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Regulation of monoamine transporters: Role of transporter phosphorylation

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ABSTRACT

Presynaptic biogenic amine transporters mediate reuptake of released amines from the synapse, thus regulating serotonin, dopamine and norepinephrine neurotransmission. Medications utilized in the treatment of depression, attention deficit-hyperactivity disorder and other psychiatric disorders possess high affinity for amine transporters. In addition, amine transporters are targets for psychostimulants. Altered expression of biogenic amine transporters has long been implicated in several psychiatric and degenerative disorders. Therefore, appropriate regulation and maintenance of biogenic amine transporter activity is critical for the maintenance of normal amine homoeostasis. Accumulating evidence suggests that cellular protein kinases and phosphatases regulate amine transporter expression, activity, trafficking and degradation. Amine transporters are phosphoproteins that undergo dynamic control under the influence of various kinase and phosphatase activities. This review presents a brief overview of the role of amine transporter phosphorylation in the regulation of amine transport in the normal and diseased brain. Understanding the molecular mechanisms by which phosphorylation events affect amine transporter activity is essential for understanding the contribution of transporter phosphorylation to the regulation of monoamine neurotransmission and for identifying potential new targets for the treatment of various brain diseases.

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1. Introduction

Overview of Monoamine Transporter Regulation: Neuronal communication is a complex and vital function of the organism. Effective communication in the brain requires precise and dynamic regulation of neurotransmitter concentrations. Many external signals in the form of neurotransmitters and hormones must be integrated and processed by neurons. Serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA) and noradrenaline/norepinephrine (NA/NE) are biogenic monoamine (MA) neurotransmitters synthesized in-vivo or de-novo from tryptophan and tyrosine respectively. Each amine controls distinct behavioral and physiological functions both in the central and peripheral nervous system. 5-HT modulates mood, aggression, motivation, appetite, sleep, cognition and sexual activity. Altered 5-HT signaling has been implicated in mental illnesses related to these biological processes (Coccaro, 1989; Sellers et al., 1992; Compagnon et al., 1993; Owens & Nemeroff, 1994). 5-HT also has important peripheral actions which include regulation of vasoconstriction, gastrointestinal and placental function. DA systems control motor function, mood, reward and cognition (Carlsson, 1987; Koob, 1998). Dysregulation of DA transmission is linked to attention deficit/hyperactivity disorder (ADHD), schizophrenia, addiction, Parkinson's disease and Tourette's syndrome (Bannon et al., 1995). NE controls arousal, mood, attention, stress-responsivenss and affective disorders (Schildkraut, 1965; Klimek et al., 1997; Leonard, 1997; Ressler & Nemeroff, 1999). NE is also the major neurotransmitter in postganglionic sympathetic synapses, and NE uptake sites and activity are compromised in cardiomyopathy, heart failure, hypertension and ischemia (Esler et al., 1981; Liang et al., 1989; Merlet et al., 1992; Bohm et al., 1995; Imamura et al., 1996; Schafers et al., 1998).

At the molecular level, MA signaling is dynamically regulated by a diverse set of macromolecules including biosynthetic enzymes, secretory proteins, ion channels, pre- and postsynaptic receptors and transporters. The presynaptically localized plasma membrane MA transporters for serotonin (SERT), dopamine (DAT) and norepinephrine (NET) exert dynamic spatial and temporal control of extracellular neurotransmitter concentration via re-uptake of released neurotransmitter from the synaptic cleft. SERT (SLC6A4), DAT (SLC6A3) and NET (SLC6A2) belong to one single gene (SLC6) family. MA transporters share a predicted structure of 12 transmembrane domains with intracellular cytoplasmic NH_2 and COOH termini. This topology was recently confirmed by the high-resolution crystal structure of a bacterial homologue, LeuT, of the mammalian MA transporters (Yamashita et al., 2005).

MA transporters are pharmacological targets for various clinically used antidepressants and psychostimulant drugs (Barker & Blakely, 1995; Jayanthi & Ramamoorthy, 2005). Mice lacking the gene encoding DAT, SERT or NET revealed that the mechanisms regulating amine biosynthesis, amine storage, receptor sensitivity and transporter expression are interdependent (Bengel et al., 1998; Gainetdinov et al., 1999b; Rioux et al., 1999; Sora et al., 2001; Hall et al., 2002; Gainetdinov & Caron, 2003; Li et al., 2004; Adriani et al., 2009). The importance of MA transporters to the regulation of neurotransmitter signaling, to disease processes and that they are a target of several drugs of abuse highlights the importance of understanding the cellular and molecular mechanisms by which various signaling pathways modify these proteins and alter their properties and function. Recent advances in the field have provided a wealth of knowledge as to how the expression and functional properties of SERT, DAT and NET are regulated at the gene and protein level. Transporter regulation can occur via phosphorylation dependent and independent post-translational modifications. Post-translational modifications can a) change intrinsic transport activity, b) alter transporter turnover, c) regulate exocytic fusion of transporter containing vesicles with the plasma membrane and d) regulate sequestration of transporter from the plasma membrane by modulating endocytic machinery pathways. Alternatively, regulation of transporter can also occur through their association with other interacting proteins by phosphorylation dependent/or independent pathways (Eriksen et al., 2010).

This review describes one molecular mechanism/modification that alters several key properties of MA transporters, MA transporter phosphorylation. We review various studies that have examined the relationship between changes in transporter phosphorylation state, transporter expression and function. Some earlier aspects of monoamine transporter phosphorylation and regulation can be found in several excellent reviews (Zahniser & Doolen, 2001; Torres et al., 2003; Melikian, 2004; Vaughan, 2004; Zahniser & Sorkin, 2004; Torres & Amara, 2007; Steiner et al., 2008; Eriksen et al., 2010). We also review recent progress in the functional analyses of transporter gene variants identified in human diseases. These data have allowed investigators to begin to identify how phosphorylation affects transporter expression levels, transport properties and drug responses. The quest/potential for developing novel pharmacological applications will be strengthened in the future by defining the role of transporter phosphorylation and refining our search for compromised signals.

2. The serotonin transporter and its functional significance

The uptake of synaptic 5-HT through Na^+/Cl^--dependent SERT is the principal process of terminating serotonergic neurotransmission. More than 15 different types of cell-surface receptors exist to transmit the specific actions of 5-HT on target cells (Glennon & Dukat, 1995), whereas a single gene (Ramamoorthy et al., 1993a) encoding the SERT appears responsible for extracellular 5-HT clearance (Blakely et al., 1991; Hoffman et al., 1991; Lesch et al., 1993b). In addition to serotonergic neurons, SERTs are expressed in peripheral tissue (Rudnick, 1977; Lesch et al., 1993b) including specialized cells of the gut (Gordon & Barnes, 2003), placenta (Balkovetz et al., 1989), lung (Paczkowski et al., 1996), adrenal chromaffin cells (Schroeter et al., 1997), blood lymphocytes (Faraj et al., 1994; Gordon & Barnes, 2003) and platelets (Jayanthi et al., 2005; Carneiro & Blakely, 2006; Carneiro et al., 2008). While clearance of synaptic and extra-synaptic 5-HT appears to be the principal function of SERT, certain cells, notably platelets, utilize SERT to acquire 5-HT from the extracellular environment for subsequent release; a function involved in the process of platelet activation (Cirillo et al., 1999; Musselman et al., 2002). Platelets and 5-HT neurons share many common properties, including vesicular monoamine transporters (VMAT), 5-HT release, pharmacological properties of SERT, identical SERT sequences, and 5-HT receptors (Owens & Nemeroff, 1994). Therefore, platelets have been widely used as a peripheral indicator of central 5-HT metabolism and SERT function (Wirz-Justice, 1988) in psychiatric disorders and vascular diseases in which 5-HT has been implicated (Meltzer et al., 1981). In the lung, SERTs efficiently clear plasma-borne 5-HT and regulate blood 5-HT levels with the aid of platelets.

Numerous SERT antagonists including the serotonin selective reuptake inhibitors (SSRIs), fluoxetine (Prozac™), paroxetine (Paxil™), citalopram (Celexa™) and sertraline (Zoloft™) are effective antidepressants. Documentation of altered SERT expression in various types of psychopathology indicates the importance of SERT expression in maintaining normal brain function (Murphy et al., 2004). Amphetamine derivatives such as fenfluramine, p-chloroamphetamine (PCA) and 3,4-methylenedioxymethampetamine (MDMA or "Ecstasy") are substrates for SERT (Rudnick & Wall, 1992). SERT in turn affords an access pathway to the cytoplasm for these drugs, where interactions with SERT and VMATs can, in concert, lead to nonvesicular 5-HT efflux (Gobbi et al., 1997; Hilber et al., 2005; Seidel et al., 2005; Fontana et al., 2009). SERT-knockout mice have provided unequivocal evidence that SERT is important for synaptic 5-HT clearance, SSRI recognition, MDMA induced hyperactivity and may contribute to the reinforcing effect of cocaine. In addition, these mice showed several compensatory changes in 5-HT levels, 5-HT synthesis

Table 1
Summary of kinase, receptor, substrates and antagonists on SERT regulation.

Regulators	Effects on SERT	References
Protein kinases		
PKC	Activation: Decreased SERT Vmax and surface SERT. Increased SERT endocytosis and phosphorylation. Biphasic effects in platelets: During initial phase, PKC phosphorylates SERT on serine residue(s) and inhibits 5-HT uptake, decreases Vmax and 5-HT affinity without affecting surface SERT. Later phase, PKC phosphorylates SERT on both serine and threonine residues, enhances SERT internalization, inhibits 5-HT uptake decreased Vmax without effecting 5-HT affinity.	(Anderson & Horne, 1992; Qian et al., 1997; Ramamoorthy et al., 1998; Ramamoorthy & Blakely, 1999; Jayanthi et al., 2005; Oz et al., 2010)
p38 MAPK	Inhibition: Decreased SERT Vmax and Km. Decreased SERT basal phosphorylation with or without changes in surface SERT proteins.	(Zhu et al., 2004a; Samuvel et al., 2005; Zhu et al., 2005; Oz et al., 2010)
PKG	Activation: Increased 5-HT uptake and SERT Vmax with no effect on 5-HT Km. Trafficking dependent and/or independent. Phosphorylation of SERT-Thr276 site is required for PKG to stimulate SERT.	(Zhu et al., 2004a; Ramamoorthy et al., 2007)
CaMKII	Inhibition: Decreased 5-HT uptake.	(Jayanthi et al., 1994)
Tyrosine kinase	Inhibition: Decreased 5-HT-uptake and Vmax.	(Helmeste & Tang, 1995; Zarpellon et al., 2008)
Phosphatases		
PP2A	Inhibition: Decreased 5-HT uptake and increased SERT phosphorylation. Associates with SERT.	(Ramamoorthy et al., 1998; Bauman et al., 2000)
Receptors		
AR_3	Activation: Increased 5-HT uptake and SERT Vmax with no effect on Km. Trafficking dependent and independent mechanisms through PKG, p38 MAPM and PP2A pathways.	(Miller & Hoffman, 1994; Zhu et al., 2007)
H_3R	Activation: Increased 5-HT uptake through NO/PKG pathway.	(Launay et al., 1994)
$\alpha2 - AR$	Activation: Decreased 5-HT uptake and Km with no effect on SERT Vmax through Ca^{2+} influx via voltage-sensitive Ca^{2+} channels.	(Ansah et al., 2003)
IL1ß	Activation: Increased 5-HT uptake and decreased Km, no effect on SERT Vmax through p38 MAPK activation.	(Zhu et al., 2006)
TNF-α	Activation: Increased 5-HT uptake, decreased Km and increased Vmax through p38 MAPK activation.	(Zhu et al., 2006)
$5-HT_{1B}$	Antagonists inhibit 5-HT clearance.	(Daws et al., 2000)
BDNF	Activation: Increased 5-HT uptake	(Benmansour et al., 2008)
Substrates		
5-HT	Upregulates surface SERT and attenuates PKC-dependent SERT phosphorylation and surface down regulation.	(Ramamoorthy & Blakely, 1999; Whitworth et al., 2002)
AMPH	Increases SERT basal phosphorylation through p38 MAPK pathway.	(Ramamoorthy & Blakely, 1999)
Fenfluramine	Attenuates PKC-dependent SERT phosphorylation and surface down regulation.	(Ramamoorthy & Blakely, 1999)
Antagonists		
Paroxitine, Citalopram, Imipramine, and Cocaine	Attenuate PKC-dependent SERT phosphorylation and surface down regulation.	(Ramamoorthy & Blakely, 1999)

and 5-HT receptor functions again highlighting the coordinated regulation of amine biosynthesis, amine storage, receptors sensitivity and transporter expression. Furthermore, these mice exhibit significant differences in basal peripheral physiology including gastrointestinal function, as well as overt behavior (Bengel et al., 1998; Sora et al., 1998; Li et al., 1999).

