise from the different methods and systems used in these studies. For example, dopamine neurons in culture may not mimic the responses of neurons in the brain. In the dopamine D2 receptor knock out model, compensatory changes may produce shifts in the normal homeostatic balance of the dopamine system. Thus, additional work will be required to establish whether auto-receptors are directly involved in the acute regulation of the dopamine transporters.

4. Evidence for regulation of function by transporter phosphorylation

4.1. Impact of carrier phosporylation on activity

Because members of the Na^+/Cl^- dependent family of transporters possess in their amino acid sequences multiple consensus sites for phosphorylation by several different protein kinases, it has been speculated that a direct phosphorylation of the transporter could regulate the activity of the transporter. Possible consequences of such phosphorylation events include altering the catalytic rate of the carrier, shifting its apparent affinity for substrate and/or inhibitors, and marking the carrier for downregulation or redistribution between surface and internal membrane compartments. A number of studies indicate that the transporters can exist as phosphoproteins and that their phosphorylation increases following activation of kinases such as protein kinase C. This has been found to be the case in both striatal synaptosomes (Vaughan et al., 1997) and in a cultured

epithelial cell line derived from porcine kidney LLC-PK_1 stably expressing dopamine transporter (Huff et al., 1997). Biochemical studies have found that the phosphorylated amino acids reside within the N-terminus of the transporter (Foster et al., 2002).

Conclusive evidence that the direct phosphorylation of the transporter alters the intrinsic activity of the transporter or triggers the downregulation and internalization of dopamine transporter has been diffcult to obtain. The removal of all the consensus sites for protein kinase C did not prevent protein kinase C-induced internalization (Chang et al., 2001). A recent study investigated the phosphorylation state of mutants where potential phosphorylation sites were removed by mutagenesis or deletion (Granas et al., 2003). The results of these experiments showed that deletion of the N-terminus of the transporter eliminated protein kinase C-induced phosphorylation of the transporter, but the mutant still transported substrate and was internalized normally following protein kinase C activation (Granas et al., 2003). Thus the transporters do become phosphorylated, but this event does not dictate whether the carrier is internalized. This implies that there must be other undiscovered phosphoproteins that regulate dopamine transporter trafficking.

Other recent experiments using COS cells transiently expressing the dopamine transporter show that dopamine transporter is phosphorylated and several dopamine transporter mutations were found to alter the regulated phosphorylation pattern of the transporter (Lin et al., 2003). Though this study did not clearly implicate direct phosphorylation of the transporter in the regulation of activity, it did highlight several phosphorylated residues in the N-terminus and other regions of the protein that might be linked to a regulatory effect.

4.2. N-linked glycosylation is important for delivery of carrier to the cell surface

The Na^+/Cl^- dependent transporters all contain sites for N-linked glycosylation in the extracellular loop and it has been shown that they do become glycosylated. Mutational studies have shown that this glycosylation is very important for the activity and stability of the transporters at the surface, but does not seem to be important for the regulation of the intrinsic transport activity (Torres et al., 2003).

5. Other agents that may regulate transport functions by a direct action

5.1. Arachidonic acid, ethanol, and nitric oxide

Several other molecules have been implicated in the regulation of dopamine transporter. Inhibitors of arachidonic acid metabolism are relatively potent inhibitors of dopamine uptake in rat striatal slices (Cass et al., 1991) an observation supported by a later study performed in striatal synaptosomes (L'hirondel et al., 1995). Others have shown in C6 rat glioma cells stably expressing the dopamine transporter that short incubations with arachidonic acid stimulate dopamine uptake while longer incubations at higher arachidonic acid concentrations inhibit both dopamine transport and binding of WIN 35,428, a tropane analog of cocaine (Zhang and Reith, 1996). The study also demonstrated that a series of compounds known to raise endogenous concentrations of arachidonic acid all inhibited dopamine transport. Because protein kinase C activation was not required, the finding suggested that arachidonic acid may act directly on the carrier.

