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Rapid regulation of dopamine transporter function by substrates, blockers and presynaptic receptor ligands

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Abstract

The extracellular actions of dopamine are terminated primarily through its binding to dopamine transporters and translocation back into dopamine neurons. The transporter thereby serves as an optimal target to regulate dopamine neurotransmission. Although acute pharmacological blockade of dopamine transporters is known to reversibly inhibit transporter function by preventing the binding of its endogenous substrate dopamine, it recently has become clear that dopamine transporter substrates, such as amphetamines, and blockers, such as cocaine, also have the ability to rapidly and persistently regulate transporter function after their direct pharmacological effect has subsided. Presynaptic receptor ligands can also regulate dopamine transporter function. This has been investigated most extensively for dopamine D2 receptors, but there is also evidence for regulation by γ-aminobutyric acid (GABA) $GABA_B$ receptors, metabotropic glutamate, nicotinic acetylcholine, serotonin, σ_2- and κ-opioid receptors. The focus of this review is the rapid, typically reversible, regulation of dopamine transporter velocity by substrates, blockers and presynaptic receptor ligands. The research discussed here suggests that a common mechanism through which these different classes of compounds regulate transporter activity is by altering the cell surface expression of dopamine transporters.

Keywords: DAT (dopamine transporter); Functional regulation; Trafficking; Presynaptic receptor; Substrate-mediated regulation

1. Introduction

Following its release, dopamine's interaction with pre- and postsynaptic receptors is terminated primarily via high affinity uptake by plasmalemmal dopamine transporters. By removing extracellular dopamine and recycling it back into the neuron, the transporter plays a key role in shaping neurotransmission mediated by the nigrostriatal and mesocorticolimbic dopaminergic pathways. Regulation of dopamine transporter function is therefore likely to have profound effects on signaling by neurons in these pathways. An extreme example of this comes from studies in mice with a targeted gene deletion of the dopamine transporter produced by homologous recombination (Gainetdinov and Caron, 2003; Giros et al., 1996). Relative to wild-type controls, dopamine transporter knockout mice have marked reductions in tyrosine hydroxylase-mediated dopamine synthesis (Jaber et al., 1999; Jones et al., 1998a), presynaptic vesicular dopamine stores (Jones et al., 1998b), and stimulated dopamine release (Benoit-Marand et al., 2000; Jones et al., 1998a). In addition, the protein levels and function of presynaptic dopamine D2 autoreceptors and postsynaptic dopamine D1 and D2 receptors are significantly down-regulated (Giros et al., 1996; Jones et al., 1999). These appear to be compensatory changes in response to the markedly enhanced concentrations of extracellular dopamine in the null mutant mice. For example, following single-pulse stimulation of the medial forebrain bundle, extracellular dopamine in the striatum is five times higher than that measured in wild-type controls and has a 300-fold longer lifetime (Jones et al., 1998a). Consistent with enhanced dopaminergic neurotransmission, dopamine transporter knockout mice are hyperactive and exhibit cognitive and sensorimotor gating deficits (Giros et al., 1996; Spielewoy et al., 2000).

Although deletion of the dopamine transporter offers a powerful tool to study the transporter's role in dopaminergic

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neurotransmission, more transient regulation of transporter activity is an important mechanism by which functional changes can occur in the native system. Of course, it has long been appreciated that monoamine transporter blockers like cocaine reversibly inhibit uptake activity by competing with substrate for binding to the transporter. However, recent studies have shown that transporter activity can be altered more persistently after exposure to and removal of transporter substrates and blockers, as well as presynaptic receptor ligands. Changes in transporter conformation due to posttranslational modification(s) and resulting changes in either affinity of the transporter for dopamine, Na^+ or Cl^-, or translocation kinetics could explain this rapid (minutes) regulation. Alternatively, changes in the number of transporters expressed at the cell surface could be the explanation. Interestingly, the bulk of the evidence supports the latter mechanism.

Thus, Na^+/Cl^- dependent neurotransmitter transporters, which include transporters for dopamine, norepinephrine, serotonin (5-hydroxytryptamine; 5-HT), γ-aminobutyric acid (GABA), and glycine, undergo trafficking to and from the cell membrane and these events are regulated by a variety of intrinsic cellular processes (Beckman and Quick, 1998; Blakely and Bauman, 2000; Hoffman et al., 1998; Reith et al., 1997; Robinson, 2002; Torres et al., 2003b; Zahniser and Doolen, 2001). Transporter trafficking is a constitutive process (Deken et al., 2003; Hicke, 1999; Loder and Melikian, 2003), allowing rapid up- and down-regulation of transporter expression at the cell surface and, thus, transport activity. For example, activation of protein kinase C pathways by phorbol esters inhibits dopamine transporter function by reducing the maximal velocity of transport (i.e., V_{max}) in human dopamine transporter-expressing *Xenopus laevis* oocytes (Zhu et al., 1997), heterologous cells expressing the human or rat forms of the dopamine transporter (Huff et al., 1997; Kitayama et al., 1994; Pristupa et al., 1998; Zhang et al., 1997) and synaptosomes prepared from rat striatum (Copeland et al., 1996; Vaughan et al., 1997). The number of dopamine transporter binding sites on the surface of the oocytes, as well as cell-surface expression measured by electrophysiology, is also reduced (Zhu et al., 1997). These effects are due, at least in vitro, to enhanced transporter internalization and are blocked by protein kinase C inhibitors such as staurosporine (Chang et al., 2001; Daniels and Amara, 1999; Melikian and Buckley, 1999). Transporter substrates and blockers are also capable of modifying dopamine transporter function, and in this review, we focus on the rapid, typically reversible, regulation in function that they produce. We also discuss the role of receptor-mediated regulation of transporter function, with particular emphasis on the dopamine D2 receptor. Several recent reviews discuss chronic regulation of transporters and its influence on various signaling pathways and, ultimately, on behavior (Gainetdinov and Caron, 2003; Torres et al., 2003b; Zahniser and Doolen, 2001). Thus, discussion of chronic regulation will not be a focus here.