Human SERT (hSERT) encodes a protein of 630 amino acids. Hydrophobicity analysis of the amino acid sequence predicts the presence of 12 hydrophobic transmembrane domains with cytoplasmic NH_2 and COOH termini. Transmembranes 3 and 4 are separated by a large, hydrophilic loop that bears two canonical sites for N-linked glycosylation (Lesch et al., 1993a; Ramamoorthy et al., 1993a). SERT is localized in the human chromosome 17q11.2 (Ramamoorthy et al., 1993a). It exists in homo-multimeric complexes (Ramamoorthy et al., 1993b; Jess et al., 1996; Chang et al., 1998; Chen et al., 1998; Kilic & Rudnick, 2000; Schmid et al., 2001a,b) and contains potential phosphorylation sites for several kinases (Hoffman et al., 1991; Ramamoorthy et al., 1993a; Miller & Hoffman, 1994).

2.1. Receptor-protein kinase mediated acute regulation of SERT activity and phosphorylation

Presynaptic receptor-second messenger-kinase/phosphatase linked pathways play a pivotal role in the regulation of SERT activity. The function of native and heterologously expressed SERT is rapidly inhibited in response to acute depletion of intracellular Ca^{2+}, inhibition of calmodulin, CaMKII, Src-kinase, p38 MAPK and activation of PKC. On the other hand, increased intracellular Ca^{2+}, activation of NOS/cGMP and MAPK pathways stimulate SERT activity. Presynaptic receptor-mediated regulation of SERT has been documented (summarized in Table 1). Activation of adenosine receptors (AR), $5-HT_{1B}$, histamine receptors (H_3R) and BDNF/TrkB stimulates 5-HT uptake (Launay et al., 1994; Daws et al., 1999,2000; Zhu et al., 2004a,b; Benmansour et al., 2008; Matheus et al., 2009; Steiner et al., 2009), whereas α2 adrenergic receptor stimulation reduces uptake of 5-HT (Ansah et al., 2003). The contribution of several of these kinase cascades to SERT regulation and phosphorylation has been demonstrated and a close temporal correlation between amine transport and surface expression has been documented (Jayanthi et al., 1994; Qian et al., 1997; Ramamoorthy et al., 1998; Ramamoorthy & Blakely, 1999; Ramamoorthy, 2002; Jayanthi et al., 2005; Samuvel et al., 2005). For example, activation of PKC and PKA, or inhibition of PP2Ac, increases SERT basal phosphorylation in HEK-293-hSERT cells (Ramamoorthy et al., 1998). Time course studies indicate that the decrease in 5-HT transport parallels that of SERT surface expression. Thus, SERT phosphorylation by the PKC-dependent pathway triggers SERT internalization and reduces 5-HT uptake. Phosphorylation of the intracellular SERT pool may slow down the recycling of SERT or

redirect SERT trafficking events which contribute to changes in surface expression. Alternatively, SERT may be phosphorylated at the cell surface providing a signal for internalization, a change in SERT catalytic properties, or both. These events may occur but at different time frames. Recent efforts have brought into sharper focus the time-dependent molecular events associated with PKC-mediated regulation of SERT that is constitutively expressed in platelets (Jayanthi et al., 2005). Jayanthi et al. demonstrated that diminished SERT catalytic activity occurs prior to an enhancement of SERT endocytic rate that ultimately reduces cell surface SERT expression. The authors provided evidence that PKC activation regulates SERT in a biphasic manner. The initial phase (1–5 min) of 5-HT uptake inhibition occurs independently of trafficking whereas the later phase (5–60 min) is associated with enhanced endocytosis. The biphasic inhibition of SERT is accompanied by sequential phosphorylation of plasma membrane resident SERT such that phosphorylation of serine (Ser) residues precedes that of threonine (Thr) residue(s). It has been suggested that the initial phosphorylation on Ser residues may be responsible for changes in the intrinsic properties and/or silencing of SERT, and that Thr phosphorylation may trigger internalization of phosphorylated SERT (Jayanthi et al., 2005).

In contrast to a PKC-dependent regulation of SERT trafficking and phosphorylation, mitogen-activated protein kinases (MAPK) activate SERT in midbrain synaptosomes and heterologous cell models. Among MAPK pathways, inhibition of p38 MAPK either pharmacologically or by specific siRNAs-mediated knockdown inhibits SERT activity. In contrast, extracellular signal-regulated kinase (ERK1/2) or c-Jun N-terminal Kinase 1 (JNK) inhibition is without effect (Samuvel et al., 2005; Oz et al., 2010). Furthermore, activation of p38 MAPK either by over expressing a constitutively active form of MAP kinase kinase 3b, an upstream kinase activator of p38 MAPK, exposure to aniosmycin, an activator of p38 MAPK, or adenosine receptor (AR$_3$) activation stimulates SERT activity (Zhu et al., 2004a; Samuvel et al., 2005; Zhu et al., 2005,2007). The degree to which p38 MAPK regulates SERT surface expression is controversial, with evidences both supportive of trafficking dependent and independent mechanisms. Zhu et al. (2004a) showed that short term incubation with the p38 MAPK inhibitor, SB203580, blocks AR$_3$ mediated increases in 5-HT uptake in RBL-2H3 cells and in CHO cells transfected with SERT and AR$_3$. However, SB203580 had no effect on AR$_3$ mediated increases in SERT surface expression. However, studies by Samuvel et al. demonstrated that p38 MAPK inhibition affects SERT insertion into the plasma membrane (Samuvel et al., 2005). Although an explanation for the discrepancy between these two studies remains unclear, many factors such as time of treatment, cell type used, differential expression of p38 MAPK isoforms in the cell model systems used, SERT expression levels and the SERT distribution ratio between plasma membrane and intracellular pool all could contribute to the observed differences. Interestingly, basal SERT phosphorylation is inhibited following p38 MAPK inhibition suggesting that constitutively active p38 MAPK is involved in maintaining SERT basal phosphorylation, expression and activity (Samuvel et al., 2005). The findings that PKC activation increases SERT internalization and phosphorylation whereas inhibition of p38 MAPK reduces basal SERT phosphorylation and plasma membrane insertion suggests that p38 MAPK and PKC must act on different phospho-sites. If direct phosphorylation is involved in both processes, then these kinases may target SERT at different cellular locations. Furthermore, PKC and p38 MAPK mediated SERT basal phosphorylation may differentially impact SERT trafficking and catalytic activity. Future efforts directed towards the identification of the specific sites of PKC and p38 MAPK mediated phosphorylation will illuminate the cellular and molecular basis of SERT regulation by these kinases. P38 MAPK is induced by stress. Numerous studies have demonstrated altered extracellular 5-HT concentrations, 5-HT synthesis/metabolism, 5-HT receptor signaling cascades, SERT binding sites and serotonergic neuronal firing in the brain in response to

stressful stimuli (Chaouloff et al., 1999). Thus, adjustments in 5-HT neurotransmission may allow an appropriate behavioral response to stress and SERT regulation by p38 MAPK may provide a novel presynaptic mechanism by which appropriate synaptic 5-HT levels are maintained during stressful conditions.

Activation of AR$_3$ in RBL cells, or in CHO-1 cells transfected with SERT and ARs stimulates 5-HT uptake through PKG activation (Miller & Hoffman, 1994; Zhu et al., 2004a). Nitric oxide (NO) stimulation of SERT expressed in HeLa and COS cells requires the cGMP pathway (Kilic et al., 2003). In platelets, histamine receptor activation stimulates 5-HT uptake via NO/PKG activation (Launay et al., 1994). Cyclic GMP analogs also stimulate 5-HT uptake in these model systems (Miller & Hoffman, 1994; Zhu et al., 2004a,b). Although studies from multiple laboratories have shown that PKG activation enhances 5-HT uptake, the issue of whether there is surface redistribution of SERT following PKG activation is both complex and controversial. It appears that under certain experimental conditions, PKG mediated SERT regulation can occur through alternative pathways. Furthermore, SERT regulation or trafficking in response to PKG may differ depending on cell type. For example, exposure of HeLa cells transiently expressing hSERT to 8-Br-cGMP resulted in increased 5-HT uptake and surface binding to SERT (Zhu et al., 2004a,b). In RBL-2H3 and CHO-1 cells co-expressing SERT and AR$_3$, activation of AR$_3$ increased 5-HT uptake and SERT surface expression via activation of PKG and p38 MAPK (Zhu et al., 2004a,b). Interestingly, inhibition of either p38 MAPK or PP2 blunted AR$_3$ stimulation of SERT activity, but not the stimulation of SERT surface density. The authors of this study proposed that AR$_3$ stimulates SERT activity via a PKG dependent enhancement of surface SERT and a separate p38 MAPK dependent enhancement of SERT intrinsic activity. Thus, PKG and p38 MAPK may work in concert to maintain a balance between the number and catalytic state of surface SERT. In contrast to these reports, studies from other laboratories demonstrated trafficking independent SERT upregulation by PKG activation (Miller & Hoffman, 1994; Kilic et al., 2003). The discrepancies between studies are not understood.

The studies described previously clearly indicate that several kinases regulate SERT activity and that regulation can be trafficking dependent or independent. These kinases also trigger SERT phosphorylation. Activation of PKG phosphorylates endogenously expressed SERT in rat midbrain as well as human SERT expressed in CHO-1 cells (Ramamoorthy et al., 2007). It would not be surprising if transporters use phosphorylation as a trigger for transporter regulation. Proof of such regulation requires the identification of kinase-specific site(s) within SERT and the demonstration of loss of kinase-mediated SERT regulation and phosphorylation when the putative site is mutated into non-phosphorylatable amino acids such as alanine. Recently, Ramamoorthy's group identified a phosphorylation site in SERT and demonstrated that phosphorylation of Thr-276 is required for cGMP-mediated SERT regulation (Ramamoorthy et al., 2007). Interestingly, this study showed that cGMP-stimulated phosphorylation of native SERT occurs only on Thr residues. Mutation of SERT Thr-276 to alanine abolished cGMP-mediated stimulation of 5-HT transport and SERT phosphorylation. Phosphorylation of Thr-276 results in addition of a negative charge that might influence SERT activity. If this hypothesis is correct, then, substitution of Thr-276 with aspartic acid (Asp), (which in its ionized form carries a negative charge) would mimic PKG effects on 5-HT uptake. Indeed mutation to Thr-276 to Asp, which would mimic phosphorylation, increased 5-HT uptake to a level equal to that of cGMP-stimulated 5-HT uptake in wild-type SERT expressing cells, and uptake was no longer sensitive to cGMP. These findings provide the first identification of a phosphorylation site in SERT and demonstrate a direct link that PKG phosphorylates SERT on Thr-276 leading to increased 5-HT uptake.

