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Dopamine Uptake Inhibition Potency Fluctuations of Cocaine at the Dopamine Transporter

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DOPAMINE UPTAKE INHIBITION POTENCY FLUCTUATIONS OF COCAINE AT THE DOPAMINE TRANSPORTER

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LIST OF ABBREVIATIONS

DA - Dopamine

DAT - Dopamine transporter

NSS - Neurotransmitter:sodium symporter

DUIP - Dopamine uptake inhibition potency

NET - Norepinephrine transporter

SERT - Serotonin transporter

GAT1 - GABA transporter

I. ABSTRACT

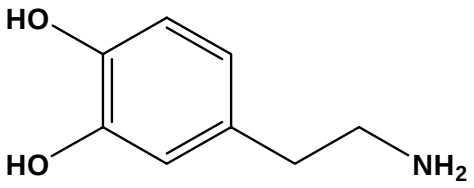
Cocaine and other dopamine transporter (DAT) inhibitors block synaptic dopamine uptake by binding to the DAT. Cocaine, a powerful psychostimulant, potentiates the dopamine mediated signal transduction in the reward areas of the brain resulting in physical dependence on the drug. Amphetamines are psychotomimetics that act as substrates at the DAT and potentiate dopaminergic neurotransmission leading to addiction. It was previously reported that a conservative glutamate-for-aspartate substitution in transmembrane 1 domain of the rat DAT protein (D79E) significantly decreased the binding affinities of several classical DAT inhibitors, but had little or no effect on the dopamine uptake inhibition potencies (DUIPs) of these blockers. This finding implied that different DAT sites/conformations/populations may be responsible for dopamine uptake and for high affinity binding of inhibitors. In the present study, the DUIPs of cocaine and other DAT ligands at wildtype DAT CHO cells fluctuated as a function of cell age, while their binding affinities remained static. Curiously, neither the DUIP nor the binding affinity of amphetamine fluctuated with cell age. The DUIP fluctuations of cocaine also extended to the N2A neuronal cells, demonstrating its physiological relevance. The loss of correlation between DUIP and binding affinity at DAT might be a unique property of DAT blockers that is not exhibited by DAT substrates. A plausible explanation for the DUIP fluctuations with cell age is the existence of two different DAT populations on the cell surface, the relative distribution of which changes with cell age under the influence of intracellular events such as DAT-protein interactions or alterations in the

phosphorylation state. Several proteins interact with the N- and C-terminal regions of the DAT. Studies employing GFP fusion polypeptides of the DAT N- or C-terminal fragments, which would act as decoys by intercepting DAT modulators, showed that the DAT N- or C-terminal interactions with intracellular proteins do not appear to be responsible for the DUIP shift. The role of phosphorylation state changes of DAT in DUIP fluctuations was also investigated. Studies employing a mutant DAT lacking the first 20 N-terminal amino acids demonstrated that N-terminal phosphorylation of DAT, specifically involving the first five serines is not associated with the DUIP shift.

II. INTRODUCTION

A. Overview

Dopamine (3,4-dihydroxyphenylethylamine) is a major catecholamine neurotransmitter in the brain that plays an important role in brain processes that control locomotor activity, reward, attention and cognition (Schultz, 2002). It is synthesized in the neurons from the amino acid tyrosine and stored in the synaptic vesicles. In response to a presynaptic action potential, dopamine is released into the synaptic cleft by exocytosis, where it binds to the presynaptic and postsynaptic dopamine receptors (Figure 1).



Dopamine

The dopamine transporter (DAT) protein mediates the reuptake of dopamine into the presynaptic neuron and is primarily responsible for regulating the intensity and duration of the dopaminergic neurotransmission. DAT is considered a specific marker of dopaminergic neurons in the central nervous system and is found in dendrites, perikarya, axons and nerve endings (Nirenberg et al., 1996, 1997). It is the site of action of psychostimulants such as cocaine, and neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). DAT has been implicated in the etiology of several neurological and psychiatric conditions such as Parkinson's disease, schizophrenia, Tourette's syndrome,

depression, attention deficit hyperactivity disorder (ADHD) and drug addiction (Torres, 2006; Bannon, 2005).

Several dopamine-mediated pathways exist in the brain. The mesolimbic and the mesocortical pathways play key roles in reward assessment. Dopaminergic projections from the ventral tegmental area (VTA) to the limbic areas (nucleus accumbens, ventral striatum and amygdala) comprise the mesolimbic pathway and projections from the VTA to the cortex (medial, prefrontal, cingulated and entorhinal cortex) comprise the mesocortical pathway (Marsden, 2006). Other pathways include the nigrostriatal pathway (substantia nigra to the caudate putamen) that is associated with locomotor activity and the tuberoinfundibular pathway (hypothalamus to the pituitary) that is associated with inhibition of prolactin release.