2. Measuring dopamine transporter function

2.1. Model and native cell systems

The function of the dopamine transporter has been studied in vitro, using heterologous cells, primary neuronal cultures, and brain tissue preparations, and in vivo, in the intact brains of rodents and primates. The cloning of the human and rat dopamine transporters (Giros et al., 1992; Kilty et al., 1991; Shimada et al., 1991) and subsequent development of model cell systems (Eshleman et al., 1995), in particular, have afforded great advances in the study of the mechanisms that underlie functional regulation of transporters. In these studies, eukaryotic cells that normally do not express transporter proteins are transfected either transiently or stably with cDNA for the human or rat forms of the dopamine transporter. The cell types used are of either non-neuronal or neuronal origin; examples of the former include *X. laevis* oocytes and human embryonic kidney 293, Madin-Darby canine kidney, African green monkey kidney (i.e., COS-7), porcine kidney epithelial (i.e., LLC-PK1) and porcine aortic endothelial cells, while examples of the latter include rat adrenal pheochromocytoma (i.e., PC12), rat C6 glioma, mouse neuroblastoma (i.e., Neuro-2a) cells and an immortalized dopaminergic cell line $1RB_3AN_{27}$. In native cells, dopamine transporters have been studied using primary neuronal cultures, synaptosomes, brain minces, or slices, and in situ in the intact brain of anesthetized or awake, behaving animals. The most commonly studied brain areas are the dorsal striatum and nucleus accumbens, which contain the nerve terminals of the dopaminergic nigrostriatal and mesolimbic neurons, respectively, in which dopamine transporters are highly localized relative to the rest of the brain (Marshall et al., 1990; Richfield, 1991).

The diverse range of available experimental systems has provided valuable insight into the function and regulation of transporters, but it has also reinforced the importance of considering cellular context when interpreting and comparing results from different studies. For example, the affinity of the transporter for dopamine is lower (3–10 fold) in most heterologous cells compared to striatal synaptosomes from the rat (Giros and Caron, 1993). In addition, the human dopamine transporter expressed in human embryonic kidney 293 cells, compared to dopamine transporters in the dorsal striatum or nucleus accumbens of the rat, appears to have unique kinetic properties. In transporter-expressing cells, dopamine is transported into the cell following an ordered binding process consisting of two Na^+ ions binding to the transporter, followed by dopamine and then one Cl^- ion binding (Chen et al., 1999; Earles and Schenk, 1999). In the rat, the kinetics of binding appears to be a partially random mechanism where either dopamine or Na^+ ions bind first followed by Cl^- binding (Amejdki-Chab et al., 1992a,b; McElvain and Schenk, 1992; Povlock and Schenk, 1997). The functional significance of these differences between dopamine transporters in heterologous cells compared to

brain are not entirely clear; they may reflect differences between rodent and human transporters, or they may reflect differences in native and non-native systems. Notably, there are variations in glycosylation state in the dopamine transporter from brain compared to transporter expressed in cells (Patel et al., 1993) and between specific brain regions in the rat (Lew et al., 1992). Glycosylation state may impact membrane expression and/or trafficking of the transporter protein (Rademacher et al., 1988), although recent studies suggest otherwise (Torres et al., 2003a). Another factor to consider in cells transfected with the transporter is that protein expression levels will vary both within and between cell lines. For example, in oocytes transfected with human dopamine transporters and dopamine D2 receptors, receptor-mediated regulation of transporter function is dependent on protein expression level (Mayfield and Zahniser, 2001). Although the various caveats of experiments in model and native systems need to be recognized, they should not preclude the use of the different techniques and they should not remove confidence that the results obtained in these various systems, when evaluated together, can elucidate the mechanisms that underlie dopamine transporter regulation.

2.2. Functional assays

The methods most often used for measuring dopamine transporter function are biochemical uptake assays, electrophysiological recording of transport-associated currents and measurement of dopamine clearance with microdialysis or voltammetry techniques. Biochemical assays quantify dopamine transporter function by determining the uptake of dopamine (most often radiolabeled) in transporter-expressing heterologous cells or brain tissue synaptosomes, minces, or slices. Uptake, which measures intracellular accumulation of dopamine, is analyzed after samples are exposed in vitro to various concentrations of substrate, blocker and/or other pharmacological agent. It can also be analyzed "ex vivo", using tissue samples taken from animals treated in vivo with drugs. One of the most important issues with this type of assay is the correct definition of nonspecific uptake. This can be accomplished by measuring uptake in the presence of a concentration of inhibitor 100–1000 times its affinity, in the absence of Na^+ (with substitution of a cation like choline to maintain osmolarity), at 4 °C, or in similar cells not expressing the dopamine transporter. Nonspecific uptake is subtracted from total uptake, both at the same concentration of substrate, to determine specific uptake by the dopamine transporter. Uptake should be measured under time and tissue conditions when the response is linear, and it is important to note that, depending on the assay temperature used, transporter trafficking may still continue to occur at a relatively rapid rate. Measuring uptake with a single subsaturating concentration of substrate will provide an indication of whether or not function has changed. However, uptake with a saturating concentration of substrate, and preferably full kinetic analysis, is needed in order to determine whether the functional change is due to a change in transporter affinity or velocity.

More recently, two-electrode voltage-clamp techniques have also been used to measure "transport-associated currents" as a real-time measure of dopamine transporter function. These currents reflect the movement of positive charges into the cell, which results from the transport of the positively charged dopamine molecule and the required co-transport of Na^+ and Cl^- ions. Transport-associated currents are relatively small and are measured as subtractive currents, i.e. before and after substrate application. Additional leak conductances are also associated with the dopamine transporter but are not as useful for quantitating changes in transporter activity. The magnitudes of steady-state dopamine transport-associated currents are directly proportional to the amount of substrate translocated and can be used as an index of changes in transporter function (Saunders et al., 2000; Sitte et al., 1998; Sonders et al., 1997). This technique is typically used in model systems (e.g., human dopamine transporter-expressing *Xenopus* oocytes or human embryonic kidney 293 cells), but current-clamp recordings also have been described recently in cultured dopaminergic neurons (e.g., Ingram et al., 2002; Prasad and Amara, 2001). In oocytes, the "on" and "off" capacitive transient components of dopamine-induced transport-associated currents have been measured at different voltages and used as a measure of cell surface area and, thus cell surface expression (Zhu et al., 1997). Using this technique, changes in cell surface expression of the dopamine transporter have been demonstrated by measuring capacitive transients before and after specific treatments.