Arachidonic acid can have other effects on the dopamine transporter; when applied to *Xenopus* oocytes expressing the human carrier, it induces a large novel nonselective cation conductance that is potentiated by dopamine and blocked by cocaine (Ingram and Amara, 2000). Dopamine potentiates this current in the absence of sodium and chloride, indicating that these currents arise from processes distinct from those associated with substrate transport. The effects of arachidonic acid were mimicked by other fatty acids with a rank order of potency correlated with their degree of unsaturation, suggesting that polyunsaturated fatty acids can have direct effects on the functional properties of the dopamine transporter.

Ethanol has been shown to enhance the activity of the dopamine transporter by increasing the number of carriers on the cell surface (Mayfield et al., 2001b). A recent mutagenesis study identified glycine-130 and isoleucine-137 as the amino acid residues responsible for this increase. When these two positions were substituted with the corresponding residues from the ethanol-insensitive norepinephrine transporter, the dopamine transporter was no longer sensitive to ethanol (Maiya et al., 2002). On the basis of these results the authors propose that this apparently intracellular domain of the dopamine transporter may interact with a cytoplasmic protein in an ethanol-dependent manner.

Several groups have shown that nitric oxide can reduce dopamine transport in striatal synaptosomes (Kiss et al., 1999; Lonart and Johnson, 1994; Pogun et al., 1994) and in C6 rat glioma cells expressing the dopamine transporter (Cao and Reith, 2002). Surprisingly nitric oxide generated by adenosine receptor activation appears to increase serotonin transporter activity in rat basophillic leukemia cells (Miller and Hoffman, 1994), suggesting that signaling pathways in different cellular environments can have distinct effects on transporter activities.

5.2. Direct substrate- and inhibitor-mediated regulation of transporters

Although it is easy to imagine how substrates, such as dopamine, could regulate carrier functions by activating presynaptic auto-receptors, there are a number of intriguing experiments to support the idea, that the transporters are regulated directly by interaction with their substrates and

inhibitors. In human embryonic kidney HEK-293 cells stably expressing dopamine transporter, addition of either of the substrates dopamine and amphetamine results in a down regulation of the activity of the transporter and the level of transporters expressed at the surface (Saunders et al., 2000). Transport inhibitors were found to block this downregulation. Uptake inhibitors by themselves have been found to up regulate the surface expression of transporters exogenously expressed in different cell types (Daws et al., 2002; Little et al., 2002). A recent study using fluorescence resonance energy transfer (FRET) to study intermolecular interactions of the dopamine transporter corroborates the effects of substrates on internalization as preincubation of cells expressing the dopamine transporter with amphetamine enhances the internalization of dopamine transporter (Sorkina et al., 2003). Another study (Daniels and Amara, 1999) did not find any effect of the substrate dopamine on activity or surface expression levels in canine kidney MDCK cells expressing a green fluorescent protein-tagged dopamine transporter, suggesting that the modulation of activity by substrates may vary in different cellular model systems. Interestingly the serotonin transporter, a member of this family of transporters also appears to be regulated by its substrate serotonin (Ramamoorthy and Blakely, 1999). However in contrast to the observations of Saunders et al. (2000), for the dopamine transporter, the internalization of the serotonin transporter following activation of protein kinase C was attenuated by co-incubation with the substrate serotonin. Because the studies were carried out in the same expression system, Saunders et al. (2000) suggest that these two transporters must be regulated in different ways.

In dopamine transporter expressing *Xenopus* oocytes it has also been found that repeated 1-min exposures to low concentrations of different dopamine transporter substrates every 5 min will lead to a decrease in the transport associated currents and this decrease could be attenuated by co-incubation with protein kinase C inhibitors. The same study also examined whether this phenomenon could be seen in vivo in intact rat brains and found that by increasing the frequency of substrate exposure to every 2 min they could observe a decrease in dopamine transporter mediated dopamine clearance in striatum but not in nucleus accumbens (Gulley et al., 2002).