In addition to the role Ser/Thr protein kinases play in the regulation of SERT, evidence suggests that a family of tyrosine protein kinases also regulate SERT activity and phosphorylation. Several

Table 2
Summary of kinase, receptor, substrates and antagonists on NET regulation.

Regulators	Effects on NET	References
Protein kinases		
PKC	Activation: Decreased NET Vmax, unaltered NE Km, decreased surface NET proteins, increased NET endocytosis and increased NET phosphorylation. Phosphorylation of Thr258/Ser259 is required for PKC-linked NET down regulation. Translocates NET from lipid-rafts to non-lipid rafts.	(Apparsundaram et al., 1998b,c; Bönisch et al., 1998; Jayanthi et al., 2004,2006)
P38 MAPK	Inhibition: Decreased NET Vmax and NE Km.	(Apparsundaram et al., 2001)
CaMKII	Inhibition: Blunted Ca^{2+}-induced stimulation of NE uptake, AMPH-induced NET down regulation.	(Uchida et al., 1997, 1998)
Tyrosine kinase	Inhibition: Decreased NET Vmax, unaltered Km.	(Apparsundaram et al., 2001)
PI-3 kinase	Inhibition: Reduced NE uptake and decreased surface NET.	(Apparsundaram et al., 2001)
Akt-1	Decreased Akt-Ser473 phosphorylation increased NE uptake and NET functional expression	(Siuta et al., 2010)
Phosphatases		
PP2A	Inhibition: Reduced NE uptake and increased NET phosphorylation.	(Bauman et al., 2000; Jayanthi et al., 2004 #8329)
Receptors		
AT1	Activation: Increased NET activity and surface expression through activation of PI-3 kinase and MAPK.	(Yang & Raizada, 1999; Savchenko et al., 2003)
NK1	Activation: Decreased NET Vmax, unaltered NE Km, decreased surface NET proteins and increased NET phosphorylation.	(Jayanthi et al., 2006)
mAChRs	Activation: Decreased NET Vmax, unaltered NE Km, decreased surface NET proteins through intracellular Ca^{2+} and PKC-dependent/independent pathways.	(Apparsundaram et al., 1998a)
Insulin	Increased NE uptake and NET Vmax, unaltered NE Km and surface NET. Ca^{2+}, PP2A, tyrosine kinase, Akt, PI-3 kinase and p38 MAPK activities are involved.	(Boyd et al., 1985; Figlewicz et al., 1993a; Apparsundaram et al., 2001)
Substrates		
NE	Long term treatment of NE down regulates NET protein.	(Zhu et al., 1998)
AMPH	Decreased surface NET. Ca^{2+} and CaMKII –dependent Syntaxin 1A interaction with NET and Rab 11 is implicated in AMPH- mediated effects. Decreased surface NET via increasing NET endocytosis, Mutations of Thr-258/Ser-259 block AMPH induced NET down regulation.	(Dipace et al., 2007; Annamalai et al., 2010; Matthies et al., 2010)
Desipramine	Decreased NE uptake and NET binding sites.	(Zhu et al., 1998; Zhu et al., 2000)

structurally distinct tyrosine kinase inhibitors such as genistein, herbimycin A and 2,5-dihydroxycinnamate inhibit 5-HT uptake in platelets (Helmeste & Tang, 1995; Zarpellon et al., 2008). Recently, Zarpellon and coworkers provided evidence that Src-family tyrosine kinases regulate SERT function and SERT phosphorylation on tyrosine residues (Zarpellon et al., 2008). In human platelets, while the Src kinase inhibitors PP2 and SU6656 inhibit 5-HT uptake and phosphorylation of tyrosine residues of SERT, the tyrosine protein phosphatase inhibitor, pervanadate, increases SERT activity and tyrosine phosphorylation of SERT. In addition, using Src-specific peptide and an *in vitro* kinase assay the authors documented the presence of Src-kinase activity in SERT immunoprecipitates from human platelets. Likewise, blots from these SERT immunoprecipitates revealed the presence of Src kinase suggesting that SERT and Src exist as a complex (Zarpellon et al., 2008). Clearly, further studies identifying the tyrosine residue (s) within SERT and the role of tyrosine phosphorylation in Src-tyrosine kinase mediated SERT regulation are warranted.

3. The norepinephrine transporter and its functional significance

NET clears NE released into the synaptic cleft in a Na^+/Cl^- dependent manner (Iversen, 1971,1978; Pacholczyk et al., 1991; Trendelenburg, 1991; Bonisch & Bruss, 2006). NET is a target for the treatment of mood and cognitive disorders (Blier, 2001; Bonisch & Bruss, 2006). NET is also a target for psychostimulants, including cocaine and amphetamines (Pacholczyk et al., 1991; Justice et al., 1998; Jayanthi et al., 2002; Binda et al., 2006). NET is selectively expressed on NE nerve terminals, thereby, enabling spatial and temporal control of the actions of NE (Moore & Bloom, 1979; Foote et al., 1983). NET is also expressed in peripheral tissue (e.g., adrenal glands, vas deferens and placenta (Schroeter et al., 2000; Jayanthi et al., 2002; Sung et al., 2003). Altered NET function is associated with attention, mood and cardiovascular disorders (Klimek et al., 1997; Rumantir et al., 2000; Shannon et al., 2000; Hahn et al., 2005; Esler et al., 2006; Kim et al., 2006; Haenisch

et al., 2008; Hahn et al., 2008). Human NET, which is localized to chromosome 16q13-q21 (16q12.2) (Brüss et al., 1993; Gelernter et al., 1993), is a 617 amino acid protein containing 12 transmembrane domains with cytoplasmic amino- and carboxy terminals. A large extracellular loop between transmembrane domains 3 and 4 contains glycosylation sites. Several canonical sites for protein kinases can be found in sequences of cytoplasmic domains (Pacholczyk et al., 1991; Justice et al., 1998; Jayanthi et al., 2002; Binda et al., 2006). Alternative splicing has been reported to regulate NET expression and function (Kitayama et al., 2001). This occurs at 3′-flanking coding and noncoding regions, resulting in different carboxy terminals. When expressed in HEK-293 cells, these isoforms of NET showed reduced functional activity and surface expression. NET knockout mice display altered seizure susceptibility and opiate/cocaine sensitivities, as well as maladaptive responses to social and cardiovascular stressors, The phenotype of these animals underscores the importance of NET in normal physiology and behavior (Bohn et al., 2000; Xu et al., 2000; Haller et al., 2002; Keller et al., 2004; Ahern et al., 2006; Keller et al., 2006; Mitchell et al., 2006). NET also effectively clears DA in brain regions (e.g. prefrontal cortex) where DAT expression is low or absent suggesting the physiological significance of NET in controlling DA transmission and DA-induced behavior (Moron et al., 2002; Madras et al., 2005; Siuta et al., 2010). NET function is highly regulated (Bonisch & Bruss, 2006; Mandela & Ordway, 2006) (summarized in Table 2). NET control of NE signaling is, thus, an important homeostatic mechanism and its dysregulation by psychostimulants is thought to contribute to the behavioral and neurochemical effects of psychostimulants (Binda et al., 2006; Dipace et al., 2007) and chronic stress (Miner et al., 2006).

3.1. Receptor-protein kinase mediated acute regulation of NET activity and phosphorylation

PKC-activation inhibits NET function. Down regulation of function involves sequestration of NET protein from the plasma membrane

(Apparsundaram et al., 1998c; Jayanthi et al., 2004). In transformed SK-N-SH cells, which endogenously express NET, activation of muscarinic acetylcholine receptors by the agonist methacholine also results in NET down-regulation. Moreover, inhibition of PKC by antagonists abolishes PKC-mediated effects but produces only partial blockade of methacholine-mediated effect. PKC activation and pathways dependent on mobilization of Ca^{2+} stores are involved in muscarinic receptor-mediated NET regulation (Apparsundaram et al., 1998a). Together these data suggest the existence of PKC-independent regulatory pathways that maintain NET surface expression and/or intrinsic transporter catalytic activity (summarized in Table 2). Jayanthi et al. provided key evidence for lipid raft-mediated endocytosis as the mechanism of NET down-regulation by PKC (Jayanthi et al., 2004). Subsequent studies by the same group identified a Thr-258/Ser-259 trafficking motif linked to substance P-mediated activation of neurokinin 1 receptor (NK1R-PKC) -induced NET down-regulation and phosphorylation (Jayanthi et al., 2006). Only Ser-259 was identified as a potential PKC site. However, phosphoamino acid analysis showed phospho-Ser and phospho-Thr residues following PKC activation (Jayanthi et al., 2006). Mutation of both Thr-258 and Ser-259 sites was required for abolition of PKC-mediated inhibition of NE transport. Mutation of Thr-258 and Ser-259 sites blocked PKC-induced phosphorylation to a significant extent (60%), but not completely. In addition, the Thr-258Ala/Ser-259Ala mutant showed enhanced basal phosphorylation suggesting that phosphorylation of this motif may influence other sites (Jayanthi et al., 2006). Other sites including Thr-19, Thr-30 and Thr-58, Ser-502, Ser-579, Thr-580 and Ser-583 are not involved in PKC-mediated NET regulation. Although Ser-259 appears to be the direct site of action of PKCε. However, based on in vitro phosphorylation assays, Ser-259 phosphorylation is not required for PKC-mediated NET down-regulation. Thr-258Ala mutation partly blunted both PKC-mediated phosphorylation and transporter down-regulation. However, the fact that the double mutation (258Ala/Ser-259Ala) partially eliminates PKC-induced phosphorylation and completely blocks NET down-regulation/internalization suggests a possible relationship between phosphorylation of the Thr-258/Ser-259 motif and transporter internalization. It is possible that PKC-mediated Thr-258 phosphorylation may have a modulatory role in NET regulation, and, in concert with Ser-259 phosphorylation, dictates NET endocytosis (Jayanthi et al., 2006).

The existence of physical complexes containing biogenic amine transporters and PP2Ac proteins has been established (Bauman et al., 2000) suggesting that modulation of transporter/phosphatase association is involved in regulated transporter phosphorylation and trafficking. In addition to NET phosphorylation, the presynaptic factor, syntaxin 1A is known to bind the N-terminal domain of NET. Truncation at the N-terminal domain of human NET disrupts NET/syntaxin 1A association and limits the ability of PKC (phorbol ester treatment) to downregulate NET function (Sung et al., 2003). Future studies exploring the idea that transporter association with these partners, per-se regulates transporter phosphorylation and function will aid our understanding of how these two mechanisms are interrelated.