Cocaine is an alkaloid, extracted from the leaves of the coca plant (*Erythroxylon coca*). It is the most potent stimulant of natural origin and is categorized as a Schedule II drug. Cocaine inhibits synaptic dopamine uptake by interfering with DAT function. It blocks the reuptake of dopamine into presynaptic neurons resulting in increased synaptic dopamine concentrations (Figure 1). Amphetamines are another class of drugs with high potential for abuse and addiction. Methamphetamine is the most commonly used amphetamine in the U.S. Amphetamines increase the synaptic dopamine concentrations by a mechanism different from that of cocaine. They act as DAT substrates and compete with dopamine for reuptake into the presyaptic neuron (Figure 1). Amphetamines also cause DAT-mediated dopamine efflux by

reversal of the transporter. The inward transport of amphetamine by the DAT increases the number of "inward-facing" transporter binding sites and thereby increases the rate of outward transport of dopamine (Kahlig and Galli, 2003). A recent study has implicated a channel-like mode of the DAT in amphetamine-mediated dopamine efflux (Kahlig et al., 2005). Amphetamines also act as weak bases at the vesicular monoamine transporters (VMAT) and release the vesicular dopamine stores, resulting in increased intracellular dopamine concentrations that also contribute to reverse transport. The resultant potentiation of dopaminergic neurotransmission in the reward areas of the brain is associated with psychostimulant addiction.

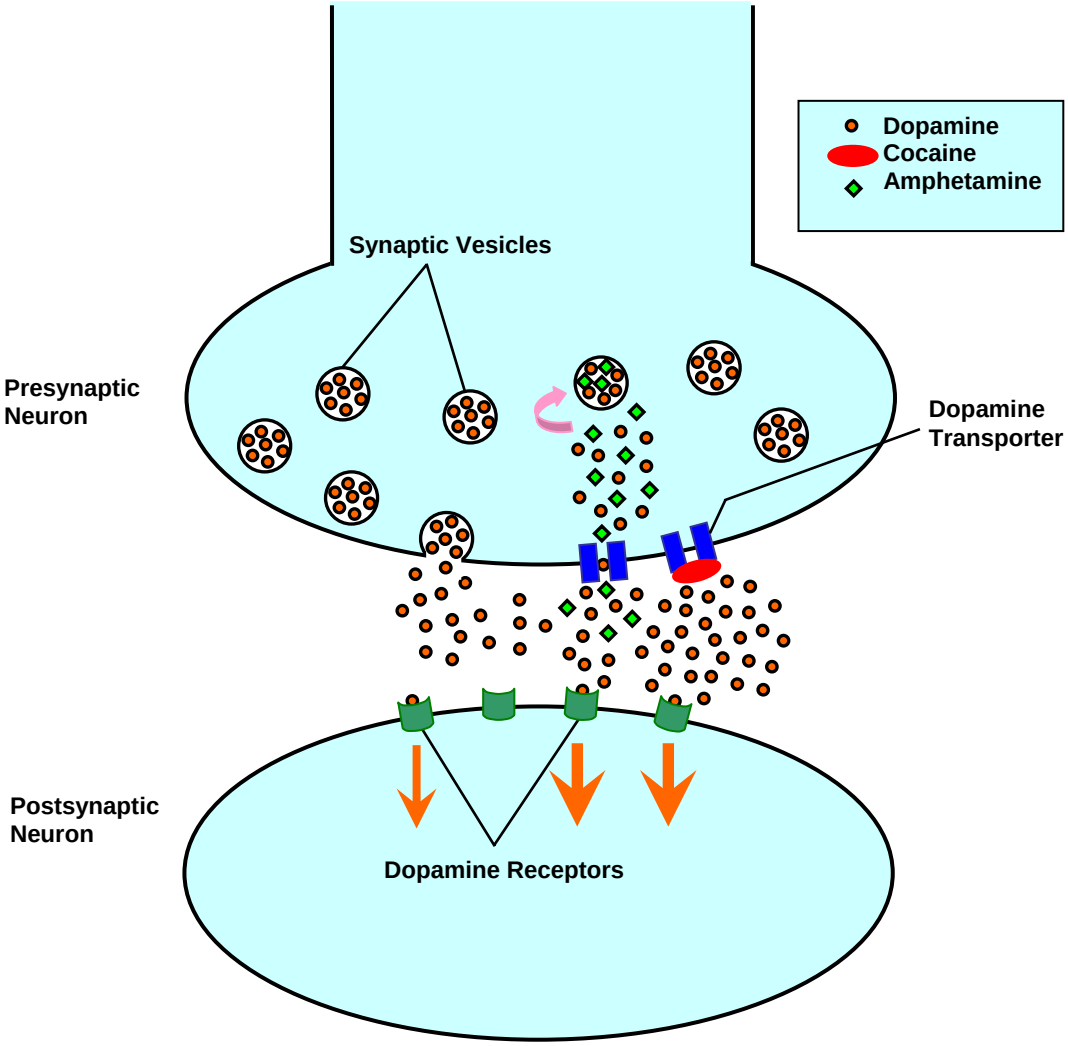


Figure 1. Modulation of the dopaminergic neurotransmission by psychostimulants.