In another experimental paradigm, rats were treated acutely with a single dose of amphetamines such as amphetamine, methamphetamine or methylenedioxymethamphetamine (MDMA) prior to preparation of striatal synaptosomes from the animals (Fleckenstein et al., 1999; Fleckenstein et al., 1997). Amphetamine pretreatment in these studies produced a reversible decrease in synaptosomal dopamine transport activity. Although it was not shown that this reduction in activity is caused by decrease in surface carriers or a change in their intrinsic acitivity, kinetic analyses did indicate that the effect was associated with a decrease in V_{max} but without change in the apparent affinity for substrate. Inhibitors of protein kinase C attenuated the effect in a manner consistent with it being a phosphorylation-dependent event (Sandoval et al., 2000). As these studies have been carried out in a complex tissue system, it cannot be excluded that such effects also could be mediated indirectly. This has been addressed in a study where antagonists of dopamine D1 and D2 receptors or antioxidants were administered before the amphetamine injection (Metzger et al., 2000). As the pre-administration of these drugs could not eliminate the effect of amphetamine injection on the transporter activity, the conclusion of this study was that none of these molecules were involved in the amphetamine-mediated down regulation of dopamine transporter.

6. Regulation of transporter trafficking and insertion

6.1. Activation of protein kinase C alters activity

Several different intracellular signaling pathways have been studied and implicated in the regulation of dopamine transporters. By far the best characterized is the stimulation of protein kinase C (PKC) activation using phorbol esters. The most consistent result from these studies is a rapid down regulation of transport activity as a result of removal of transporters from the cell surface. This effect has been shown in striatal synaptosomes (Copeland et al., 1996; Vaughan et al., 1997), in COS cells (Kitayama et al., 1994; Pristupa et al., 1998), in C6 rat glioma cells (Zhang et al., 1997), in rat pheochromocytoma PC12 cells (Melikian and Buckley, 1999), in LLC-PK_1 cells (Huff et al., 1997), in MDCK cells (Daniels and Amara, 1999), in insect sf9 cells (Pristupa et al., 1998), and in *Xenopus* oocytes (Zhu et al., 1997). The protein kinase C isozyme responsible for the effect in *Xenopus* oocytes has also been characterized to be one of the conventional protein kinase C isoforms, that are sensitive to the inhibitor Gö6976 (Doolen and Zahniser, 2002).

Two groups have explored the cell biological mechanism of these changes in surface expression in greater detail. Using canine kidney MDCK cells stably expressing green fluorescent protein-tagged dopamine transporters it was possible to visualize a very distinct internalization of the transporter and an accumulation of intracellular vesicles containing the transporter following treatment with the protein kinase C activator phorbol 12-myristate 13-acetate (PMA). This internalization is inferred to be clathrin-mediated, as a dominant negative mutant of dynamin inhibited this process (Daniels and Amara, 1999). Using specific antibodies to monitor dopamine transporter trafficking in a stably transfected PC12 cells line Melikian and Buckley (1999) have also observed a robust removal of transporter molecules from the surface following protein kinase C activation.

Once internalized it appears that the fate of the transporter depends on the cell system in which the transporter is expressed. Using sucrose gradients for subcellular fractionation in human dopamine transporter transfected PC12 cells, it was found that after PMA treatment the transporter co-

localized with markers of the endosomal recycling compartment and was subsequently recycled back to the surface (Melikian and Buckley, 1999). In MDCK cells, rather than being recycled the internalized transporter transits to the lysosomes where it is ultimately degraded (Daniels and Amara, 1999). Melikian's group has extended their analysis of the mechanisms for both the basal and protein kinase C-mediated recycling of dopamine transporter in PC12 cells (Loder and Melikian, 2003). In these cells the transporters are internalized and recycled back to the surface very robustly. This constitutive process was found to be independent of transporter expression levels. In contrast, it was found that the internalization stimulated by protein kinase C activation varies with different transporter expression levels and protein kinase C activation increases the removal from the surface, but also inhibits the recycling back to the surface. It was therefore suggested that the constitutive and regulated trafficking of the transporter might be mediated by independent mechanisms.