Microdialysis and voltammetry have been used to measure changes in local extracellular dopamine concentrations and to relate these changes to dopamine transporter function, typically by using pharmacological methods. Microdialysis measures local concentrations of dopamine within discrete brain regions. This is accomplished by perfusion of artificial cerebral spinal fluid through a dialysis probe, followed by subsequent separation of dopamine and other neurochemicals in the resulting perfusate. Dopamine, the concentration of which is the net result of release and uptake, collects in the perfusate via exchange diffusion. A quantitative version, no-net flux microdialysis, allows a precise estimation of basal, extracellular dopamine levels using perfusion of dopamine concentrations that bracket the expected concentration. The in situ level is therefore the concentration at which the dopamine inflow is equal to the outflow—the concentration of no net-flux (Justice, 1993). In vivo probe recovery, which is referred to as the extraction fraction, is used to assess the effects of experimental manipulations on transporter-mediated uptake. Changes in the extraction fraction have been shown to reflect alterations in neurotransmitter uptake that are not confounded by release or metabolism (Parsons and Justice, 1994; Smith and Justice, 1994). Thus, no-net flux provides a rigorous method for measuring functional changes that result from

alterations in transporters. Microdialysis is used both in vitro, typically in brain slices, and in vivo, in either anesthetized or awake, behaving animals. Voltammetry, which is also used in vitro with cells, tissue minces or brain slices and in vivo in anesthetized or behaving animals, assesses changes in local neurotransmitter concentrations by measuring electrical currents that result from the oxidation and reduction reactions induced by application of small voltages through an electrochemical electrode. Voltammetric measures of dopamine, which include signal rise time, maximal amplitude, and decay time, are obtained in the striatum and nucleus accumbens after its stimulated release (e.g., Garris and Wightman, 1994) or following its exogenous application (e.g., Zahniser et al., 1998). Detailed kinetic analyses have indicated these measures are reflective of uptake by the dopamine transporter and can be disassociated from release and diffusion (Nicholson, 1995; Wightman et al., 1988; Zahniser et al., 1999). In vivo microdialysis and voltammetry are particularly appealing because they offer measurement of transporter function in the native system, even while animals are behaving, but they suffer from their indirect nature and often are unable to fully elaborate the specific mechanisms that underlie observed effects.

3. Regulation of function by substrates

Substrates for the dopamine transporter, which bind to the transporter and are translocated across the membrane, can produce marked down-regulation of transporter function subsequent to their binding. The substrates that have been studied most frequently include amphetamines, dopamine and tyramine. Those with the most potent and consistent effects appear to be methamphetamine and amphetamine.

3.1. Amphetamines

In striatal synaptosomes from rats sacrificed 1 h after a single subcutaneous injection of methamphetamine, [^{3}H]dopamine uptake is reduced, compared to saline-injected controls, by ~35% and ~65% after doses of 5 and 15 mg/kg, respectively (Fleckenstein et al., 1997). This acute effect is due to reduced dopamine transporter V_{max} with no apparent change in the transporter's affinity for dopamine (i.e., K_m), suggesting that uptake is reduced due to a loss of transporters or a kinetic inactivation and not due to residual methamphetamine in the tissue. Functional down-regulation is reversible and is not observed when rats are sacrificed 24 h after methamphetamine injection. Furthermore, it occurs in the absence of changes in dopamine transporter protein levels or changes in binding of the cocaine analog [^{3}H]WIN 35,428 (Kokoshka et al., 1998) and appears unrelated to methamphetamine-induced hyperthermia and neurotoxicity (Metzger et al., 2000). Other psychostimulants that act as transporter substrates that have been reported to reduce dopamine transporter function after a single in vivo injection include amphetamine and methylenedioxymethamphetamine (i.e., MDMA or "ecstasy"; Fleckenstein et al., 1999; Metzger et al., 1998).

Investigation of the time course of methamphetamine-induced down-regulation of dopamine transporters using rat striatal synaptosomes incubated in vitro with a range of drug concentrations reveals that 1 or 10 μM methamphetamine for 30–60 min or as little as 5 min, respectively, reduces [^{3}H]dopamine uptake by 40–60% (Kim et al., 2000; Sandoval et al., 2001). This effect is likely not due to changes in membrane potential or residual methamphetamine and is blocked by the protein kinase C inhibitors bisindolylmaleimide I and NPC15437 (Kim et al., 2000; Sandoval et al., 2001). Amphetamine-induced reductions in transporter function can also occur on a relatively rapid time scale. For example, incubation of striatal synaptosomes with 10 μM amphetamine for 60 min reduces [^{3}H]dopamine uptake by ~30% compared to vehicle control (Kim et al., 2000). In human dopamine transporter-expressing oocytes, transport-associated currents are reduced, compared to baseline, by up to 45% after seven 1-min exposures and up to 75% by thirteen 1-min exposures to 2 μM amphetamine (Gulley et al., 2002). Amphetamine-induced decreases in transporter function are completely blocked by pre-exposure to the protein kinase C inhibitor bisindolylmaleimide I, but not by its inactive analog bisindolylmaleimide V (Gulley et al., 2002). It is not clear whether methamphetamine- and amphetamine-induced regulations require only binding of the substrate to the transporter or actual translocation of the substrate.

Methamphetamine- and amphetamine-induced decreases in dopamine transporter activity, which are characterized by changes in transporter V_{max} rather than K_m, are suggestive of drug-induced changes in transporter trafficking. Support for such a mechanism comes from studies of exposure of dopamine transporter-expressing cells to amphetamine. In human embryonic kidney 293 cells expressing fluorescently tagged human dopamine transporters, exposure to 2 μM amphetamine for 60 min reduces both [^{3}H]dopamine uptake and transport-associated currents (Saunders et al., 2000). Confocal microscopy indicates this functional inhibition is due to trafficking of transporter away from the cell surface, as florescence moves from the outer cell membrane, where the majority of dopamine transporters are localized before incubation with amphetamine, and accumulates intracellularly. Internalization of the transporter is evident as early as 20 min after amphetamine exposure and is prevented by co-incubation with transporter blockers such as cocaine and mazindol (Saunders et al., 2000). Amphetamine-induced internalization can also be blocked by insulin, which activates phosphatidylinositol 3-kinase (Carvelli et al., 2002). A role for this signaling pathway in dopamine transporter trafficking was further supported in studies where constitutively active phosphatidylinositol 3-kinase was transiently transfected in human dopamine transporter-expressing hu-

man embryonic kidney 293 cells. Compared to control cells transfected with an empty vector, phosphatidylinositol 3-kinase activation results in a ~ 30% increase in [^{3}H]dopamine uptake and prevents amphetamine-induced transporter internalization (Carvelli et al., 2002). When considered together with results that show amphetamine-induced reductions in transporter function are the result of dynamin-dependent, clathrin-mediated endocytosis (Saunders et al., 2000) and are not due to nascent transporter failing to leave the cytosol or de novo synthesis of new transporter protein (Carvelli et al., 2002), drug-induced changes in transporter trafficking are the likely mechanism for the down-regulation of transporter function produced by the amphetamines.