Dopamine, synthesized in the presynaptic neuron, is stored in the synaptic vesicles. Following the arrival of an action potential, dopamine is released into the synaptic cleft by Ca^{2+}-mediated exocytosis. Dopamine binds to the dopamine receptors on the postsynaptic neurons and transmits neuronal signals. Neurotransmission is terminated primarily by reuptake of dopamine into the presynaptic neuron via the DAT. Cocaine blocks the dopamine reuptake and increases synaptic dopamine concentrations. Besides competing with dopamine for reuptake, amphetamine promotes dopamine efflux.

B. Statement of the Problem

Cocaine abuse and addiction remains a major problem in the U.S. According to the 2004 National Survey on Drug Use and Health, approximately 34.2 million Americans have experimented with cocaine. There are more than 2.5 million chronic cocaine users and around 3 million occasional cocaine users in the U.S. The tolerance factor of cocaine leads to compulsive and addictive use within the first few uses. Even after long periods of abstinence, the memory of euphoria associated with cocaine can trigger tremendous craving and relapse to drug use. Cocaine addiction is rapidly progressive and can lead to severe medical, psychiatric, and psychosocial problems. It can even contribute to the spread of human immunodeficiency virus (HIV) infections, as a consequence of needle sharing. The euphoria associated with cocaine use is reported to be intensely pleasurable and results from the mesolimbic dopamine neurotransmission. The DAT has remained a primary target, with interactions at serotonin transporter (SERT) and norepinephrine transporter (NET) playing a secondary role, in cocaine abuse and addiction. There are no effective medications currently available to treat cocaine addiction. Drugs touted as therapies for cocaine addiction have proven to be addictive themselves. It would be beneficial to identify a compound that blocks cocaine's binding to the DAT while allowing dopamine reuptake. No crystallographic or high resolution data are available for any of the mammalian neurotransmitter transporter proteins. Molecular 3-D models of the DAT have been developed based on sequence homology with other protein families and data from site-directed mutagenesis

studies. Therefore, definitive information about the conformation of the DAT or the binding sites of the DAT ligands is not available.

It was previously demonstrated that replacement of the aspartic acid residue at position 79 in transmembrane domain (TM) 1 of rDAT with glutamic acid (D79E) significantly decreased binding affinities of certain DAT inhibitors, while dopamine uptake inhibition potencies (DUIPs) remained unaffected. Thus, the point mutation decoupled the binding affinities of the DAT blockers from their respective DUIPs (Wang et al., 2003). In the course of the above study, it was observed that the DUIP of cocaine at wildtype (WT) DAT fluctuated over a wide range. Moreover, the DUIP of cocaine did not match its binding affinity value at the WT DAT. Several studies record a similar loss of correlation between DUIP and binding affinity of cocaine at the WT DAT (Pristupa et al., 1994; Eshleman et al., 1999; Wang et al., 2003). Cocaine DUIP values differ by several fold at the WT DAT as a function of the assay conditions (Reith and Coffey, 1994; Eshleman et al., 1999). Considering the fact that the same stably transfected WT DAT CHO cell line and similar assay conditions were employed in all the experiments, similar DUIP values for cocaine would be expected. One of the factors that differed between the assays was the cell passage number. Therefore, the nature of the DUIP fluctuation phenomenon was investigated by studying the influence of cell passage number on the DUIP of cocaine. The DUIP fluctuation phenomenon was further characterized by studying whether this phenomenon extended to the other DAT blockers and DAT substrates. The physiological relevance of the DUIP fluctuation phenomenon was also

investigated. The specific hypothesis to be tested here is that the DUIP of cocaine and other DAT ligands at WT DAT fluctuates as a function of cell passage.