These observations suggest that a major mechanism for acute regulation of transport activity is through changes in carrier trafficking, but they also raise the question of why this might be a good strategy for the dopamine transporter. As mentioned previously the transport cycle is relatively slow. Dopamine transporters are thought to be expressed abundantly in regions close to the active synaptic zone but are also expressed on dendrites and the soma of presynaptic neurons. It has been suggested that the buildup of dopamine following burst firing is a result of this slow activity of the transporter and a saturation of the transport process (Prasad and Amara, 2001). For these reasons the regulation of the carrier number can have a profound effect on dopamine clearance.

6.2. Protein kinase C activation increases dopamine transporter mediated efflux

There are other curious effects of protein kinase C on the dopamine transporter. Surprisingly, inhibitors of protein kinase C applied to rat striatal slices inhibit amphetamine-induced efflux (Kantor and Gnegy, 1998). In this preparation, efflux was calcium-independent and could be blocked by transport inhibitors, consistent with the idea the efflux was mediated by the dopamine transporter. This efflux of dopamine could also be triggered by protein kinase C activators even in the absence of the substrate amphetamine (Cowell et al., 2000; Kantor and Gnegy, 1998). Although extracellular calcium is not required, an extension of these studies showed that depletion of intracellular calcium diminished the effect (Kantor et al., 2001). These effects appear distinct from those addressing protein kinase C effects on uptake, where protein kinase C activation leads to an internalization of the carriers. These efflux studies have been carried out at a shorter time period, where the redistribution of the transporter has not yet taken effect, perhaps accounting for the apparent discrepancy. It is also intriguing to consider that this effux is mediated by a conformation of the carrier that exists in the early phases of carrier internalization.

6.3. Receptor stimulation leads to increase of dopamine transporter mediated efflux

Other investigators found similar effects as several different receptor systems can modulate amphetamine-facilitated efflux through dopamine transporter. Activation of either σ_2 receptors in striatal tissue (Izenwasser et al., 1998) or nicotinic acetylcholine receptors in rat prefrontal cortex (Drew et al., 2000) leads to an increase in amphetamine stimulated efflux; both of these effects appear to be mediated through activation of protein kinase C (Derbez et al., 2002; Drew and Werling, 2001).

A recent study has found that dendrodendritic release of dopamine is mediated through a calcium-independent extrasynaptic mechanism and appears to involve a reversal of the dopamine transporter (Falkenburger et al., 2001). The signal triggering this reversal was proposed to be mediated by activation of metabotrobic glutamate receptors and depolarization of the membrane. It is tempting to speculate that this reverse transport of dopamine could involve the same mechanism, as the enhancement of efflux seen following protein kinase C and receptor activation discussed above.

6.4. Actions of other kinases: protein kinase A and tyrosine kinases

Although a number of laboratories have examined the contributions of protein kinase A to the regulation of the dopamine transporter activity, most have failed to implicate this kinase in the acute regulation of dopamine transporter in several different experimental paradigms (Copeland et al., 1996; Daniels and Amara, 1999; Tian et al., 1994; Zhu et al., 1997). Moreover, one group has been unable to demonstrate a protein kinase A-mediated increase in the phosphorylation state of the dopamine transporter in striatal synaptosomes under conditions where protein kinase C activation could be shown to increase phosphorylation (Vaughan et al., 1997). However, there has been at least one system where an action of protein kinase A has been inferred. Forskolin, an activator of adenyl cyclase was shown to stimulate dopamine transport in striatal tissue suspensions, an effect that was blocked by the protein kinase A-selective inhibitor, H89. This effect was acute, occurring within the first minutes of application and disappearing after 15 min (Batchelor and Schenk, 1998).