3.2. Dopamine and tyramine

Dopamine and tyramine can also reduce dopamine transporter function, but conflicting reports exist in the literature for the effects of dopamine. Exposing human dopamine transporter-expressing oocytes to 10 μM tyramine for 1 min up to 13 times in an hour leads to a modest, but consistent reduction in transport-associated currents of ~ 40% compared to baseline (Gulley et al., 2002). Both 3 and 10 μM dopamine reduce transport-associated currents in oocytes, with the lower concentration decreasing currents by >70% after thirteen 1-min exposures and the higher concentration decreasing currents by >65% after seven 1-min exposures (Gulley et al., 2002). Similar to the effects of amphetamine in human embryonic kidney 293 cells expressing fluorescently tagged human dopamine transporters, 60-min exposure to 10 μM dopamine reduces subsequent uptake of [^{3}H]dopamine (Saunders et al., 2000). In contrast, this same relatively high dopamine concentration has no effect, after a 40-min exposure, in Madin-Darby canine kidney cells expressing green fluorescent protein-tagged human dopamine transporters (Daniels and Amara, 1999). Whether or not the discrepancy in the results from dopamine transporter-expressing canine kidney cells is due to differences in the model systems employed or other aspects of experimental design is not clear.

Recently, however, the ability of locally applied dopamine to alter subsequent transporter function in vivo has been described (Gulley et al., 2002). Using voltammetry to monitor exogenous dopamine clearance in anesthetized rats, brief, repeated applications of dopamine (~ 15 pmol) were shown to markedly inhibit the ability of the transporter to clear subsequent applications of dopamine in the dorsal striatum. In these studies, dopamine signal parameters were increased by up to 250% above baseline, an effect consistent with inhibition of dopamine transporter function. Furthermore, these changes were seen consistently when dopamine was applied every two, but not every 3 or 5 min. They were not seen after repeated vehicle application, and they were reversed when the time between dopamine applications was increased to five min (Gulley et al., 2002). The importance of a 2-min application interval for dopamine-induced regulation of the transporter in this study is unclear, but it may provide clues about the time course of constitutive dopamine transporter recycling in vivo. It is just as likely, however, that this time interval simply represents an arbitrary point at which dopamine-induced functional changes could be easily observed. Regardless, these studies emphasize that the regulation of the dopamine transporter by its endogenous substrate can occur in vivo. However, because of the relatively high concentrations of dopamine and the long exposure times used in all of the studies to date, the physiological relevance of dopamine-induced down-regulation of the dopamine transporter to native dopaminergic neurotransmission remains to be explored.

Substrate-induced internalization may also underlie dopamine-induced decreases in dopamine transporter function. When human embryonic kidney 293 cells expressing the fluorescently tagged human dopamine transporter are exposed to 100 μM dopamine, there is an increased localization of the transporter in the cytosol versus the cell membrane (Saunders et al., 2000). In addition, 10 μM dopamine decreases [^{3}H]WIN 35,428 binding in human dopamine transporter-expressing oocytes by ~ 37%, which is similar in magnitude to the effect of this treatment on transport-associated currents (Gulley et al., 2002). Specific binding of [^{3}H]WIN 35,428 to intact oocytes measures dopamine transporters present on the cell surface because it is dependent on the presence of relatively high Na^+ concentrations (Reith and Coffey, 1993; S. Doolen and N.R. Zahniser, unpublished observations), and the Na^+ concentration inside oocytes is low (~ 6 mM; Barish, 1983) relative to the extracellular superfusion buffer (96 mM). Thus, it appears that dopamine, like amphetamines, can down-regulate dopamine transporter function and this effect may be due to substrate-induced internalization of the transporter.

3.3. Regional specificity and physiological significance

Although substrate-induced regulation of dopamine transporters has been shown in both model and native systems, there is some indication that transporters may be differentially regulated in certain brain regions. Specifically, methamphetamine-induced decreases in [^{3}H]dopamine uptake are observed in synaptosomes prepared from dorsal striatum, but not in those from nucleus accumbens (Kokoshka et al., 1998). Likewise, repeated application of exogenous dopamine at 2-min intervals inhibits dopamine transporter function in dorsal striatum, but not in nucleus accumbens (Gulley et al., 2002). This resistance to dopamine-induced regulation, which is also observed in the nucleus accumbens of awake, unrestrained rats (Kiyatkin et al., 2000), may be due in part to differences in dopamine transporter function in the nucleus accumbens versus the striatum. For example, the rate of uptake is slower in the nucleus accumbens (Cass et al., 1992; Garris and Wightman, 1994; May and Wightman, 1989; McElvain and Schenk, 1992; Wu et al., 2001), which likely reflects a lower density of transporters compared to the

striatum (Cass et al., 1993b; Marshall et al., 1990; Mennicken et al., 1992; Richfield, 1991). One possibility is that, because of the reduced uptake capacity in the nucleus accumbens, it is physiologically important for the transporter to be resistant to substrate-induced regulation. However, the basis for why substrate-mediated down-regulation of function would be less likely to occur in nucleus accumbens, than in dorsal striatum, remains to be established and is a particularly intriguing question.

The physiological significance of substrate-induced *down-regulation* of function, which may seem to be a paradoxical response to persistent elevations in neurotransmitter levels, is also unclear. Because the primary function of transporter proteins appears to be neurotransmitter clearance, it might be expected that repeated substrate treatment would lead to an up-regulation of function, such as has been seen with the GABA transporter GAT1 (Beckman et al., 1999). On the other hand, dopamine can be cytotoxic (Rabinovic et al., 2000), and down-regulation of the dopamine transporter induced by relatively high extracellular concentrations of dopamine may represent a protective mechanism for dopaminergic neurons. Consistent with this suggestion is the finding that dopamine applied to striatal synaptosomes can inhibit dopamine transporter function through its oxidation and promotion of free radicals (Berman et al., 1996).