Studies were extended further to identify the factors responsible for the DUIP fluctuation. DAT inhibitors may have more than one binding site with different affinities at each site (Madras et al., 1989; Boja et al., 1991; Boja et al., 1992; Rothman et al., 1994; Reith and Coffey, 1994; Gracz and Madras, 1995). Two distinct inhibitor-binding DAT conformations or populations may exist and the DAT conformation or population responsible for inhibitor high-affinity binding may be less responsible for dopamine uptake (Wang et al., 2003). DAT function and trafficking can be regulated by several factors such as protein-protein interactions, oligomerization state of the transporter and posttranslational modifications. Several proteins have been shown to interact with the N- and C-terminus of DAT (reviewed in Torres et al., 2006). The specific hypothesis to be tested here is that intracellular factors interacting with the DAT N- or C-terminus are responsible for the DUIP fluctuation of cocaine at the WT DAT.

C. Literature Review

1. Structure of the DAT

DAT belongs to the Na^+/Cl^--dependent family of neurotransmitter transporters, also referred to as the neurotransmitter:sodium symporter (NSS) family. This family includes the transporters for biogenic amines (norepinephrine, serotonin and dopamine), amino acids (GABA, glycine, proline and taurine) and osmolytes (betaine and creatinine). A 620 (human) or 619 (rat) amino acid DAT sequence is predicted from cloned cDNAs (Kilty et al., 1991; Giros et al., 1992) with an apparent molecular mass of approximately 80,000 daltons (Patel et al., 1994). DAT is composed of 12 transmembrane (TM) domains connected by alternating intracellular and extracellular loops, with the N- and C-termini located on the intracellular side of the membrane (Edvardsen and Dahl, 1994; Nirenberg et al., 1996; Hersch et al., 1997). It has a large second extracellular loop that has potential N-glycosylation sites (Vandenbergh et al., 1992; reviewed in Surratt et al., 1993). Stability and plasma membrane trafficking of the DAT is strongly influenced by glycosylation (Reith et al., 1997).

Approaches such as site-directed mutagenesis, chimeric transporters and substituted cysteine accessibility method (SCAM) have been extensively employed to identify the residues and the domains that participate in substrate binding and translocation, and inhibitor binding. The rat DAT (rDAT) amino acids F69, F105, F114, F155, T285, F319, F331, P394, F410, S527, F530, Y533, and S538 and the human DAT (hDAT) amino acids V55 and S528 appear to be involved selectively with substrate uptake or efflux (reviewed in Volz and Schenk,

2005). Substitution of F69, F331 and F530 by alanine decreases the K_m and V_{max} of dopamine uptake (Lin et al., 1999). Mutation of P394 to alanine does not affect plasma membrane expression, dopamine uptake or WIN 35,428 binding but does affect the apparent affinity of dopamine efflux at the DAT (Lin et al., 2000a, Itokawa et al., 2002). Substitution of S528 by alanine increases the efflux of intracellular dopamine at the DAT without affecting the K_m and V_{max} of dopamine uptake (Chen and Justice, 2000). Mutation of Y533 has been reported to selectively reduce the V_{max} of dopamine uptake at the DAT (Itokawa et al., 2000). Substitution of V55 by alanine decreases the Km for dopamine uptake at the DAT (Lin and Uhl, 2003).

Na^+ and Cl^- are transported as co-substrates along with dopamine. W84 and D313 have been implicated in controlling the ability of Na^+ to drive the DAT between inward- and outward facing conformations (Chen et al., 2002; Chen et al., 2003). Reports provide evidence for the role of N85 and N466 in Cl^- binding (Edvardsen and Dahl, 1994; Syringas et al., 2000).

rDAT amino acids residues W84, F154, P235, F252, S253, P287, F361, F456, F542, P544, and P545 and the hDAT amino acids W84, N182 and F168 may be involved selectively with cocaine analog binding (reviewed in Volz and Schenk, 2005). Substitution of F154, F252, F361, F456, F542 by alanine decreases WIN 35,428 binding affinities at the DAT (Itokawa et al., 2000; Lin and Uhl, 2002; Lin et al., 1999). Substitution of F168 by leucine does not affect dopamine uptake at the DAT but increases WIN 35,428 binding (Lee et al., 2000). The W84A mutation increases WIN 35,428 binding affinity without

decreasing dopamine uptake affinity, suggesting that this tryptophan may sterically hinder the binding of cocaine analogs at the DAT (Lin et al., 2000b). Substitution of P235, P287, P544, P545 by alanine reduces WIN 35,428 binding without affecting dopamine uptake affinities (Lin and Uhl, 2002; Lin et al., 2000a). Mutation of S253 decreases WIN 35,428 binding affinity without affecting dopamine uptake (Itokawa et al., 2000). Substitution of the N182 by histidine does not affect dopamine uptake but does increase WIN 35,428 binding at the DAT (Lee et al., 2000).