Other kinases have also been investigated for their role in dopamine transporter regulation. Pathways involving protein tyrosine kinases also have been linked to the regulation of the dopamine transporter, as inhibitors of these kinases decrease the transporter activity. It has been speculated that this effect is mediated by an alteration in the number of transporters on the surface because incubation

with these inhibitors leads to a decrease in V_{max} (Doolen and Zahniser, 2001; Simon et al., 1997). In a study that supports the involvement of protein tyrosine kinases in transporter regulation, natural ligands and activators of receptor tyrosine kinases like insulin were shown to increase the activity of the norepinephrine transporter (NET) in human neuroblastoma SK-N-SH cells (Apparsundaram et al., 2001). This work also implicated another family of kinases in the regulation of NET, as inhibition of the p38 stress activated mitogen activated protein kinase (MAPK) would eliminate the increase in activity observed. Interestingly it was found that these effects were not due to a change of density of transporters on the surface but were the result of an increase in their turnover rate. The results of the various studies suggest that kinase activation may regulate transporter function acutely through at least two different mechanisms—by altering the intrinsic activity and/or the surface expression of the carriers. Pharmacological evidence to support the regulation of dopamine transporter activity by phosphatidylinositol-3-kinase has also been reported (Carvelli et al., 2002). In this study it was found that acute treatment with inhibitors of phosphatidylinositol-3-kinase leads to an enhanced internalization of the transporter by a clathrin-dependent mechanism. Recent work also implicates phosphatidylinositol-3-kinase as well as the MAPK kinase-1/2 MEK1/2 in the regulation of dopamine transporter. In this study inhibitors of both kinases decrease dopamine transporter transport activity through a mechanism that appears to involve changes in carrier surface expression. Several potential sites for phosphorylation by kinases in the dopamine transporter N-terminus were proposed to be involved in mediating this regulation (Lin et al., 2003).

6.5. Role of phosphatases

A number of phosphatases have been considered as possible players in the phosphorylation dependent events that regulate dopamine transport. In striatal preparations inhibitors including okadaic acid, an inhibitor of serine/threonine phosphatases have been found to decrease the activity of the transporter and also to enhance its phosphorylation (Vaughan et al., 1997). The pharmacology of this effect and also in vitro biochemical studies support that it is most likely protein phosphatase 1 (PP1) that is responsible for this dephosphorylation (Foster et al., 2003). As is the case for the protein kinase C-activated increase in phosphorylation, the majority of phosphorylation sites that become dephosphorylated by PP1 are located in the N-terminus of the transporter (Foster et al., 2002). Because the effects of okadaic acid have not been as thoroughly investigated as the effect of protein kinase C, it is not possible to conclude whether the decrease in transporter activity results from a direct phosporylation of the dopamine transporter or a phosporylation site in other proteins involved in transporter redistribution. The effect of okadaic acid and PMA are additive in some cell systems (Vaughan et al., 1997) but not in others (Granas et al., 2003) suggesting that the mechanism for the decrease in activity could be mediated through different pathways. Interestingly in a study of the related serotonin transporter (SERT), it was found that another okadaic acid sensitive protein phosphatase 2A (PP2A) forms a PMA-sensitive physical complex with SERT (Bauman et al., 2000). This study also found PP2A existed in a complex with both the dopamine transporter and the norepinephrine transporter in native tissue.

7. Regulation of dopamine transport activity by protein–protein interactions

7.1. Oligomerization

Recent studies have investigated whether the transporters work as single units or they form complexes with each other and whether this could have an effect on transport activity. Several different studies have addressed these issues, and it has in fact been possible to show that the transporters do exist as complexes or oligomers (Hastrup et al., 2001; Sorkina et al., 2003; Torres et al., 2003).

It has been difficult to determine whether oligomerization is important for the activity of the transporters or for the stability of the transporters, however, oligomerization has clearly been established to have a role in facilitating the trafficking of the transporters to the surface. Several reports have shown that mutants in which the interaction domains have been mutated are not efficiently delivered to the cell surface (Hastrup et al., 2001; Torres et al., 2003). To further support this idea it was also found that peptides containing the interaction domain could interfere with the trafficking of the wild type transporter to the surface (Torres et al., 2003). The two studies found two different regions of the transporter to be involved in the interaction. Javitch's group found an interaction between the individual transporters at transmembrane domain 6 (Hastrup et al., 2001) and Caron's group (Torres et al., 2003) found an interaction at transmembrane domain 2.