4. Regulation of function by blockers

Compared to substrate-induced down-regulation, the acute regulation by the transporter blocker cocaine appears to be in the opposite direction—an up-regulation of dopamine transporter activity. However, acute functional regulation by other blockers has been reported only infrequently, so generalizations about the effects of blockers should be made with care. Nevertheless, up-regulation of dopamine transporters in response to repeated cocaine administration has been described in both humans and animals, but in many cases transporter binding, rather than function, has been measured (see Zahniser and Doolen, 2001). For example, postmortem analysis of the brains of human cocaine abusers reveals increases in dopamine transporter sites, as measured by [^{3}H]WIN 35,420 binding in the caudate, putamen, and nucleus accumbens (Little et al., 1993; Staley et al., 1994) and these are positively correlated to the severity of cocaine use at the time of death (Little et al., 1999). Nonetheless, recent in vitro studies utilizing synaptosomal preparations of cryoprotected tissue from deceased human cocaine abusers confirm these binding changes have functional consequences, as [^{3}H]dopamine uptake is increased two-fold in drug users compared to age-matched, drug-free controls (Mash et al., 2002). In vivo single-photon emission computed tomography in recently abstinent cocaine users suggests that dopamine transporters are up-regulated within 96 h of the last drug administration (Malison et al., 1998). Thus, although not all clinical studies report increases in dopamine transporter binding sites (Hurd and Herkenham, 1993; Wilson et al., 1996) and a number of factors may influence the results obtained in human imaging studies (Volkow et al., 2002) or postmortem analysis (Mash et al., 1996), investigation of dopamine transporters in humans suggests that recent exposure to cocaine may up-regulate the transporter. In rats, cocaine-induced up-regulation of dopamine transporter binding in dorsal striatum and nucleus accumbens, as measured by radioligand binding, is observed from a few hours to 3 days after low to high doses (5–40 mg/kg/day) given over multiple days (5–14 days; Koff et al., 1994; Letchworth et al., 1997; Pilotte et al., 1994). Increases in striatal and nucleus accumbens dopamine transporter binding in monkeys are dependent on the length of time the animals self-administered cocaine and the duration of their abstinence before [^{3}H]WIN 35,428 binding is measured (Farfel et al., 1992; Letchworth et al., 2001). Increases in dopamine transporter function may, in fact, underlie reports of ‘‘neurochemical tolerance’’ to cocaine-induced increases in dopamine levels in monkeys self-administering cocaine (Bradberry, 2000).

Cocaine-induced increases in dopamine transporter function have also been reported in rats. In vivo voltammetry and no-net flux microdialysis in rats given 10–30 mg/kg cocaine for 4–10 days indicates uptake is enhanced in the dorsal striatum and nucleus accumbens after 1–3 days of abstinence (Kalivas and Duffy, 1993; Ng et al., 1991; Parsons et al., 1991; Segal and Kuczenski, 1992; Thompson et al., 2000b). In both rodents and monkeys, however, there are other reports suggesting chronic cocaine, rather than up-regulating transporters, down-regulates or has no effect on, dopamine transporter binding and function (Benmansour et al., 1992; Cass et al., 1993a; Chefer and Shippenberg, 2002; Jones et al., 1996; Kalivas and Duffy, 1990; Kimmel et al., 2001; Peris et al., 1990; Pilotte et al., 1996; Sharpe et al., 1991). Some of the discrepancies in these studies may be the result of methodological issues, including the length of withdrawal from repeated cocaine and whether dopamine transporters were assessed in dorsal striatum versus nucleus accumbens.

After only a single cocaine administration, functional analysis also suggests up-regulation of dopamine transporters can occur. For example, 1 h following intraperitoneal injection of 30 mg/kg cocaine, ex vivo analysis of [^{3}H]dopamine uptake in striatal synaptosomes shows a modest, but statistically significant, increase of ~17% above control levels (Fleckenstein et al., 1999). Interestingly, this same dose of cocaine was found in another study to elevate [^{3}H]dopamine uptake even more in synaptosomes from the nucleus accumbens; the V_{max} was increased by 56% above control, with no change in K_m (Daws et al., 2002). Acute, functional up-regulation might also underlie the seemingly contradictory results from in vivo voltammetry experiments that show cocaine, which blocks the transporter’s ability to remove dopamine, can augment dopamine transporter-mediated clearance of exogenously applied do-

pamine (Cass et al., 1993b; David et al., 1998; Daws et al., 2002; Sabeti et al., 2002; Zahniser et al., 1999). Mazindol, another dopamine transporter blocker, was also reported to paradoxically up-regulate transporter function after systemic injection of 1–20 mg/kg given at least 30 min before in vivo voltammetry or microdialysis measurements of dopamine clearance (Ng et al., 1992; Stamford et al., 1986).

Cocaine-induced increases in the function of dopamine transporters have also been demonstrated in model systems, which have been especially useful for defining a potential mechanism for cocaine's effect. In heterologous cells transfected with the human dopamine transporter, [^{3}H]dopamine uptake is increased, compared to control, by 30–40% in cells incubated with 1 or 10 μM cocaine for as little as 60 min and as long as 48 h (Daws et al., 2002; Little et al., 2002). In Neuro-2A cells expressing the human dopamine transporter, incubation with 1 μM cocaine for 24 h leads to a ~46% increase in [^{3}H]dopamine uptake (Zhang et al., 1998). These effects, which are also observed in a human transporter-expressing mouse neuroblastoma cell line (Ho and Segre, 2000), are potentiated by co-incubation with 1% ethanol; cocaine plus ethanol increases uptake ~77% above control (Ho and Segre, 2001). Biotinylation experiments and confocal microscopy of immunofluorescently labeled human dopamine transporter-transfected cells confirm that the effects of cocaine are due to increases in cell surface expression of the transporter (Daws et al., 2002).

Unlike the regional specificity observed with substrate-induced down-regulation, cocaine-induced up-regulation of dopamine transporters has been noted in both the dorsal striatum (Fleckenstein et al., 1999) and nucleus accumbens (Daws et al., 2002). The effect of cocaine on [^{3}H]dopamine uptake actually appears to be more potent in the nucleus accumbens. However, estimates of turnover kinetics of the dopamine transporter protein suggest that repeated systemic administration of cocaine increases the half-life of the dopamine transporter in striatum, but not nucleus accumbens (Kimmel et al., 2003). Increases in transporter half-life might have a net effect of increasing dopamine uptake. In any case, it will be very interesting to compare the results just discussed for cocaine with those from future experiments investigating the consequences of acute exposure to other transporter blockers, in particular more selective dopamine transporter blockers like GBR 12909, on dopamine transporter function and expression. As long as increased transporter activity is observed, it seems unlikely that the effects are due to residual drug binding to the transporter.

5. Regulation of function by presynaptic receptor ligands

Neurotransmitters and drugs that bind to a number of different presynaptic receptors on dopaminergic neurons have the capacity to regulate dopamine transporter function as well. Dopamine receptors and the D2 subtype in particular, have been the focus of several studies that, when taken together, suggest that activation of dopamine D2 receptors up-regulates transporter function both in vivo and in vitro. Activation of $GABA_B$, metabotropic glutamate, nicotinic acetylcholine, 5-HT, σ_2, and κ-opioid receptors has also been shown to regulate acutely dopamine transporter function.