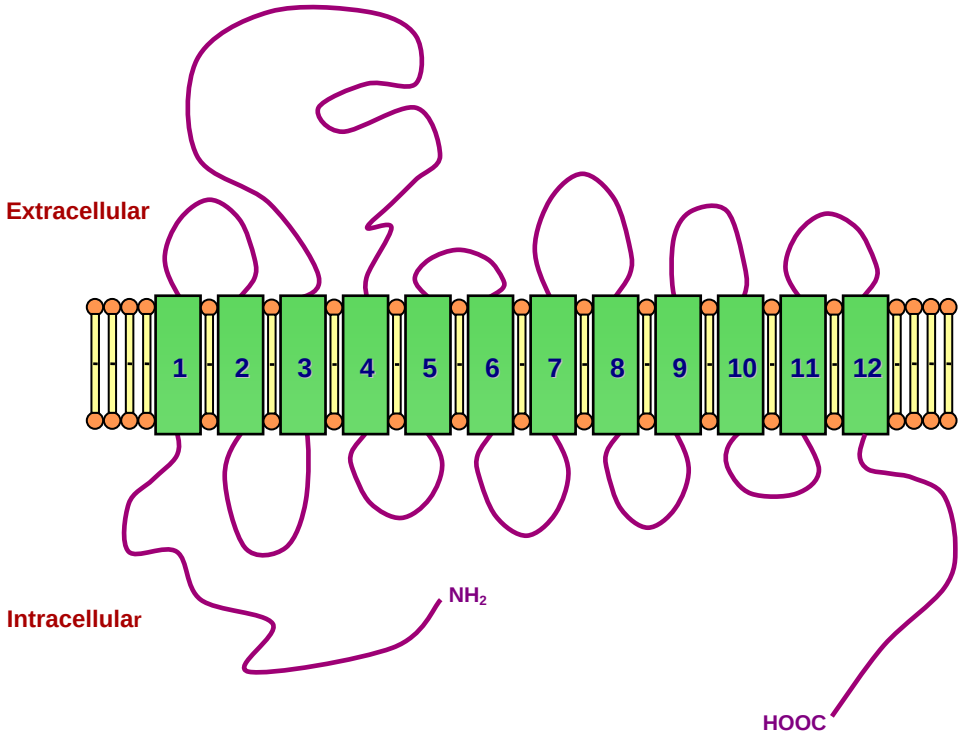


Figure 2. Topology of the dopamine transporter.
The dopamine transporter has 12 transmembrane domains with the N- and C-termini located on the intracellular side of the membrane. The large second extracellular loop has several sites for glycosylation.

Recently, the crystal structure of a bacterial leucine transporter from Aquifex aeolicus ($LeuT_{Aa}$), a homologue of the NSS family of transporters, has been reported (Yamashita et al., 2005). This high resolution structure of $LeuT_{Aa}$ may be very useful in understanding the structure and function of the DAT and other mammalian transporters in the NSS family.

2. Mechanism of Dopamine Reuptake

The dopamine reuptake process is driven by coupling dopamine influx with the inward flow of Na^+ and Cl^- as these ions move down a gradient across the cell membrane. The Na^+ gradient is generated by the Na^+K^+-ATPase (Giros et al., 1992). DAT promotes the transport of dopamine together with Na^+ and Cl^- ions as co-substrates with a stoichiometric ratio of 2:1:1 for sodium:chloride:dopamine (Chen and Reith, 2003). According to the alternating access transport mechanism, binding of dopamine, Na^+ and Cl^- to DAT on the extracellular side induces a conformational change in the transporter allowing the exposure of the binding site to the intracellular side of the membrane. Dopamine and the ions are translocated into the cell and the empty transporter returns to the outward facing conformation to initiate another transport cycle. Reorientation of SERT to its outward facing conformation requires binding and outward transport of intracellular K^+ (Rudnick, 1997). In addition to the classic substrate transport mechanism, transporters can exhibit channel-like activity. Simultaneous measurement of dopamine transport-associated current and dopamine flux in hDAT-expressing Xenopus oocytes revealed that charge

movement during substrate translocation was greater than would be expected for a transport mechanism with fixed stoichiometry of 2 Na^+ and 1 Cl^- ions per dopamine molecule. Besides the transport-associated current, hDAT also mediates a constitutive leak current, the voltage and ionic dependencies of which differ markedly from those of the transport-associated current (Sonders et al., 1997). Recent studies also provide evidence for the existence of a chloride channel within the DAT (Ingram et al., 2002; Carvelli et al., 2004).