Diverse biologic stimuli including neuronal activity, peptide hormones, and trophic factors have been implicated in NET regulation. Insulin regulates NE transport (Boyd et al., 1985,1986; Figlewicz et al., 1993a,b; Apparsundaram et al., 2001) and MAPKs differentially regulate NET function by trafficking dependent and independent mechanisms (Apparsundaram et al., 2001). PP2Ac blockers are also known to abolish insulin-mediated NET regulation (Apparsundaram et al., 2001). Raizada and colleagues demonstrated that acute angiotensin II (AngII) treatments elicit a rapid increase in NE transport in cultured brainstem neurons (Boyd et al., 1985; Sumners & Raizada, 1986; Lu et al., 1996). Later studies from Blakely's group using a newly developed ectodomain NET antibody, mouse

brainstem and superior cervical ganglion neurons (SCG) primary cultures, further confirmed that Ang II treatments increase surface NET and activity rapidly (Savchenko et al., 2003). In addition, when neuronal activity was mimicked by KCl-evoked depolarization, increased surface NET expression was seen. Although the involvement of phosphatidylinositol 3-kinase (PI-3 kinase) and MAPK following long-term AngII receptor activation has been demonstrated (Yang & Raizada, 1999), future studies are warranted to elucidate the pathways by which acute AngII receptor activation alters NET surface expression and delineate the convergence between these and those engaged by depolarization-evoked increases in neuronal activity. Recent studies using neuronal Rictor Null mice established that Akt (protein kinase B)-linked NET regulation contributes to altered DA-transmission and schizophrenia-like behaviors (Siuta et al., 2010). Rictor Null mice exhibit diminished Akt-Ser473 phosphorylation and enhanced NET functional expression within the cortex. NE levels are elevated and lower DA content is lower in the cortex. Interestingly, the NET blocker, nisoxetine reverses hypodopaminergia condition and schizophrenia-linked behaviors (Siuta et al., 2010). Thus, NET activities regulate NE as well as DA homeostasis, thereby, affecting both NE and DA-linked behaviors.

4. The dopamine transporter and its functional significance

DAT is a major regulator of DA neurotransmission. DAT activity requires Na^+ and Cl^- to clear synaptic DA. Human DAT. which is located in chromosome 5p15.3 (Giros et al., 1992), encodes 620 amino acids with 12 putative transmembrane domains and a large second extracellular loop with several putative N-glycosylation sites. Both amino- and carboxy-terminals are intracellular with several predicted protein kinase sites (Kilty et al., 1991; Shimada et al., 1991). DAT is primarily expressed in DA neurons. As observed with other MA transporters, it is also found in peripheral systems including lymphocytes (Amenta et al., 2001). DAT-knockout mice display increased extracellular DA concentrations relative to wild types as well as altered synthesis of DA and other amines (Giros et al., 1996). DA receptor function and expression is also abnormal (Gainetdinov et al., 1999a; Jones et al., 1999; Fauchey et al., 2000; Ralph et al., 2001; Ghisi et al., 2009). DAT knockout mice are hyperactive, exhibit dwarfism, cognitive problems, sleep dysregulation, alterations in gut motility, skeletal abnormalities and behavioral inflexibility (Giros et al., 1996; Gainetdinov & Caron, 2003; Caron & Gainetdinov, 2010).

The DAT is a well-established target of various psychoactive compounds including cocaine, amphetamines and PCP derivatives. Whereas cocaine and methylphenidate are DAT activity blockers and inhibit DA uptake, amphetamine and methamphetamine (METH) are substrates that are transported by DAT and that trigger DA release via a DAT-dependent mechanism. Neurotoxins such as 6-hydroxydopamine and MPP+ enter DA neurons by the activity of DAT and produce DA neuronal damage. Altered DAT expression and function has been documented in human cocaine and METH addicts and in Parkinson's disease (Volkow et al., 2001; Mash et al., 2002; Weintraub et al., 2005). The dependence on DAT for normal DA clearance and signaling suggests that DAT dysfunction may contribute to various brain disorders associated with dysregulation of DA transmission including schizophrenia, affective disorders and addiction. Data from animal models, human neuroimaging and genetic studies suggest altered DAT availability or function in attention deficit hyperactivity disorder (ADHD) and two ADHD medications, Ritalin™ and Adderall™ target DAT (Volkow et al., 2001; Logan et al., 2007). The DAT coding variant Ala-559Val identified in ADHD is associated with abnormal DAT function and regulation (Mazei-Robison & Blakely, 2005; Mazei-Robison et al., 2008). Decreased mesocorticolimbic DA transmission is implicated in certain symptoms of depression (Nestler & Carlezon, 2006) and medications with proven antidepressant efficacy in humans (e.g., bupropion, nomifensine, amineptine) are DA uptake

Table 3
Summary of kinase, receptor, substrates and antagonists on DAT regulation.

Regulators	Effects on DAT	References
Protein kinases		
PKC	Activation: Decreased DAT Vmax, unaltered DA Km, decreased surface DAT proteins, increased DAT endocytosis and increased striatal DAT phosphorylation on N-tail serines. Regulates AMPH-induced DA efflux. Triggers DAT degradation in some models and associates with DAT. Residues 587 – 596 located at DAT-carboxy terminal is required for basal and PKC-stimulated DAT internalization.	(Boudanova et al., 2008; Chang et al., 2001; Chen et al., 2009; Daniels & Amara, 1999; Holton et al., 2005; Johnson et al., 2005b; Melikian & Buckley, 1999; Vaughan et al., 1997; Zhu et al., 1997)
ERK1/2	Inhibition: Decreased DAT Vmax, unaltered DA Km and decreased surface DAT proteins.	(Moron et al., 2003)
CaMKII	Associates with DAT, facilitates N-tail DAT phosphorylation and mediates AMPH-induced DAT mediated DA efflux.	(Fog et al., 2006)
Akt	Involved in insulin and AMPH-mediated DA efflux.	(Garcia et al., 2005; Wei et al., 2007)
PI-3-kinase	Inhibition: Decreased DAT Vmax, unaltered DA Km and decreased surface DAT proteins.	(Carvelli et al., 2002; Lute et al., 2008)
Cdk5	Inhibition: Decreased DAT Vmax without altering surface DAT.	(Price et al., 2009)
Tyrosine kinase	Inhibition: Decreased DAT Vmax and decreased surface DAT proteins.	(Doolen & Zahniser, 2001)
Phosphatases		
PP2A	Inhibition: Decreased DA uptake and increased DAT phosphorylation.	(Bauman et al., 2000)
PP1	Dephosphorylates phospho-DAT, effects on DAT functional properties are not known.	(Foster et al., 2003)
Receptors		
D$_2$R	Activation: Increased DAT activity and surface expression through activation of ERK1/2. Associates with DAT.	(Mayfield & Zahniser, 2001; Lee et al., 2007)
D$_3$R	Activation: Regulates DAT in a biphasic manner through activation of ERK1/2 and PI-3 kinase: Earlier activation (1-3 min) increases DAT activity, surface DAT and DAT exocytosis. Prolonged activation (30 min) decreases DAT activity, surface DAT and DAT exocytosis, increased DAT endocytosis.	(Zapata et al., 2007)
NK1R	Activation: Decreased DAT Vmax, unaltered DA Km, decreased surface DAT proteins and increased DAT phosphorylation.	(Granas et al., 2003)
KOR	Activation: Increase DA-uptake	(Thompson et al., 2000)
mGluR5	Activation: Decreased DAT Vmax, PKC and/or CaMKII inhibitors prevent mGluR5 effect, phosphatase inhibitor augments mGluR5-effect.	(Page et al., 2001)
TrkB	Activation: Increased DAT Vmax, DA Km and surface DAT through MAPK and PI-3 kinase.	(Hoover et al., 2007)
Insulin	Increased DA-uptake and surface DAT. Requires Akt, PI-3 kinase activity.	(Garcia et al., 2005; Wei et al., 2007; Williams et al., 2007a)
Substrates		
DA	DA down regulates surface DAT in a PKC dependent manner.	(Chi & Reith, 2003; Richards & Zahniser, 2009)
AMPH	Affects in a biphasic manner: Increased surface DAT at early exposure followed by decreased surface DAT. Increased DAT phosphorylation that is sensitive to cocaine and other DAT antagonists. Akt, PKC, PI-3 kinase, CaMKII and Syn 1A are involved in AMPH-mediated DA efflux and phosphorylation. Insulin attenuates AMPH- effect.	(Saunders et al., 2000; Khoshbouei et al., 2004; Cervinski et al., 2005; Garcia et al., 2005; Gorentla & Vaughan, 2005; Johnson et al., 2005b; Fog et al., 2006; Binda et al., 2008)
Antagonists		
Cocaine	Increased DAT Vmax and surface expression. Regulates DAT-serine phosphorylation and DAT-PP2A association.	(Daws et al., 2002; Little et al., 2002; Ramamoorthy et al., 2010 Samuvel et al., 2008)
GBR 12909	Decreased basal and PKC-stimulated DAT phosphorylation. Prevents PKC-mediated DAT down regulation.	(Gorentla & Vaughan, 2005)

inhibitors. Thus normal regulation and expression of DAT is important for maintaining DA homoeostasis.

4.1. Receptor-protein kinase mediated acute regulation of DAT activity and phosphorylation

As described above for SERT and NET, the function of native and heterologously expressed DAT is rapidly altered in response to acute activation and/or inhibition of several protein kinases including PKA, PKC, PI-3 kinase, ERK1/2, Akt, CaMKII, cyclin-dependent kinase (Cdk 5), tyrosine kinases and protein phosphatase PP1/PP2Ac (Carvelli et al., 2002; Foster et al., 2003; Moron et al., 2003; Melikian, 2004; Vaughan, 2004; Sorkina et al., 2005; Bolan et al., 2007; Hoover et al., 2007). In addition, G-protein coupled receptor (GPCR) and non-GPCR receptor regulation of DAT has been documented (Yang et al., 1997; Page et al., 2001; Granas et al., 2003; Savchenko et al., 2003; Bolan et al., 2007; Hoover et al., 2007; Zapata et al., 2007) (summarized in Table 3).

The most thoroughly studied kinase is PKC. Studies in various cell lines transfected with DAT have shown that activation of PKC by phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) decreases dopamine transport capacity (Reith et al., 1997; Zhu et al.,

1997; Daniels & Amara, 1999; Melikian & Buckley, 1999; Chang et al., 2001; Doolen & Zahniser, 2002; Sorkina et al., 2005; Chen et al., 2009; Eriksen et al., 2009). A similar down-regulation has also been reported in synaptosomal preparations (Copeland et al., 1996; Vaughan et al., 1997; Foster et al., 2002, 2008). DAT down-regulation in response to PKC activation has been attributed to dynamin-dependent endocytosis (Daniels & Amara, 1999; Melikian & Buckley, 1999; Saunders et al., 2000; Sorkina et al., 2005; Eriksen et al., 2009) although an initial rapid trafficking-independent inactivation of the transporter might occur at the plasma membrane (Mazei-Robison & Blakely, 2005) as reported for SERT (Jayanthi et al., 2005). While recycling of internalized DAT was shown in PC12 cells, the internalized DAT was subject to lysosomal degradation in HeLa and MDCK cells (Daniels & Amara, 1999; Melikian & Buckley, 1999). Using PC12 cells and site-directed mutagenesis, the importance of DAT-carboxy terminal residues 587–596 in basal and PKC-stimulated internalization has been documented (Holton et al., 2005; Boudanova et al., 2008). DAT ubiquitination is also a critical step for PKC-dependent DAT endocytosis. Miranda et al. showed that DAT is constitutively ubiquitinated and that ubiquitination is enhanced in response to PMA stimulation (Miranda et al., 2005). The ubiquitination was

dependent on the presence of lysines (Lys) located in the intracellular DAT N-terminus (Lys-19, Lys-27, and Lys-35) and mutation of these residues to arginine abolished ubiquitination and phorbol ester stimulated DAT down-regulation (Miranda et al., 2007). Interestingly, ubiquitination was most apparent in endosomes suggesting that ubiquitination may be a signal for endocytosis. Evidence from both in-vitro and ex-vivo studies have demonstrated that DAT exists in a phosphorylated form and basal DAT phosphorylation is stimulated by PKC activation and phosphatase inhibition (Huff et al., 1997; Vaughan et al., 1997; Granas et al., 2003; Cervinski et al., 2005). Thus, both PKC and PP1/2A/PP1 play a pivotal role in the turnover of DAT phosphorylation.