Recent work using fluorescence resonance energy transfer (FRET) between populations of transporters tagged with either cyan or yellow fluorescent protein to determine where in the cell oligomerization took place established that oligomers exist in intracellular compartments and on the plasma-membrane (Sorkina et al., 2003). It appears that dopamine transporter oligomers form initially in the endoplasmic reticulum and persists as they traffic to the cell surface and during endosomal recycling.

7.2. Proteins that stabilize or restrict dopamine transporter surface expression: PICK1, hic5, syntaxin, α-synuclein

The functional impact of the formation of dopamine transporter dimers or homomultimers has not yet been fully

elucidated. However, several different proteins have now been established to interact with the carrier and to modulate dopamine transport activity. A yeast two-hybrid screen revealed an interaction between the dopamine transporter and the protein PICK1 (protein that interact with C-kinase) (Torres et al., 2001) and also with another protein called Hic-5 (Carneiro et al., 2002). PICK1 was originally identified as a substrate of protein kinase C. The interaction of PICK1 with the dopamine transporter appears to be important for stabilizing the transporter at the surface and thereby increasing its activity. Surprisingly, it was not possible to link the protein to the protein kinase C mediated down-regulation of the transporter. In studies exploring the interaction of the dopamine transporter with Hic-5 which also associates with several other proteins, Hic-5 decreases the amount of transporter expressed on the cell surface and has been proposed to serve as a scaffolding protein.

Another group has investigated dopamine transporter binding to α-synuclein, a protein linked to cell death of dopamine neurons and to the neurodegeneration observed in Parkinson's disease. Like PICK1, α-synuclein stabilizes the dopamine transporter on the surface, leading the study's authors to speculate this persistence of the transporter could cause accelerated oxidative stress and cell death (Lee et al., 2001). Surprisingly in mice where the α-synuclein gene has been knocked out, it has been found that the transporter activity is not altered, but this negative result could be a consequence of long-term adaptive changes (Abeliovich et al., 2000). The effect of α-synuclein on dopamine transport activity has been further complicated by a recent study that replicated the interaction of the two proteins, but obtained an opposite functional effect in which co-expression of α-synuclein inhibited dopamine transport (Wersinger and Sidhu, 2003). Because α-synuclein is implicated in cell death, its level of expression could affect transport activity by an indirect mechanism, and might provide an explanation for the discrepancies between the two studies.

Another protein that interacts with these families of transporters is the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein syntaxin 1A. Syntaxin 1A is a pre-synaptic protein that is involved in vesicular docking and release. Three members of the Na^+/Cl^--dependent carrier family, the GABA transporter 1 (Deken et al., 2000; Quick et al., 1997), the serotonin transporter (Haase et al., 2001) and the norepinephrine transporter (Sung et al., 2003) have been found to interact with this protein, and this interaction appears important for the trafficking of the transporter to the surface. Interestingly, syntaxin 1A may also directly modulate transporters by directly inhibiting their turnover rate (Deken et al., 2000; Sung et al., 2003). Although the reason behind this phenomenon is not clear, the result suggest a single interacting protein can have multiple opposing actions—one that enhances surface expression and the other that reduces activity.