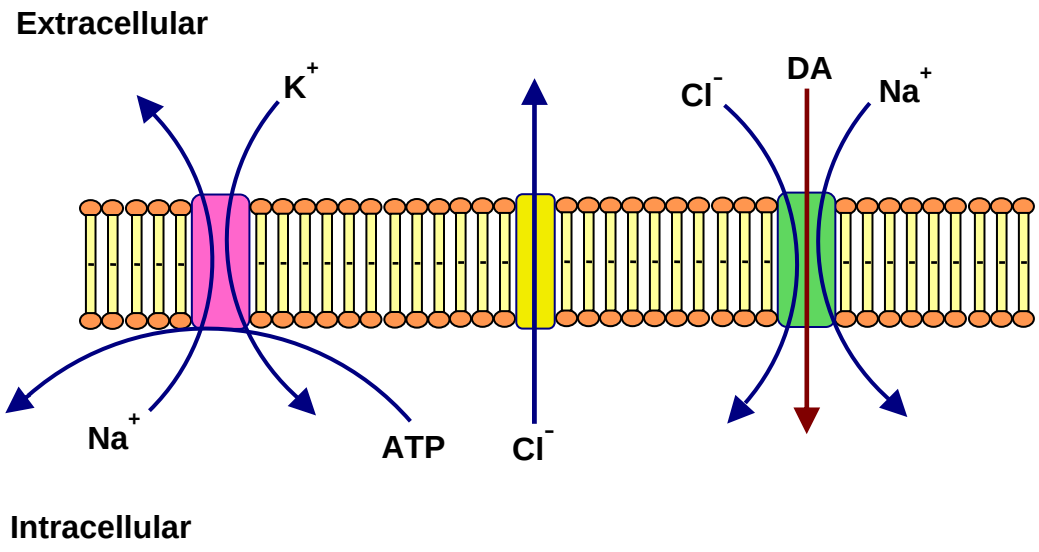


Figure 3. Dopamine transport via the DAT.
The Na^+K^+-ATPase (pink) generates the Na^+ and K^+ gradients across the plasma membrane by moving three Na^+ ions out of, for every two K^+ ions into, the cell. The resultant transmembrane electric potential (negative inside) leads to Cl^- redistribution. The DAT (green) translocates one dopamine molecule along with two Na^+ ions and one Cl^- ion as co-substrates in one transport cycle.

3. DAT Inhibitors and Substrates

Cocaine is a tropane derivative that binds to the dopamine transporter and inhibits dopamine uptake. Cocaine binds to the NET and SERT in addition to DAT (Giros and Caron, 1993); serotonergic mechanisms may also play a role in cocaine addiction (Rocha et al., 1998). However, decades of studies have established that binding of cocaine to the DAT is primarily responsible for its reward and reinforcement effects (Kuhar, 1992; Wise, 1996). A study employing a knockin mouse line carrying a triple mutant (L104V/F105C/A109V) of the DAT that is functional but insensitive to cocaine inhibition showed that blockade of the DAT is necessary for cocaine reward (Chen et al., 2006). Cocaine can augment dopamine release by mobilizing a synapsin-dependent reserve pool in mice. Synapsins are phosphoproteins that interact with the surface of synaptic vesicles and segregate synaptic vesicles into the reserve pool. Secretory vesicles can exist as a releasable pool that is available for immediate exocytosis and a reserve pool that is spatially segregated and mobilized after prolonged synaptic activity (Pieribone et al., 1995; Duncan et al., 2003). Pretreatment with α-methyl-p-tyrosine, which depletes the readily releasable pool of dopamine, did not affect cocaine's ability to augment dopamine release. However, the mechanism by which cocaine promotes this synapsin-mediated dopamine release is unknown (Venton et al., 2006).

Several analogs of cocaine have been synthesized. WIN 35,428 (12 β-carbomethoxy-3β-(4-fluorophenyl)-tropane) is a cocaine analog that is extensively used in DAT binding assays. Other drugs classified as DAT

inhibitors and employed in the present work include mazindol, methylphenidate benztropine and the amphetamines.

Mazindol, an imidazoisoindol derivative, is a tricyclic compound that binds to all the monoamine transporters and inhibits substrate uptake. Studies have shown that mazindol exhibits addictive potential (Chait et al., 1987). It is clinically used as an anorexiant for the management of obesity and as an orphan drug for the treatment of Duchenne muscular dystrophy.

Methylphenidate (Ritalin®) is typically used in the symptomatic treatment of ADHD. Its binding affinity for the DAT is almost two times higher than that of cocaine and exhibits reinforcing effects similar to cocaine but much milder (Bergman et al., 1989).