Several other kinase pathways affect DAT function and surface expression. Inhibitors of PI-3 kinase or Akt, as well as over-expression of a dominant negative Akt mutant in heterologous cells, decrease DAT activity and surface levels (Carvelli et al., 2002; Garcia et al., 2005). A physiological role of this pathway in regulating DAT levels was supported by studies in rats in which insulin was depleted using the diabetogenic agent, streptozotocin. Following insulin depletion, Akt function and DAT surface expression were markedly reduced (Williams et al., 2007b). Other studies (Carvelli et al., 2002; Moron et al., 2003) have demonstrated that DAT transport properties are sensitive to modulation of MAPK modulation. Pharmacological blockade of constitutively active MAPK reduces DAT activity and surface levels (Moron et al., 2003). However, changes in surface expression are not always associated with kinase-induced alterations in DAT activity. For example, Cdk5 inhibition reduces DAT activity without affecting the levels of surface DAT protein (Price et al., 2009). Another study provided evidence that PKC induced internalized DAT can be recycled back to the cell surface by PKA-dependent mechanisms (Pristupa et al., 1998).

D2 and D3 DA receptors (D_2R and D_3R) are located on DA neurons. Although their role in the presynaptic regulation of DA release is well recognized, early studies examining their contribution to DAT regulation yielded disparate results. Using voltammetry to monitor instantaneous rates of DA clearance or electrophysiology, several studies showed that D_2R/D_3R agonists increase DAT function whereas a downregulation of function occurs in response to receptor antagonists or gene deletion (Meiergerd et al., 1993; Dickinson et al., 1999; Mayfield & Zahniser, 2001). However, radioligand uptake studies using tritiated DA revealed no such effects. Recent works by Shippenberg and colleagues have provided an explanation for this seeming paradox. Using confocal microscopy in conjunction with the fluorescent DAT substrate, 4-[4-(diethylamino)-styryl]-N-methylpyridinium iodide (ASP^+) to monitor DAT activity in real time or tritiated tyramine in uptake studies, these investigators showed that activation of either D_2R or D_3R is sufficient to upregulate DAT function in heterologous expression systems (Mayfield & Zahniser, 2001; Bolan et al., 2007; Zapata et al., 2007). However, these effects were not observed in [^{3}H]DA uptake studies. Importantly, and in contrast to DA, ASP^+ and tyramine do not bind to or activate DA receptors. Thus, by using these substrates the measurement of basal DAT activity is not confounded by substrate-induced receptor activation, thereby, enabling detection of agonist-evoked increases in transporter function. These investigators went on to show that the short form of the D_2R, which is located on DA nerve terminals, is associated with DAT and its activation increases transporter activity by an ERK1/2 dependent mechanism. Using co-immunoprecipitation and GST fusion protein pull-down experiments in striatal tissue extracts, Liu and co-workers provided evidence that D_2Rs directly interacts with DAT (Lee et al., 2007). Interestingly, the D_3R also regulates DAT trafficking and DA uptake. However, this effect varies as a function of the duration of receptor activation and appears to involve multiple kinase cascades. Thus, transient receptor activation (1–3 min) of D_3R increased DA uptake via ERK1/2 and PI-3 Kinase-dependent mechanisms. DAT cell surface expression and the rate of DAT exocytosis were also increased.

In contrast, prolonged (30 min) D_3R activation reduced DA uptake and DAT cell surface expression. This was accompanied by a decreased rate of DAT exocytosis and an increased rate of endocytosis. Together, these findings demonstrate that D_2R and D_3R regulate DA transmission by affecting DAT function as well as DA release. Insulin increases DA uptake and DAT cell surface levels through activation of PI-3 Kinase and Akt. Non-GPCRs can also regulate DAT function and expression. BDNF stimulates DA uptake and surface expression through activation of TrkB-receptor linked PI-3 kinase and ERK1/2 activation (Hoover et al., 2007). Systemic administration of the endocannabinoid, anandamide, increases extracellular DA levels in the nucleus accumbens, a brain region implicated in mediating the abuse liability of various psychoactive drugs (Solinas et al., 2006). Although these effects were initially attributed to alterations in DA release evidence that anandamide and other cannabinoids inhibit DA uptake in native tissue and heterologous expression systems has been presented (Chen et al., 2003; Price et al., 2007). A recent study has shown that the interaction of anandamide with DAT occurs via a cannabinoid receptor-independent mechanism and is associated with a redistribution of DAT from the plasma membrane to the cytosol (Oz et al., 2009). However, there is no direct evidence demonstrating that modulation of DAT phosphorylation by these kinases, receptors or receptor linked kinase cascades is a prerequisite for their effects on DAT trafficking and function. Another study from Page et al. has shown that activation of metabotropic glutamate receptors (mGluR5) down regulates DA uptake through the activation of both PKC and CaMKII in striatal synaptosomes (Page et al., 2001).

DAT phosphorylation was reviewed extensively by Foster et al. (2006). DAT-Ser phosphorylation was identified using p-Ser antibody (Vrindavanam et al., 1996). It is, however, important to note that the cytoplasmic domains of DAT contain putative consensus sites for several protein kinases (Kilty et al., 1991; Shimada et al., 1991). Mutatagenesis (Chang et al., 2001; Lin et al., 2003) studies revealed phosphorylation sites(s) Ser-7 for PKC, Thr-62, Ser-581 and Thr-612 for PI-3 kinase and Ser-12 and Thr-595 for ERK1/2. Using enzymatic cleavage, phospho-peptide mapping, amino acid analysis and truncation studies Vaughan and coworkers (Foster et al., 2002) identified a cluster of six serines located at the distal end of the DAT N-tail that are phosphorylated following PKC activation or phosphatase inhibition (Foster et al., 2002; Granas et al., 2003; Cervinski et al., 2005). Another cluster of serines and threonines located at the DAT N-tail more proximal to the membrane contain consensus motifs for PKC, PKA, and proline-directed kinases (Gorentla et al., 2009). DAT C-terminal Ser and Thr residues have been suggested as potential phosphorylation sites (Chang et al., 2001) for PKC, PKA, and proline-directed kinases (Gorentla et al., 2009). Whether these kinases directly phosphorylate DAT is unclear. While the cluster of N-tail serines are phosphorylated by PKC (Foster et al., 2002), elimination of PKC-mediated N-tail DAT phosphorylation by mutagenesis and truncation failed to prevent PKC mediated DAT internalization suggesting that N-terminal phosphorylation of DAT is not essential for internalization (Chang et al., 2001; Granas et al., 2003). Proline-directed kinases (e.g. RK1/2, p38 MAPK, and JNK) phosphorylated N-DAT on the proximal Thr residue. These kinases require a proline immediately following the phosphoacceptor site. Vaughan and colleagues verified the presence of pThr on the N-terminal tail of DAT and identified Thr-53 as the phosphorylation site for ERK1/2 using recombinantly expressed N-peptide domain of rDAT and heterologously expressed DAT in $LLCPK_1$ cells (Gorentla et al., 2009). These findings are also supported by the fact that MEK inhibitor U0126 is able to inhibit DAT phosphorylation (Lin et al., 2003). However, the functional implication of DAT Thr-53 and other phosphorylation sites is yet to be determined. Furthermore, using recombinantly expressed N- and C-terminal peptide domains of rDAT (N-DAT and C-DAT) and in vitro phosphorylation and dephosphorylation assays, Gorentla et. al estabilished that PKCα, PKA, PKG, and CaMKII were most efficacious

in phosphorylating N-DAT serines. Other kinases (PKCβI, PKCβII, PKCγ, and Akt) had less of an effect. The lower probability for these kinases to act on DAT, could be due to the absence of DAT-interacting proteins or other in-vivo conditions that are absent in the in vitro preparation used (Gorentla et al., 2009). PKCβII and Akt regulate DAT (Garcia et al., 2005; Johnson et al., 2005b), but regulation may be due to phosphorylation of targets upstream of DAT rather than by direct phosphorylation. DAT substrates and antagonists regulate DAT phosphorylation (described in the later part under activity dependent MA transporter phosphorylation).

4.2. Altered DAT phosphorylation; an animal model of addiction

While cocaine inhibits both SERT, and NET, several lines of evidence have shown that cocaine produces its primary reinforcing effects by binding to DAT and blocking the reuptake of DA into presynaptic terminals, thereby potentiating DA neurotransmission in mesocortico-limbic reward pathways (Ritz et al., 1987; Robinson & Berridge, 1993; Koob et al., 1998; McFarland & Kalivas, 2001). The importance of DAT to DA homeostasis and the reinforcing effects of cocaine is supported by a number of studies of cocaine-induced behavior, amine synthesis, storage, release, DA receptor expression changes resulting from constitutive gene deletion, as well as changes described in human cocaine addicts (Giros et al., 1996; Fumagalli et al., 1998; Jones et al., 1998; Rocha et al., 1998; Sora et al., 1998, 2001). Long-term changes in DAT levels, kinetics, or regulation would be expected to greatly influence spontaneous and drug-induced behaviors. Ramamoorthy and coworkers addressed potential changes in the regulation and phosphorylation of DAT using the rat-cocaine self-administration model of drug reinforcement. Their study provided evidence that environmental conditions experienced during abstinence from protracted drug use impact the nature of abstinence-induced changes in dynamic DAT regulation (Samuvel et al., 2008; Ramamoorthy et al., 2010). Specifically, in rats with a history of cocaine self-administration followed by three weeks of abstinence in which animals are not re-exposed to the environment associated with drug administration, cocaine-experienced rats exhibited elevated DAT activity and Vmax in both the caudate putamen (CPu) and nucleus accumbens (NAcc) (Samuvel et al., 2008). Furthermore, increased surface DAT density, a higher level of DAT-PP2Ac association along with decreased levels of p-Ser DAT phosphorylation were observed in the CPu, but not in the NAcc. Increased DA affinity was evident only in the NAcc (Samuvel et al., 2008). By contrast, in rats with a history of repeated cocaine self-administration followed by three weeks of daily extinction trials in which they were placed in the self-administration environment but drug was not available, DA uptake was significantly higher in the CPu relative to saline controls. In spite of elevated DAT activity in this region, total and surface DAT density and the DAT-PP2Ac interaction remained unaltered, although p-Ser-DAT phosphorylation was elevated. In contrast to the CPu, DAT activity, levels of total and surface DAT, p-Ser-DAT phosphorylation, and DAT-PP2Ac interactions remain unchanged in NAcc (Ramamoorthy et al., 2010). These studies demonstrate that abstinence from cocaine produces marked and persistent alterations in DAT function, expression and catalytic properties. Furthermore, following re-exposure to stimuli associated with drug administration alterations in DAT-trafficking and catalytic regulatory cascades differ from those that occur in their absence. Further understanding of key signaling pathways involved in cocaine-induced DAT neuroplasticity may aide in the development of environmentally based interventions for addiction, as well as pharmacotherapies that restore DAT function.