8. Conclusions

Both genetic and pharmacological studies have established that the dopamine transporter serves a pivotal role in limiting dopamine-mediated neurotransmission and ultimately in determining behavior. As the dopamine transporter is a major target for drugs used in treating depression, nicotine addiction, ADHD and for psychostimulant drugs of abuse it is important to understand the precise mechanisms that regulate its functions. It is now well recognized that the transporter can be regulated both at an acute level (as summarized in Table 1) and also chronically at the level of gene activation. Furthermore it is understood that the most commonly observed way of dynamically regulating transporter activity is through the removal and recycling of the protein from the cell surface. Multiple intracellular and

Table 1
Summary of molecules involved in the regulation of the activity and/or number of available active dopamine transporters

Molecule	
Dopamine	↓ (−)[a]
Amphetamine	↓[b]
Methamphetamine	↓[c]
MDMA (Ecstacy)	↓[d]
Cocaine	↑[e]
Ethanol	↑[f]
Dopamine D2 Receptor	↑ (−)[g]
Protein kinase C	↓[h]
Protein phosphatase 1/2A	↑[i]
Protein kinase A	↑[j]
Protein tyrosine kinases	↑[k]
Arachidonic Acid	↓ (−)[l]
Reactive Oxygen Species	↓[m]
Nitric Oxide	↓[n]
PICK1	↑[o]
Hic5	↓[p]
α-Synuclein	↑↓[q]

↑, up-regulation; ↓, down-regulation; (−), this study showed no change of transporter activity following application of this molecule.

[a] (Gulley et al., 2002; Saunders et al., 2000; (Daniels and Amara, 1999)).
[b] (Saunders et al., 2000; Sorkina et al., 2003).
[c] (Fleckenstein et al., 1997b).
[d] (Fleckenstein et al., 1999).
[e] (Daws et al., 2002; Little et al., 2002).
[f] (Mayfield et al., 2001b).
[g] (Cass and Gerhardt, 1994; Dickinson et al., 1999; Mayfield and Zahniser, 2001a; Meiergerd et al., 1993; (Prasad and Amara, 2001)).
[h] (Copeland et al., 1996; Daniels and Amara, 1999; Huff et al., 1997; Kitayama et al., 1994; Melikian and Buckley, 1999; Pristupa et al., 1998; Vaughan et al., 1997; Zhang et al., 1997; Zhu et al., 1997).
[i] (Foster et al., 2003; Granas et al., 2003; Vaughan et al., 1997).
[j] (Batchelor and Schenk, 1998).
[k] (Doolen and Zahniser, 2001; Simon et al., 1997).
[l] (Cass et al., 1991; L'hirondel et al., 1995; Zhang and Reith, 1996; (Ingram and Amara, 2000)).
[m] (Berman et al., 1996; Fleckenstein et al., 1997a).
[n] (Cao and Reith, 2002; Kiss et al., 1999; Pogun et al., 1994).
[o] (Torres et al., 2001).
[p] (Carneiro et al., 2002).
[q] (Lee et al., 2001; Wersinger and Sidhu, 2003).

extracellular signaling events trigger and modulate these processes, but surprisingly little is known about the mechanism by which this is achieved. Undoubtedly future experiments will elucidate the mechanism of and proteins involved in regulation of transporter trafficking. Although it has been shown that protein kinase C activation and subsequent phosphorylation events play a role in regulated internalization, current data does not support the direct phosphorylation of the transporter as an obligate step in the process. It therefore remains to be determined what proteins are the specific targets for phosphorylation. Other acute regulators have been shown to mediate intrinsic changes to transporter activity, and it remains to be demonstrated whether any of these changes are mediated by mechanisms involving carrier phosphorylation, allosteric effects of transporter ligands, the formation of dopamine transporter multimers or interactions with other proteins yet to be identified.

Several proteins have been shown to interact with dopamine transporter and have effects on the activity of the carrier, and future proteomics and genomics experiment will very likely discover even more proteins involved in dopamine transporter regulation. The transporter has been shown to form homo oligomers and this seems to be important for the stability and trafficking of the transporter, but it remains to be shown whether this multimerization could have other functional effects.

The current knowledge of how the dopamine transporter is regulated has been greatly enhanced, but several aspects of the regulation remain to be elucidated. Future research should provide answers to some of the remaining questions and may even lead to the development of drugs with novel mechanisms of action that can help alleviate neuropsychiatric diseases and conditions like affective disorders and drug addiction.

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