Benztropine (3α-diphenylmethoxytropane; Cogentin®) and its analogs have high affinity for the dopamine transporter and inhibit dopamine uptake but demonstrate behavioral profiles that are distinctive from cocaine. Benztropine differs from cocaine in that it has a diphenylmethoxy group attached to the C-3 position of the tropane ring (Newman and Kulkarni, 2002). It is an anticholinergic agent that is clinically used in the treatment of Parkinsonism. Benztropines are being extensively studied as potential agents in the treatment of cocaine addiction.

Amphetamines (alpha-methylphenethylamines) are structurally similar to dopamine and act as substrates at the dopamine transporter. Examples of amphetamines include (+)-amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA; ecstasy). Though amphetamines act

at the NET and the SERT, the interaction of amphetamines with the DAT is thought to mediate the acute behavioral and reinforcing effects of these psychostimulants (Giros et al., 1996). Amphetamines are used in the treatment of ADHD and narcolepsy.

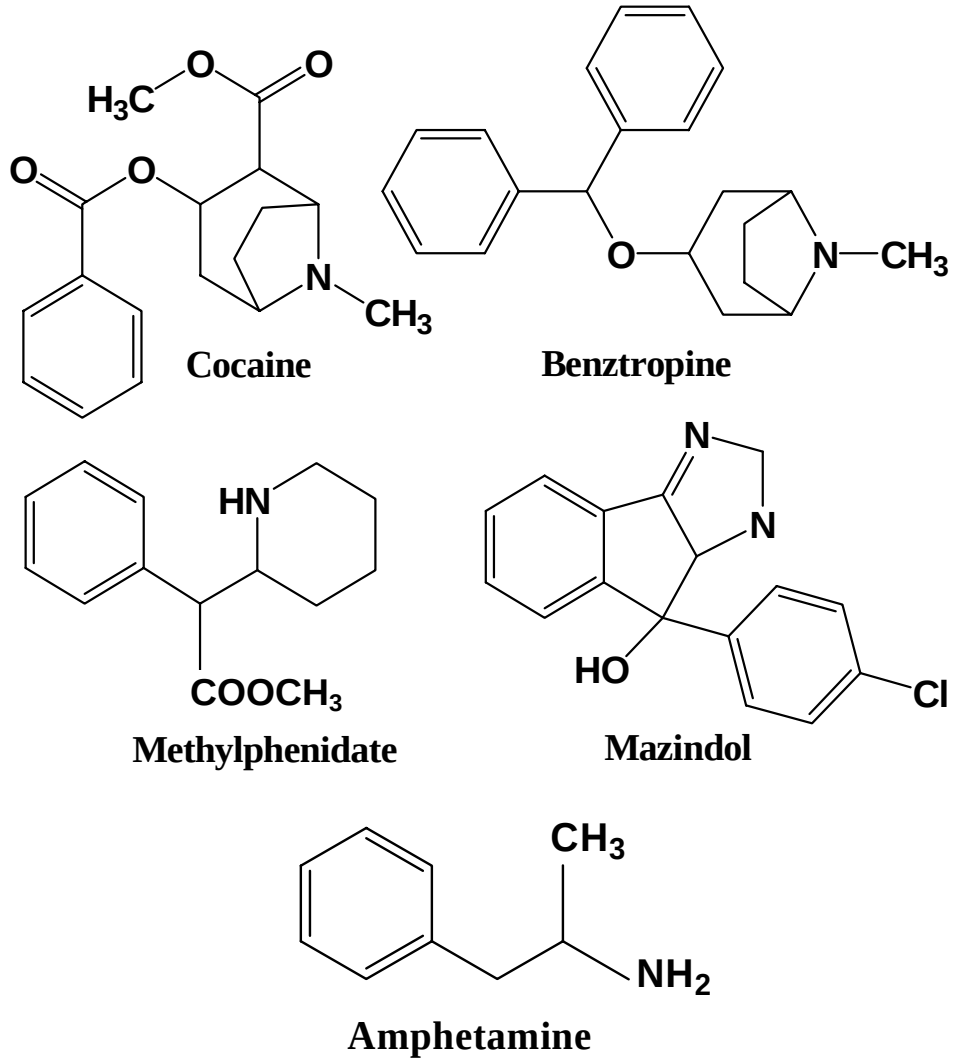


Figure 4. Chemical structures of DAT ligands.
Cocaine, benztropine, methylphenidate and mazindol are DAT blockers. Amphetamine is a DAT substrate.