5. Activity-dependent regulation of monoamine transporter phosphorylation

Increasing evidence indicates that MA transporters are regulated by their substrates. This feedback loop provides a mechanism by which changes in extracellular neurotransmitter concentrations can rapidly modulate neurotransmitter transport capacity. Transporter substrates are known to regulate transporter function and expression (Ramamoorthy & Blakely, 1999; Chi & Reith, 2003; Gulley & Zahniser, 2003). Ramamoorthy and Blakely provided evidence that SERT substrates such as 5-HT, amphetamines, and fenfluramine control PKC-dependent SERT phosphorylation and surface redistribution (Ramamoorthy & Blakely, 1999). The inhibitory effect of 5-HT occurs in the presence of 5-HT receptor antagonists, suggesting that the effect of 5-HT on PKC-mediated SERT phosphorylation is not dependent on 5-HT-receptor activation. Furthermore, like 5-HT transport, 5-HT effects on PKC-induced SERT phosphorylation requires extracellular Na^+ and Cl^- and can be blocked by SERT antagonists, SSRIs and cocaine. Intriguingly, PKC induced decreases in cell surface SERT density can be abolished by the presence of 5-HT during PKC activation. Together, these findings suggest that SERT, in the presence of actively transporting substrates, reduce the opportunity for PKC-linked SERT phosphorylation and transporter internalization. In addition, d-amphetamine in the presence of Na^+ and Cl^- increases basal SERT phosphorylation (Ramamoorthy & Blakely, 1999). Interestingly, p38 MAPK inhibition by PD169316, not only inhibited SERT basal phosphorylation but also blunted the ability of D-amphetamine to influence phosphorylation suggesting that p38 MAPK governs the influence of D-amphetamine on basal phosphorylation and cell surface expression (Samuvel et al., 2005). Interestingly, amphetamines substitute for 5-HT in suppressing PKC-mediated SERT phosphorylation. This action could override homeostatic transporter sequestration processes and contribute to psychostimulant sensitization by increasing the number of psychostimulant targets available to a subsequent stimulus. On the other hand, non-permeant SERT ligands including SSRIs and cocaine, that prevent 5-HT permeation, block the effect of 5-HT. Thus SSRIs may have therapeutic utility in disease states, not only by preventing 5-HT uptake but also by shifting the cellular distribution of SERT.

In striking contrast to SERT, the DAT substrates DA and AMPH decrease DA uptake and cell surface DAT in a PKC dependent manner (Gorentla & Vaughan, 2005; Richards & Zahniser, 2009). However, amphetamine regulates DAT in a biphasic manner. DAT cell surface delivery is increased with in 30 s treatment of amphetamine and returned to control levels at 2.5 min. At later time points, DAT internalization is increased (Johnson et al., 2005a). A recent study demonstrated that METH is more potent than AMPH in inducing DAT-mediated DA efflux and DA efflux induced by the DAT substrate, METH as well as by AMPH is sensitive to PKC and CaMKII inhibition (Goodwin et al., 2009). Vaughan and coworkers reported that METH decreases DAT function in parallel with an increase in DAT basal phosphorylation in both striatal tissue and the LLC-PK1 cells (Cervinski et al., 2005). Interestingly, the DAT inhibitor, cocaine, and the PKC inhibitor, BIM, prevent METH effects on DAT phosphorylation. The authors also reported that removal of the N-terminal tail serine-cluster abolished both basal, PKC and METH induced DAT phosphorylation but not PKC- or METH induced transporter down regulation. Interestingly, mutation of PKC-mediated N-tail DAT phosphorylation sites (Ser-2, Ser-4, Ser-7, Ser-12 and Ser-13) into Ala blunts AMPH induced DAT-mediated DA efflux without affecting DA uptake (Khoshbouei et al., 2004). Substitution of Ser-7 and Ser-12 to Asp so as to mimic phosphorylation restored DA efflux to that of wild types. The authors proposed that phosphorylation of N-tail serine residues shifts DAT to a conformation that favors AMPH-induced DA efflux without affecting DA uptake. CaMKII has been implicated in AMPH-induced DA efflux (Fog et al., 2006). Recent studies from the Gether laboratory have provided evidence that CaMKIIa binds to the DAT C-tail facilitating phosphorylation of N-terminal Ser residues and triggering AMPH-induced DAT-mediated DA efflux (Fog et al., 2006). Furthermore, studies from Galli and coworkers established the requirement of DAT-Syn1A interactions for CaMKII to facilitate AMPH evoked DAT-mediated DA efflux (Binda et al., 2008). In this

study, blocking CaMKII activity decreased the AMPH- induced DAT-Syn1A association and DA efflux. Interestingly, the DAT-Syn1A interaction promotes transporter channel-like activity which has been implicated in AMPH-induced DA efflux (Sung et al., 2003; Dipace et al., 2007; Binda et al., 2008; Carvelli et al., 2008). Thus, DAT-CaMKII interactions modulate DAT phosphorylation and facilitate DA efflux by regulating DAT-Syn1A interactions. However, whether CaMKII triggers N-tail DAT phosphorylation Ser residues in intact cell models are unknown. Insulin has been shown to oppose AMPH induced-decreases in DAT surface expression by inhibiting Akt, a kinase downstream of PI-3 kinase (Carvelli et al., 2002; Garcia et al., 2005; Lute et al., 2008). AMPH causes a time-dependent decrease in the activity of Akt, and this effect is blocked by the DAT inhibitor cocaine, suggesting that AMPH must interact with DAT to inhibit Akt. The ability of AMPH to decrease Akt activity was blocked by CaMKII inhibition suggesting that DAT activity mediates the amphetamine-induced inhibition of Akt through a CaMKII-dependent mechanism (Wei et al., 2007). AMPH-mediated effects on DAT properties have been discussed in a previous review (Fleckenstein et al., 2007) and the reader is also referred to a very recent review of AMPH-induced reverse transport and trafficking of DAT and NET (Robertson et al., 2009).

Substitution of Thr-62 to Ala or Asp in DAT results in reduced V_{max} and lower K_m for DA. Interestingly mutation of Thr-62 to Asp causes higher basal DA efflux (Guptaroy et al., 2009). Because Thr-62 is a potential canonical site for PKC, PKG and PKA, Gnegy and coworkers suggest that phosphorylation of DAT Thr-62 may favor DAT to an inward-facing conformation during the transition cycle between inward- and outward-facing conformations (Guptaroy et al., 2009). In the view of high conservation of Thr-62 residue with SERT (Thr-81) and NET (Thr-58), Gnegy and coworkers further suggest that perhaps, the phosphorylation of Thr at this position in SERT and NET influence amine efflux and influx through regulating transporter protein conformational switch (Guptaroy et al., 2009). However, recent studies from Sitte and colleagues reported that while mutation of Thr-81 residue in SERT into Ala diminishes PCA stimulated 5-HT efflux, substituting Thr-81 to Asp also diminishes PCA stimulated 5-HT efflux and failed to rescue from the effect of Thr-81Ala mutation. Furthermore the authors also document that substitution of Thr-58 in NET and Thr-62 in DAT to Ala or Asp resulted in the failure to observe amphetamine-induced amine efflux. The authors have proposed that Thr-81 in SERT and similar positions in NET and DAT may support a network of interactions of N-terminus with internal loops lining the inner vestibule which in turn influences conformational cycle between inward- and outward-facing conformations (Sucic et al.). However, N-tail DAT-Thr-62 or SERT-Thr-81 or NET-Thr-58 phosphorylation in intact cell models and their role in conformational changes or transporter functional regulation remain to be examined. Recently, it was postulated that modification of Thr-276 in SERT via PKG-dependent phosphorylation alters the TM5 conformation that leads to increased catalytic activity (Zhang et al., 2007). It is possible that conformational changes resulting from phosphorylation or dephosphorylation of specific residue(s) or motif may dictate the ability of AMPH to influence transporter trafficking and efflux with or without the influence of transporter interacting proteins.

In LLC-PK1 cells stably expressing DAT, several DAT blockers including cocaine, mazindol and methylphenidate have no effect on basal or PKC-induced DAT phosphorylation. However, another DAT blocker, GBR 12909, suppresses both (Gorentla & Vaughan, 2005). Consistent with its effects on phosphorylation, GBR 12909 prevents PKC-induced DAT down regulation whereas other DAT blockers are without effect. Cocaine mediated upregulation of DA uptake and surface DAT has also been documented (Daws et al., 2002; Little et al., 2002). These observations indicate that substrates and inhibitors have distinct effects on DAT phosphorylation and expression, and indicate distinct molecular mechanisms of action. Although, several cellular

candidates that are involved in DAT mediated DA influx and efflux have been identified, the influence of multiple signaling cascades in the regulation of DAT which is occupied with substrates and/or inhibitors remains to be determined.

NET proteins are down regulated following acute and chronic treatment (3 days) with either NE and AMPH or the NET uptake inhibitor, desipramine, perhaps through changes in protein expression and/or NET turnover (Zhu et al., 1998). Amphetamine but not cocaine treatment decreased the number of NET binding sites providing further evidence that NET ligands affect transporter expression (Zhu et al., 2000). In the CAD, catecholaminergic cell line, a CaMKII dependent (KN93-sensitive) NET-SYN1A association has been implicated in mediating acute AMPH-mediated NET downregulation. Deletion of amino acids 28-47 from the N-terminus accelerates AMPH-mediated decrease in surface NET through a CaMKII-dependent increase in NET–SYN1A interaction (Dipace et al., 2007). However, a similar decrease in surface NET was achieved when CAD cells expressing wild type-hNET or hNETΔ_{28-47} were exposed to AMPH for 60 min. How amino acids located in positions 28–47 regulate these processes is unknown. Intriguingly, in human placental trophoblast, HTR cells, mutation of the PKC-sensitive motif Thr-258/Ser-259 prevented acute AMPH-mediated NET downregulation and endocytosis (Annamalai et al., 2010) but did not perturb cocaine mediated NET upregulation (Jayanthi et al., 2010). The NET-specific blocker desipramine prevented AMPH-induced NET downregulation. However, inhibition of PKC, CaMKII, p38 MAPK or depletion of Ca^{2+} was without effect. Although, the results are consistent in both CAD and HTR cells with regard to acute AMPH exposure decreasing surface NET density, it is reasonable to speculate that the underlying molecular mechanisms linked to AMPH-mediated NET downregulation may be distinctly different in CAD and HTR cells and perhaps specific to the CNS and periphery. Recently, using ex vivo rat cortical preparations and mouse superior cervical ganglion neurons, Matthies et al. demonstrated that acute AMPH exposure triggers NET to accumulate in recycling endosomes in a Rab-11 dependent manner (Matthies et al., 2010). Thus studies exploring the effects of substrates and inhibitors on NET phosphorylation and functional expression are just emerging.

Collectively these studies indicate the potential for endogenous amine substrates and psychostimulants (substrates or blockers) in concert with other regulatory cascades to modulate the retention time of MA transporters at the plasma membrane via activity dependent-transporter phosphorylation. It is also possible that state of transporter phosphorylation may modulate how endogenous and psycho-active compounds interact with transporters. Substrate translocation or inhibitor occupancy may influence protein equilibrium to establish a conformational set to favor and/or hinder transporter phosphory-lation or transporter association with other regulators. Thus, control of transporter cell surface expression by endogenous amines may provide a unique homeostatic mechanism in the neuron to fine-tune transport capacity to match the changing demands imposed by fluctuation in synaptic amine concentrations that occur in response to multiple signals. It is important to note that while SERT substrates induce neurotransmitter clearance in the synapse by increasing surface transporters, the opposite occurs for DAT. The physiological relevance of this divergent regulation has yet to be determined. Substrate mediated amine transporter regulation not only serves to regulate the level and duration of available synaptic amines to receptors, but also regulates the neuronal accumulation of neurotox-ins such as METH, MDMA, MPTP and 6-hydroxydopamine leading to nerve terminal damage and neurodegeneration.