5.1. Dopamine receptors

It is well known that activation of presynaptic dopamine D2 receptors on dopamine neuronal terminals, or the so-called terminal "autoreceptors", regulates dopamine synthesis and release in an inhibitory manner (Langer, 1997; Starke et al., 1989). Recent evidence suggests that they may modulate dopamine transporter function as well, but in a stimulatory manner. Thus, activation of all three types of autoreceptors localized on dopamine neuronal terminals would work in concert to reduce extracellular concentrations of dopamine and thereby limit postsynaptic dopamine receptor stimulation.

In minces from rat striatum, the velocity of uptake of 1 μM dopamine, measured by voltammetry, is increased by application of 100 nM quinpirole, a dopamine D2 receptor agonist (Batchelor and Schenk, 1998; Meiergerd et al., 1993). Quinpirole also dose-dependently increases [^{3}H]dopamine uptake into synaptosomes prepared from the nucleus accumbens of ovariectomized and estrogen-primed female rats (Thompson et al., 2000a, 2001). The ability of *N*-(*p*-isothiocyanatophenethyl) spiperone, a selective and irreversible alkylating antagonist of dopamine D2 receptors, to block quinpirole-induced increases in [^{3}H]dopamine uptake confirms the role of dopamine D2 receptor activation in this effect of quinpirole (Thompson et al., 2001). Decreases in dopamine transporter half-life in the striatum are also observed after quinpirole treatment, but this same treatment had the opposite effect in the nucleus accumbens (Kimmel et al., 2001). However, agonists selective for the dopamine D3 receptor, which are members of the dopamine D2 receptor family, have been shown to increase dopamine clearance in the nucleus accumbens (Zapata and Shippenberg, 2002).

Experiments utilizing acute or chronic treatment with dopamine D2 receptor antagonists provided some of the first evidence to suggest dopamine D2 receptor-mediated up-regulation of transporter function. In this case, antagonism of dopamine D2 receptors attenuated dopamine transporter activity. For example, in rats pre-treated with the selective dopamine D2 receptor antagonist pimozide for 10 days, cocaine-induced increases in dopamine uptake in the nucleus accumbens are blocked (Parsons et al., 1993). In vivo voltammetry in the striatum, nucleus accumbens and prefrontal cortex of anesthetized rats showed that local application of the selective dopamine D2 receptor antago-

nist raclopride produces the same effect in all three brain regions: reduced clearance of exogenously applied dopamine (Cass and Gerhardt, 1994). Importantly, the dopamine D1 receptor antagonist SCH 23390 had no effect on clearance, suggesting selective regulation by dopamine D2 receptors. Local application of the dopamine D2 receptor antagonist haloperidol (10 μM) inhibits the clearance of exogenous dopamine by ~60% in the dorsal striatum (Rothblat and Schneider, 1997), whereas acute systemic haloperidol (0.5 mg/kg) inhibits striatal uptake of dopamine released by electrical stimulation by ~42% (Benoit-Marand et al., 2001). The effects of haloperidol on the uptake of electrically evoked dopamine appear to be at least partially dependent on stimulation frequency. At low frequencies, haloperidol concomitantly up-regulates dopamine release and down-regulates dopamine uptake, whereas with high frequency stimulation, dopamine uptake is reduced but release is not affected (Wu et al., 2002). Administration of the irreversible dopamine D2 receptor antagonist *N*-(*p*-isothiocyanatophenethyl) spiperone also significantly reduces exogenous dopamine clearance in nucleus accumbens (Thompson et al., 2001). Similarly, in rat brain slices containing the substantia nigra, the selective dopamine D2 receptor antagonist sulpiride (50 μM) significantly reduces clearance of locally applied dopamine (Hoffman et al., 1999).

The inhibitory effect of dopamine D2 receptor antagonists on dopamine transporter function suggests that, under normal conditions when these drugs are not present, dopamine interacts with dopamine D2 receptors to enhance the uptake of extracellular dopamine. This hypothesis, which is consistent with the role played by these receptors as a short inhibitory feedback loop that regulates extracellular dopamine through modulation of its synthesis and release, is largely supported by studies in mice with a targeted gene deletion of these receptors. In dopamine D2 receptor knockout mice, the clearance of exogenously applied dopamine is reduced by ~50%, compared to wildtype control mice with a normal number of dopamine D2 receptors (Dickinson et al., 1999). Furthermore, local application of raclopride inhibits dopamine uptake in wildtype mice but has no effect in the knockout mice. In vivo microdialysis measurements indicated basal and potassium-stimulated dopamine concentrations are similar in these knockout and wildtype mice (Dickinson et al., 1999). In contrast to these effects, striatal uptake of dopamine released after electrical stimulation in D2 knockout mice has been reported to be increased in vitro (Schmitz et al., 2002) and not changed in vivo (Benoit-Marand et al., 2001), relative to uptake in wildtype mice. The discrepancy with Dickinson et al. (1999) may be due to differences in experimental techniques, namely measurement of clearance of locally applied exogenous dopamine as opposed to clearance of endogenous dopamine stimulated by release. It is also notable that all three studies used dopamine D2 receptor knockout mice generated by different strategies. Regardless, a lack of dopamine D2 receptor-mediated regulation of dopamine transporter function was also observed in mesencephalic neurons maintained in primary culture (Prasad and Amara, 2001). As has often been the case with synaptosomes, however, it may be difficult with cultured neurons to detect significant dopamine D2 receptor regulation using [^{3}H]dopamine uptake experiments. Again, the reason for this is unclear but the longer time course commonly used for uptake measurements may be relatively insensitive for detecting dopamine D2 receptor regulation of dopamine transporter function, compared to the faster electrochemical clearance measurements. Thompson et al. (2000a, 2001), who observed dopamine D2 receptor regulation in synaptosomes from nucleus accumbens, measured [^{3}H]dopamine uptake after only 20 or 60 s of incubation. However, another difference is that they used tissue from ovariectomized and estrogen-primed female rats. It also may be the case that dopamine D2 receptor-mediated dopamine transporter regulation is not present until neurons are fully mature.