6. Role of protein phosphatases in the regulation of monoamine transporters

The state of a phosphoprotein is the balance between phosphory-lation and dephosphorylation rates catalyzed by protein kinases and protein phosphatases. While research from different laboratories has

Table 4
Effects of human SERT coding variants on SERT expression and regulation.

hSERT variants	Effect on SERT activity (*versus* wild type)				
	Basal	PKG-activation	P38 MAPK-activation	PKC-activation	Surface expression
hSERT-WT	100%	Stimulation	Stimulation	Inhibition	100%
Thr4Ala[1]	Higher	Insensitive	Insensitive	More-sensitive	Similar to WT
Gly56Ala[1,3*#]	Higher	Insensitive	Insensitive	More-sensitive	Similar to WT
Lys201Asn[2]	Higher	NT	NT	NT	Similar to WT
Glu215Lys[1]	Similar to WT	Insensitive	Insensitive	Similar to WT	Similar to WT
Leu255Met[1]	Similar to WT	Similar to WT	Similar to WT	Similar to WT	Similar to WT
Ser293Phe[1]	Higher	Similar to WT	Similar to WT	Similar to WT	Similar to WT
Pro339Leu[1]	Lower	Similar to WT	Similar to WT	Similar to WT	Lower
Leu362Met[1]	Higher	Similar to WT	Similar to WT	Similar to WT	Similar to WT
Ile425Val1[1, 4]	Higher	Insensitive	NT	Similar to WT	Similar/higher to WT
Ile425Leu[3]	Higher	Less-sensitive	Less-sensitive	< = Less-sensitive	Higher
Phe465Leu[3*]	Higher	Less-sensitive	Less-sensitive	< = Less-sensitive	Higher
Leu550Val[3]	Higher	Less-sensitive	Less-sensitive	< = Less-sensitive	Higher
Lys605Asn[1]	Similar to WT	Insensitive	Insensitive	More-sensitive	Similar to WT
Pro621Ser[1]	Similar to WT	Insensitive	Insensitive	More-sensitive	Similar to WT

NT: Not tested; < = comparable or slightly reduced sensitivity in comparison with WT; *: Less sensitive to PP2 inhibition with calyculin and fostriecin in comparison with WT; #exhibits elevated basal phosphorylation.
[1] (Prasad et al., 2005) [2](Rasmussen et al., 2009); [3](Prasad et al., 2009)[4](Kilic et al., 2003; Zhang et al., 2007).

provided much information regarding the role of protein kinases in the regulation of MA transporter functional expression and phosphorylation, very little is known about MA-transporter dephosphorylation and its role in regulating transporter function. The PP1/PP2A inhibitor, okadaic acid (OK) down regulates SERT, DAT and NET activity (Bauman et al., 2000; Foster et al., 2003). Treatment with OK or another potent PP1/PP2A inhibitor, calyculin A, results in rapid increase in SERT phosphorylation in parallel with the decrease in 5-HT uptake (Ramamoorthy et al., 1998; Bauman et al., 2000). Similarly these inhibitors also promote DAT phosphorylation and functional down regulation in striatal synaptosome preparations (Vaughan et al., 1997). Studies have shown that PP2Ac and phosphatase activity present with MA transporter as a complex (Bauman et al., 2000). Interestingly, the associations can be regulated by kinases. In addition, Foster et al established that PP1 dephosphorylates phospho-DAT in striatum and cell preparations (Foster et al., 2003). While it is not known whether MAPK phosphates regulate DAT phosphorylation, Amara's group demonstrated that MAPK phosphates, MKP3 functions as a positive regulator of DAT by stabilizing DAT at the cell surface. The latter effect is due to interference with dynamin-dependent internalization and a delay in DAT degradation (Mortensen et al., 2008). It has been proposed that the association of PP2Ac or the activity of PP1 may govern the stoichiometry of amine transporter phosphorylation as well as the duration of the phosphorylated form of the transporter. Thus, coordinated phosphorylation and dephosphorylation provides an important trigger for transporter protein expression and trafficking.

7. Role of lipid rafts in the regulation of monoamine transporters

Cholesterol depletion reduces SERT, NET and DAT activity. Recent studies provided evidence for lipid raft localization and raft-mediated internalization of SERT, NET and DAT (Scanlon et al., 2001; Jayanthi et al., 2004; Magnani et al., 2004; Samuvel et al., 2005; Adkins et al., 2007; Foster et al., 2008; Matthies et al., 2009). PKC activation stimulates lipid raft-mediated internalization of native NET expressed in placental trophoblasts (Jayanthi et al., 2004). Interestingly, PKC-, but not p38 MAPK-mediated SERT regulation in rat brain involves raft-mediated distribution (Samuvel et al., 2005). The presence of NET and SERT in lipid rafts suggests that signaling machinery specific to lipid rafts may be linked to PKC-mediated transporter down regulation. Similar to SERT and NET, the presence of DAT in lipid-rafts has been demonstrated in striatal tissue and cell models (Adkins et al., 2007; Foster et al., 2008). Using fluorescence correlation

spectroscopy, fluorescence recovery after photobleaching and biochemical approaches Adkins et al. demonstrated that depletion of cholesterol or disrupting the cytoskeleton increased lateral mobility of DAT, suggesting that association of the DAT with lipid microdomains in the plasma membrane and/or the cytoskeleton serves to regulate both the lateral mobility of the transporter and its transport capacity (Adkins et al., 2007). It is interesting to note that Foster et al. described that while activation of PKC did not alter DAT distribution between lipid rafts and non-rafts, the majority of PKC stimulated phosphorylation occurs at DAT located in lipid rafts (Foster et al., 2008).

Raft- associated sorting has been proposed to underlie several cellular processes including signal transduction, protein sorting and membrane trafficking (Chamberlain & Gould, 2002). GPCRs, tyrosine kinase receptors, several channel proteins, many components of GPCR signal transduction proteins including adenylyl cyclase, Akt, PLC, activated PKC, PP2Ac, non-receptor tyrosine kinases, and other signaling molecules such as syntaxin 1A (a SNARE protein), alpha-synuclein and the PKC binding protein PICK1 are associated with lipid rafts (Simons & Toomre, 2000; Chamberlain & Gould, 2002). The localization of receptors that regulate MA transporter function and potential transporter-interacting proteins as well as transporters in lipid raft microdomains, raises the possibility that lipid rafts may act as morphological "conveyers" of signal transduction by placing various signal transduction molecules near the transporter molecule. For example, transporter-interacting proteins may "guide" targeting of the transporter to lipid rafts, and phosphorylation of a "motif/site" within the transporter may act as a "signal" for fostering protein–protein interactions and redistribution. Thus, protein redistribution from plasma membrane microdomains may be one of the several mechanisms by which synaptic plasticity and neurotransmitter homeostasis are maintained (Simons & Ikonen, 1997; Parton & Richards, 2003). More interestingly, antidepressants and antipsychotic drugs are colocalized in raft-like domains, suggesting that lipid-raft localized amine transporters SERT and NET as well as 5-HT$_3$ receptors may functionally interact with antidepressants and antipsychotic drugs therein, and this interaction may play a role in the pharmacological effect of these drugs (Eisensamer et al., 2005).

8. Dysregulation of amine transporters in human disease

Several lines of evidence suggest that variants of the MA-transporter gene may play important roles in a number of neuropsychiatric disorders. Systematic screening for DNA sequence variations

in the coding regions of SERT, NET and DAT uncovered several amino acid changes in these proteins. We emphasize here those variations exhibiting abnormal regulation. Comprehensive details of these coding variants can be found in the original publications (Hahn et al., 2003; Lin & Uhl, 2003; Ozaki et al., 2003; Hahn et al., 2005; Prasad et al., 2005; Sutcliffe et al., 2005; Mazei-Robinson & Blakely, 2006; Hahn & Blakely, 2007; Hahn et al., 2008; Mazei-Robison et al., 2008; Carneiro et al., 2009; Prasad et al., 2009).

8.1. The effect of SERT coding variants on SERT regulation in disease states

A recent search for sequence variants in the SLC6A4 gene, encoding SERT, uncovered 15 variants in genomic DNA from a population of 450 individuals in the DNA Polymorphism Discovery Resource database 2(Ozaki et al., 2003). Of these, six were synonymous and the remaining nine resulted in an amino acid change (Thr-4Ala, Gly-56Ala, Leu-255Met, Ser-293Phe, Pro-339Leu, Leu-362Met, Ile-425Val, Lys-605Asn and Pro-621Ser) (summarized in Table 4). Murphy and colleagues reported that two unrelated probands and their family members with Obsessive-Compulsive Disorder (OCD) contained the Ile-425Val coding variant carried on the 5-HTTLPR/1/1 background (Ozaki et al., 2003). In functional studies, this variant has been found to exhibit elevated 5-HT uptake activity without any change in SERT surface expression and is insensitive to stimulation by nitric oxide donors in transfected cells (Kilic et al., 2003). Recently, a thorough study reveals that PKG activator, 8-Br-cGMP stimulates the activity of wild type SERT, but not Ile-425Val, and the activity of wild type SERT stimulated by 8-Br-cGMP was similar to that of the Ile-425Val mutant. In addition, more prolonged treatment with inhibitors of soluble guanylyl cyclase or PKG decreased activity of the Ile-425Val mutant, but not the wild type, indicating that Ile-425Val mutant transporters could exist in high activity forms due to sustained targeting by PKG. In addition, mutation of the PKG phosphorylation site Thr-276 into alanine not only prevented activation of wild type hSERT through the PKG pathway but also blocked the inhibition of Ile-425Val-5-HT uptake activity by inhibitors of the pathway (Zhang et al., 2007). Furthermore, the elevated 5-HT uptake activity found in Thr-276Asp is similar to the level of 5-HT uptake activity found in Ile-425 V. In addition, double mutants containing both Ile-425Val and Asp at 276 did not lose activity when treated with a soluble guanylyl cyclase inhibitor (ODQ) in contrast with Ile-425Val alone, in which activity decreased over the incubation time. ODQ also had no effect on Thr-276Asp (Ramamoorthy et al., 2007; Zhang et al., 2007). Collectively, these findings suggest that mimicking PKG phosphorylation by mutating Thr-276 into Asp-276 results in an elevated 5-HT transport phenotype that parallels that of Ile-425Val and is insensitive to PKG-linked SERT modulation.

Other human variants such as Gly-55Ala, Ile-425Leu, Phe-465Leu and Leu-550Val are linked to autism (Prasad et al., 2005; Sutcliffe et al., 2005). Autism linked hSERT variants Ile-425Val, Gly-55Ala, Ileu-425Leu, Phe-465Leu and Leu-550Val exhibit elevated 5-HT transport (Prasad et al., 2009). Remarkably, human SERT variants, Thr-4Ala and Gly-56Ala failed to show 5-HT uptake stimulation when tested with 8-Br-cGMP and the p38 MAPK inhibitor, anisomycin, typical of wild type hSERT. Importantly, in a physiologically relevant human cell model, such as the Epstein–Barr virus (EBV)-transformed lymphocyte which natively expresses the most common of these variants (Gly-56Ala), a loss of 5-HT uptake stimulation by PKG and p38 MAPK activators was seen as was insensitivity to PP2A inhibition and enhanced sensitivity to PKC mediated regulation. Furthermore, HeLa cells transfected with the Gly-56Ala variant exhibit elevated basal phosphorylation and, unlike wild type hSERT, could not be further phosphorylated after 8-Br-cGMP treatments (Prasad et al., 2005). Other coding variants associated with autism such as Ile-425Leu, Phe-465Leu and Leu-

550Val exhibit low sensitivity to PP2A inhibition, but unaltered response to PKG or p38 MAPK stimulation. These results suggest that the effect of the Gly-56Ala or Ile-425Val mutation may shift the balance of SERT toward the phosphorylated form, possibly by elevated/sustained phosphorylation of PKG-dependent-Thr-276 residue due, in part, to an altered role of PP2A.