Although, as just discussed, there are some inconsistencies in the literature and the reasons for these discrepancies have not yet been reconciled, the majority of studies indicate that activation of dopamine D2 receptors up-regulates dopamine transporter function. Similar to transporter substrates and blockers, a potential mechanism for dopamine D2 receptor-mediated regulation is via changes in transporter trafficking. Indeed, experiments in oocytes co-expressing the human dopamine transporter and the short form of the human dopamine D2 receptor suggest that activation of dopamine D2 receptors results in an increased number of dopamine transporter binding sites localized on the cell surface (Mayfield and Zahniser, 2001). Specifically, increases as large as 70% in dopamine uptake and transport-associated currents were observed when oocytes were exposed to 0.1–10 μM dopamine alone (“maximal dopamine D2 receptor activation”) relative to co-exposure with dopamine and 1 μM sulpiride (“maximal dopamine D2 receptor blockade”). Conditions of maximal dopamine D2 receptor activation were associated with ~60% increases in the cell-surface binding of [^{3}H]WIN 35,428 to the dopamine transporter, and this effect was blocked by pertussis toxin pre-treatment. These results suggest that dopamine D2 receptor-mediated up-regulation of transporter function is due to increases in cell-surface expression of the transporter and that normal dopamine D2 receptor signaling via the inhibitory GTP-binding protein $G_{i/o}$ is required for this regulation. Furthermore, it is noteworthy that the effect of dopamine D2 receptor activation is opposite to dopamine substrate-induced regulation. This suggests that dopamine can regulate the transporter indirectly via activation of dopamine receptors, as well as directly by being a substrate. The fact that neither amphetamine nor tyramine have a high affinity for dopamine D2 receptors also supports a disassociation between substrate-induced and dopamine D2 receptor-mediated effects on transporter function.

5.2. Other presynaptic receptor systems

While dopamine D2 receptor-mediated regulation of dopamine transporters has received the most attention, reports in the literature suggest that several other neurotransmitter receptors, potentially localized on dopaminergic neuronal terminals, can also regulate transporter function. For example, activation of presynaptic $GABA_B$ receptors, which couple to intracellular signaling pathways common to dopamine D2 receptors, may also rapidly up-regulate dopamine transporter function. Indirect evidence for this comes from electrochemical studies in slices containing substantia nigra (Hoffman et al., 1999). In these experiments, co-superfusion with the $GABA_B$ agonist baclophen blocked the dopamine D2 receptor antagonist-induced reduction in dopamine clearance. However, to our knowledge, this has not been explored in brain regions containing dopaminergic neuronal terminals.

Activation of group 1 metabotropic glutamate (mGlu) receptors by a 5-min application of the selective agonist (*S*)-3,5-dihydroxyphenylglycine also leads to a reduction in [^{3}H]dopamine uptake in striatal synaptosomes (Page et al., 2001). This inhibitory effect appears to be mediated by phosphorylation of the transporter through activation of Ca^{2+} calmodulin-dependent kinase II and protein kinase C, triggered by agonist binding at subtype five of mGlu receptors ($mGlu_5$). This regulatory interaction provides yet another potentially important way in which glutamate can directly regulate dopaminergic neurotransmission.

The effects of nicotinic acetylcholine receptor activation on dopamine transporter function also appear to be mediated in part through protein kinase C. In brain slices of the prefrontal cortex, activation of these receptors by nicotine or other cholinergic agonists increases amphetamine-stimulated [^{3}H]dopamine release (Drew et al., 2000). These changes in amphetamine-stimulated release of dopamine are thought to reflect increases in transporter function because amphetamine causes the reverse transport of dopamine from inside to the outside of the cell via the dopamine transporter (Raiteri et al., 1979; Sulzer et al., 1993). This type of regulation requires activation of α-4/β-2 subunit-containing nicotinic receptors and is not observed in the dorsal striatum or nucleus accumbens. Furthermore, protein kinase C inhibitors, as well as Na^+ channel blockers, Ca^{2+} chelators and Ca^{2+} channel blockers, significantly inhibit nicotinic receptor-mediated increases in amphetamine-stimulated dopamine release (Drew and Werling, 2001; Huang et al., 1999). It may seem paradoxical that activation of protein kinase C via mGlu and nicotinic acetylcholine receptors would have opposite effects on dopamine transporter function. However, one possible explanation involves differences between dopamine uptake by the transporter and amphetamine-stimulated dopamine release by reverse transport. Regulation of inward versus outward transport of dopamine is likely governed by different mechanisms. Specifically, Kantor and Gnegy (1998) showed that activation of protein kinase C by phorbol esters increases amphetamine-induced dopamine release from rat striatal slices, whereas similar phorbol ester treatment reduces the number of dopamine transporters expressed at the cell surface (see Section 1). It also is noteworthy that the effects of nicotine are not dependent on Ca^{2+} calmodulin-dependent kinase II, whereas the effects of mGlu receptor agonists are dependent it (Drew and Werling, 2001). Still another factor to consider is the problem of disassociating influences on amphetamine-mediated dopamine release from effects on more traditional, exocytosis-mediated dopamine release. Unless particular steps are taken, for example measuring dopamine using no-net flux microdialysis or under low Ca^{2+} concentrations (e.g., Drew and Werling, 2001), separating the two is difficult.

Changes in amphetamine-stimulated dopamine release have also suggested regulation of dopamine transporters by 5-HT and σ_2 receptor agonists. For example, *R*(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin (*R*(+)-8-OH-DPAT), a 5-HT_{1A} receptor agonist, inhibits amphetamine-stimulated dopamine release in the dorsal striatum and nucleus accumbens of freely moving rats (Ichikawa et al., 1995). In contrast, activation of 5-$HT_{2A/2C}$ receptors with the selective agonist DOI leads to an increase in amphetamine-stimulated dopamine release in the prefrontal cortex, dorsal striatum and nucleus accumbens (Ichikawa and Meltzer, 1995; Kuroki et al., 2003). Pentazocine, a selective σ_2 receptor agonist, also enhances amphetamine-stimulated dopamine release and this effect has been demonstrated in PC12 cells expressing the human dopamine transporter (Weatherspoon and Werling, 1999) and in brain slices containing the striatum (Izenwasser et al., 1998). Increases in amphetamine-stimulated dopamine release that are mediated by σ_2 receptors appear to be dependent on activation of protein kinase C and voltage-dependent Ca^{2+} channels, but independent of Ca^{2+} calmodulin-dependent kinase II activity (Derbez et al., 2002).

In vitro voltammetry and in vivo no-net flux microdialysis, which more precisely assess changes in dopamine uptake compared to measures of amphetamine-stimulated dopamine release, have been used to demonstrate κ-opioid receptor-mediated regulation of dopamine transporter function (Thompson et al., 2000b). Specifically, a single injection of the κ-opioid receptor agonist U-69593 (0.10 or 0.32 mg/kg) increases in vitro dopamine uptake into nucleus accumbens minces obtained 1–4 h after drug exposure; this effect is blocked by pre-treatment with the κ-opioid receptor antagonist nor-binaltorphimine (10 mg/kg). When U-69593 is given repeatedly over 5 days, however, both in vitro and in vivo measures of uptake indicated dopamine transporter function is decreased by the drug. This functional down-regulation is region-specific, as it is observed in nucleus accumbens, but not dorsal striatum. Co-administration of U-69593 also blocks repeated cocaine-induced increases in dopamine transporter function in both nucleus accumbens and medial prefrontal cortex (Thompson et al., 2000b; Chefer et al., 2000). These effects of κ-opioid receptor activation on dopamine transporter activity may help to

explain the ability of κ-opioid agonists to antagonize some of the persistent alterations in mesocorticolimbic dopaminergic neurotransmission that result following repeated cocaine administration (Chefer et al., 2000). Although the signaling mechanism by which κ-opioid receptors produce these effects is unknown, Carvelli et al. (2002) have shown that mitogen activated kinases and phosphatidylinositol 3-kinase, which are signaling systems that can be activated by κ-opioid receptor agonists, regulate DAT activity and trafficking.