8.2. The effect of NET coding variants on NET regulation in disease states

Nonsynonymous single nucleotide polymorphisms (SNPs) in the human NET (hNET) gene that influence transporter function can contribute to disease, such as the nonfunctional transporter, Ala-457Pro, identified in orthostatic intolerance. The hNET gene polymorphism with an Ala-457Pro mutation in the coding region has been identified in an individual with the autonomic disorder orthostatic intolerance (OI) (Shannon et al., 2000). The presence of the hNET-Ala-457Pro allele tracked with elevated heart rates and plasma NE levels in family members suggesting that genetic or acquired deficits in NE inactivation may underlie hyperadrenergic states that lead to orthostatic intolerance. hNET-Ala-457Pro lacked transport activity and had reduced surface expression compared to wild type hNET. Defects in biosynthetic progression and trafficking have been attributed to reduced functional expression of hNET-Ala-457Pro and dominant-negative effect on wild type hNET (Hahn et al., 2003). Similar to the Ala-457Pro variant, several other hNET variants (e.g., Ala-369Pro and Asn-292Thr) were found to be defective in glycosylation, retained intracellularly, lacked NE transport function and exhibited a dominant negative effect on wild type hNET. The most intriguing finding was that another variant, Phe-528Cys, had higher transport capacity and exhibited insensitivity to down-regulation by PKC. Phe-528Cys variant showed decreased potency for the tricyclic antidepressant desipramine (Hahn et al., 2005).

8.3. The effect of DAT coding variants on DAT regulation in disease states

Multiple, rare, hDAT coding variants have been described and characterized. An analysis of function and regulation of five naturally occurring coding variants, Val-55Ala, Arg-237Gln, Val-382Ala, Ala-559Val and Gln-602Gly, expressed in COS-7 and SH-SY5Y revealed that all variants, except Val-382Ala, exhibit levels of surface protein expression and DA transport activity comparable to wild type hDAT (Mazei-Robison & Blakely, 2005). Val-382Ala, divergent at the most highly conserved residue among reported hDAT variants, exhibited altered substrate selectivity for DA versus NE, diminished surface expression and decreased sensitivity to phorbol ester induced internalization (Mazei-Robinson & Blakely, 2006). Although a single variant within the hDAT gene is unlikely to play a major role in the ADHD, Mazei-Robison et al. recently identified a pedigree containing two male children diagnosed with ADHD who share a rare DAT coding variant, Ala-559Val (Mazei-Robison et al., 2008). This variant has only been isolated once previously, in a female subject with bipolar disorder. Although hDAT Ala-559Val supports normal DAT protein and cell surface expression, as well as normal DA uptake, the variant exhibited anomalous DA efflux from DA-loaded cells. The Ala599Val variant exhibited increased sensitivity to intracellular Na^+, but not intracellular DA, and displayed exaggerated DA efflux at depolarized potentials. In contrast to wild type DAT, amphetamine and methylphenidate, two of the most commonly used ADHD medications blocked hDAT Ala-559Val-mediated DA efflux rather than inducing the efflux as occurs with wild type DAT. It has been hypothesized that AMPH-induced changes in intracellular signaling pathways and hDAT phosphorylation (Fog et al., 2006) may be constitutively promoted in hDAT Ala-559Val, and AMPH application may restore an efflux-incompetent conformation. In support of this idea, elevated basal hDAT A559V phosphorylation can be attenuated by AMPH (Mazei-Robison et al., 2008). Furthermore, recent studies demonstrated that hDAT A559V exhibits elevated Ser-7 and Ser-
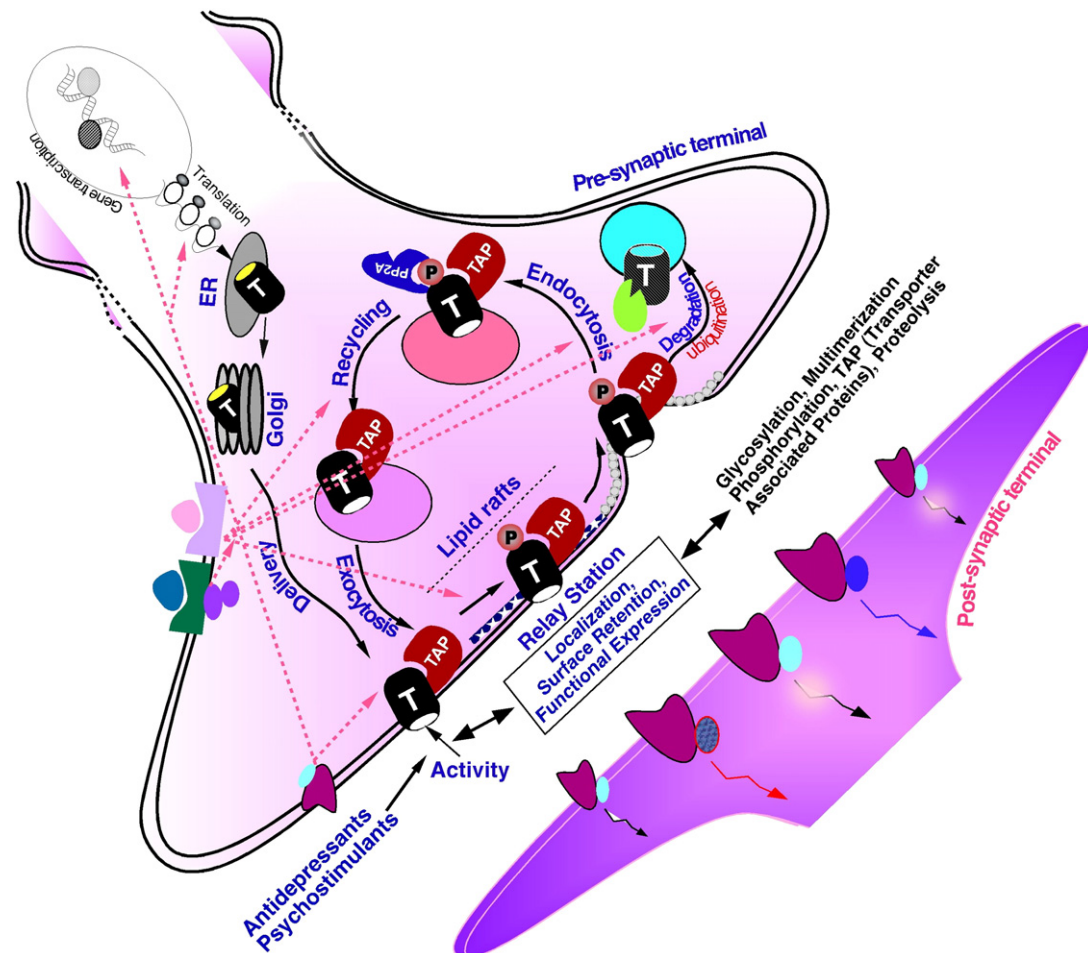
Fig. 1. Hypothetical representation of functional regulation of MA transporters. Based on published reports, MA-transporters are subject to regulation at the levels of transcription, translation and functional delivery. Presynaptic homo- and hetero- receptors can trigger specific signaling cascades and hence MA transporter regulation to maintain normal amine neurotransmission. At the gene level, transporter gene transcription can be regulated by many cellular signals and transcription factors. At the protein level, MA transporters may be organized as a complex in the plasma membrane motifs (lipid rafts versus non-lipid rafts) that can be regulated by several cellular mechanisms, such as phosphorylation, protein-protein interactions, and ubiquitination. Transporter activity can be regulated through trafficking dependent and/or independent mechanisms. Kinase/phosphatase mediated post translational modifications such as phosphorylation regulate transporter catalytic functions (amine influx/efflux), surface residency or both. At the plasma membrane level, lipid raft microdomains, in which MA transporters along with other signalosomes are located, may serve as morphological "conveyers" of signal transduction in the milieu of various incoming signals. Phosphorylated transporters may be subjected to trafficking dependent or independent regulatory mechanisms. Transporters may enter into a regulated endocytic pathway and, thus, internalized transporters may be subjected to degradation or recycling depending on the signals and cell system. Internalized transporters could be dephosphorylated by associated protein phosphatases allowing the return of nonphosphorylated transporters to the cell surface. MA transporters such as endogenous amines and psychostimulants (substrates or blockers) in concert with other regulatory cascades can modulate the retention time of MA transporters at the plasma membrane via activity dependent-transporter phosphorylation. Thus, disease linked human coding variants can influence activity dependent regulation, thereby, producing an abnormal phenotype. (T: Transporters; TAP: Transporter Associated Proteins; PP2A: Protein Phosphatase 2A; P; phosphorylation; ER: Endoplasmic Reticulum).

13 phosphorylation and support anomalous DA efflux in transfected HEK-293 cells (Bowton et al., 2010). Interestingly, blockade of D_2R-linked CaMKII signaling attenuated anomalous DA efflux found in hDAT A559V in transfected HEK-293 cells and DA neurons. These studies provided further elucidation of the potential role of presynaptic DA autoreceptor signaling in the regulation of DAT mediated DA influx (Bolan et al., 2007; Zapata et al., 2007) and efflux (Bowton et al., 2010) for normal DA homeostasis.

Based on recent findings with naturally occurring rare MA transporter coding variants, it is tempting to speculate that altered MA transporter phosphorylation could be one of the molecular mechanisms contributing to functional deficits in transporter biosynthesis, activity, regulation and sensitivity that have been identified in human disease.

9. Conclusion

In general, accumulated evidence suggests that while activation of PKC down regulates MA transporter function, activation of MAPKs and tyrosine kinases upregulate MA transporters. As shown in Fig. 1,

regulation can occur at several subcellular locations of MA transporters to ensure response to external signals for proper MA transporter functional expression to regulate aminergic neurotransmission and behavior. It is noteworthy that the intrinsic transport capacity of a transporter molecule governs its own plasma membrane expression and function. There appears to be multiple mechanisms by which transporter substrates and antagonists can influence transporter function and expression. The capacity of a transporter to fine-tune its function in response to extracellular neurotransmitter would maintain a constant level of neurotransmitter at the synaptic cleft. On the other hand interactions with psychostimulants would interfere and compromise the normal activity-dependent regulation of MA transporters and may cause or initiate neuroplastic changes associated with drug addiction. SSRIs and other agents may have therapeutic utility in disease states, not only by preventing amine uptake but also by shifting the cellular distribution of MA transporters.

The past few years have been an active period of research with regard to coding variants of MA transporters and their impact on normal transporter regulation. These investigations strongly support

the idea that naturally occurring human disease linked coding variants exhibit altered amine-transport phenotypes as a consequence of altered transporter phosphorylation. Much progress has been made in identifying the contribution of kinase cascades, transporter interacting proteins and phosphorylation to MA transporter regulation in a variety of systems using receptor/kinase-activators or inhibitors. Fundamental questions, however, remain as to whether such regulation is important for MA signaling and MA-dependent behaviors. Translating information obtained from in vitro and ex vivo studies to in vivo settings is a challenging question and must be addressed in the next decade. Developing genetically modified mice bearing deficits in specific regulatory elements and/or introducing naturally identified mutants should facilitate systems level analysis of transporter response in the face of neuronal excitability and receptor-second-messenger activation and aid our understanding of the contribution of that particular regulatory cascade in amine signaling, behavior and psychiatric illnesses.

In summary, investigations from several laboratories provide clear evidence that MA transporters are principle players in regulating normal and abnormal amine signaling in the CNS and periphery and, hence, are key players in the control of complex behavioral and physiological functions. Future studies are needed to delineate cis/trans acting elements and signals for acute and chronic amine transporter regulation and their physiological relevance. Many of the tools needed to address these issues are already in place and should allow for rapid progress in understanding the role of cellular regulation of amine transporters in amine signaling and behavior. The results from this work will aid in the development of novel targets for manipulating MA transporter regulation and more effective therapies for the treatment of depression, addiction and other psychiatric disorders.

Acknowledgments

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