Taken together, studies of the effects of receptor ligands point to a diverse set of presynaptic receptors that all have the potential to modulate dopamine transporter function. All of the described receptor systems are thought to be present on dopaminergic neurons in the striatum and/or nucleus accumbens, so their potential to regulate dopamine neurotransmission is not surprising. Some clues as to the mechanisms that underlie their regulatory effects have emerged, but there has yet to be confirmation that changes in trafficking of the dopamine transporter play a role.

6. Summary and future directions

Using model and native cell systems and measuring dopamine transporter function both in vitro and in vivo, acute exposure to transporter substrates, transporter blockers, and a variety of presynaptic receptor ligands has been shown to have the capacity to regulate rapidly dopamine transporter function (Fig. 1). At the appropriate concentrations or doses, transporter function is reduced by substrates and enhanced by the blocker cocaine-effects opposite to those produced by direct interaction of substrates and blockers with the transporter. These regulatory effects appear to be due to trafficking of the transporter away from the cell membrane to intracellular locations or recruitment of transporters from the inside of the cell to the membrane, respectively. However, it remains to be unequivocally demonstrated whether decreased cell-surface expression reflects increased internalization or reduced recruitment of internal transporters back to cell surface. Presynaptic receptor ligands also can regulate dopamine transporter function. Acute activation of dopamine D2, $GABA_B$, and κ-opioid receptors increases transporter uptake/clearance, whereas acute activation of $mGlu_5$ and chronic activation of κ-opioid receptors produces the opposite effect. Nicotinic acetylcholine, $5\text{-}HT_{2A/2C}$, and σ_2 receptor agonists increase reverse transport induced by amphetamine; $5\text{-}HT_{1A}$ receptor agonists decrease reverse transport. Whether dopamine transporter function is inhibited or enhanced seems to be dependent on the receptor system activated and the mechanisms for the effects are not clear.

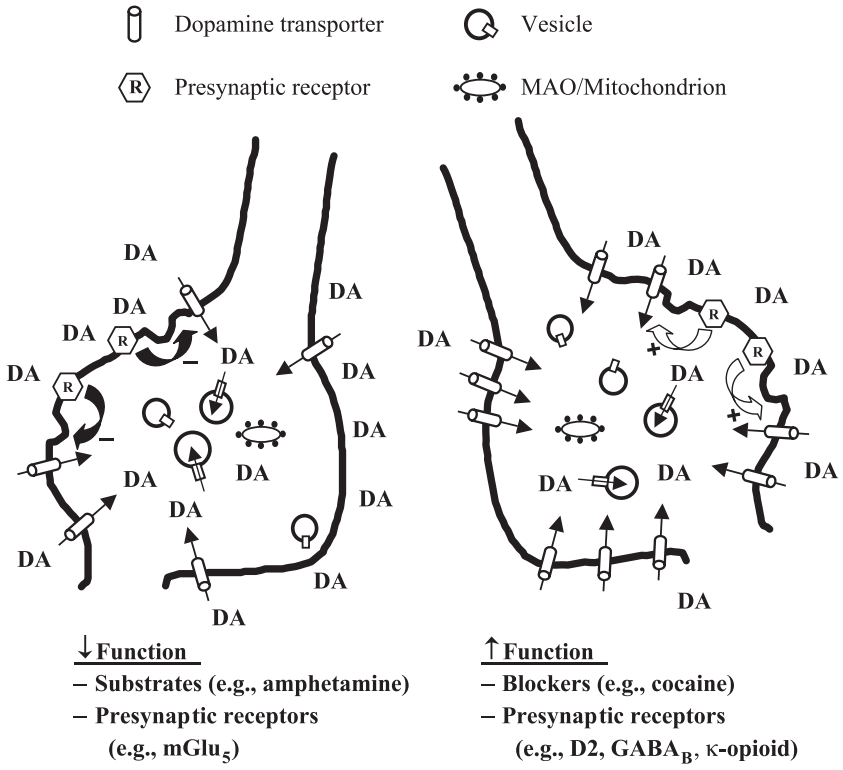

Fig. 1. Schematic diagram of the acute regulation of dopamine transporter function by substrates, blockers and presynaptic receptor ligands. Shown are two dopamine neurons and their respective down- and up-regulation of dopamine transporter function. The relative locations of presynaptic receptors, dopamine transporters and other components of the nerve terminal are for illustration purposes only and should not imply that data suggest this precise localization. The effects of nicotinic acetylcholine, 5-HT, and σ_2 receptor ligands on dopamine release induced by amphetamine-stimulated reverse transport, which are discussed in Section 5.2, are not pictured here. DA—dopamine; MAO—monoamine oxidase.

In the past 10 years, our understanding about the dopamine transporter has evolved substantially beyond the notion that it is a "synaptic vacuum cleaner" that is either "on" or "off" and otherwise relatively static. Some of the important challenges in future studies will be to determine the physiological relevance of dopamine transporter regulation. Does this occur during "normal" dopaminergic neurotransmission or is it more important when drugs are administered? The fact that dopamine can both down-regulate dopamine transporter function directly, by being a substrate, and up-regulate function indirectly, via D2 autoreceptor activation, raises the question of whether these actions occur sequentially or are competitive. Additionally, brain regional differences will be critical to elucidate and understand. Whether all transporter blockers result in up-regulation, similar to that observed following exposure to cocaine, is another critical question to answer and may have important implications for the drug abuse field. Whether the cellular and/or signaling mechanisms underlying substrate-, blocker- and presynaptic receptor ligand-induced regulation of dopamine transporter function are common or distinct also needs to be addressed. A recent, very intriguing study with the GABA transporter GAT1 showed that this transporter traffics in vesicles similar to, but distinct from, synaptic vesicles (Deken et al., 2003). This observation further supports the suggestion that both release and reuptake may be highly and rapidly regulated ways that transmission by neurons can be shaped.

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