DAT expression at the cell surface can be regulated by DAT substrates and inhibitors. In both HEK 293 cells expressing hDAT and in rat striatal synaptosomes, persistent exposure to dopamine (1 hr) decreased DAT surface expression and dopamine uptake activity. The presence of cocaine during dopamine pretreatment prevented the down-regulation of surface DAT. This suggests that dopamine translocation by the DAT is a prerequisite for the dopamine-mediated decrease in surface expression of DAT (Chi and Reith, 2003). In HEK 293 cells and N2A cells stably expressing hDAT, acute treatment with cocaine (≥ 5 min) increased DAT surface expression and dopamine uptake activity. Similar results were obtained with synaptosomes prepared from the nucleus accumbens of rats sacrificed 30 minutes after a single cocaine injection (Daws et al., 2002; Little et al., 2002). Persistent exposure to amphetamine (> 20 min) decreases DAT surface expression and dopamine uptake activity (Gulley et al., 2002; Saunders et al., 2000; Zahniser and Sorkin, 2004). A recent study showed that amphetamine increases the DAT surface expression rapidly within one minute by recruiting more transporter proteins to the plasma membrane (Johnson et al., 2005a).

4. Regulation of DAT Function and Expression

DAT Oligomerization

Increasing evidence suggests that the NSS family members are organized into oligomeric complexes. Coimmunoprecipitation studies employing differentially tagged monomers demonstrated that SERT and NET can exist as homo-oligomers (Kilic and Rudnick, 2000; Kocabas et al., 2003). Fluorescence resonance energy transfer (FRET) studies provided evidence for the homo-oligomerization of SERT and GABA transporter (Schmid et al., 2001). Similar to other transporters of the NSS family, DAT also exists as an oligomeric complex and oligomerization may be essential for the proper trafficking of the DAT to the plasma membrane. Two loss-of-function DAT mutants (Y335A and D79G) that expressed at the cell surface decreased the transport activity of WT DAT without affecting its cell surface expression. This suggested the formation of oligomeric complexes of WT DAT with the DAT mutant at the cell surface. The same study showed that an intact leucine heptad repeat in the TM2 domain of the DAT is required for oligomerization. Substitution of amino acids in this repeat by mutagenesis resulted in a mutant transporter devoid of uptake activity because of its intracellular retention. This mutant did not inhibit the function of the WT DAT. Coimmunoprecipitation and immunofluorescence studies demonstrated that an HA epitope-tagged DAT with the leucine heptad repeat mutation did not associate with a histidine epitope-tagged WT DAT (Torres et al., 2003). DAT can be oxidatively cross-linked to form homodimers that retain transporter functionality; the cross-link is between C306 at the extracellular end of TM6 in

each of the two DAT molecules (Hastrup et al., 2001). The intracellular end of TM6 domain of the DAT has a dimerization motif (GV/IXXGV/IXXA/T) that is found in other neurotransmitter proteins as well. This motif was originally identified in glycophorin A and was found to be essential for dimerization. Mutation of the first glycine of the dimerization motif to valine greatly diminished DAT surface expression and completely blocked substrate transport. Mutation of the second glycine completely inhibited surface expression. Hence, a functional dimerization motif in the TM6 domain of the DAT is essential for efficient trafficking of the transporter. The data suggested that the TM6 domain contributes to the dimerization interface and that oligomerization may be required for cell surface trafficking (Hastrup et al., 2001). Studies employing FRET revealed that DAT exists as oligomers in all cellular compartments in which it is localized (Sorkina et al., 2003). DAT can also exist as tetramers and the TM4 (C243) domain is present at a homodimeric interface distinct from the TM6 (C306) interface (Hastrup et al., 2003). The same study also showed that DAT inhibitors such as the cocaine analog MFZ 2-12, benztropine and mazindol disallowed the cross-linking of C243 suggesting that these uptake inhibitors are not simply neutral blockers but by themselves produce conformational changes of the DAT. Oligomerization of the DAT protein may thus play an important role in the proper functioning and trafficking of the transporter.

Posttranslational Modifications

DAT is a heavily glycosylated protein with three or four potential consensus sequences (depending on species) for N-linked glycosylation (sugars are attached to the amide nitrogen of asparagine residues) in the large second extracellular loop. In general, N-linked glycosylation is essential for proper folding and stability of proteins. Differences in apparent molecular mass of the DAT were observed in the striatal tissue of 4, 14 and 60 day old rats. These differences were eliminated by N-deglycosylation suggesting that differential glycosylation of DAT occurs during development and aging (Patel et al., 1994). Alterations in the glycosylation pattern of DAT were also observed in different brain regions (nucleus accumbens versus striatum) and in different species (Lew et al., 1991; Patel et al., 1993). Non-glycosylated DAT does not transport dopamine as efficiently as WT DAT. A sharp reduction in dopamine uptake V_{max} greater than that accounted for by the fall in surface DAT levels was observed with DAT mutants lacking the glycosylation sites (N181, N188 and N205). Inhibiting DAT N-glycosylation increased the DUIP of cocaine-like drugs without changing their affinity for the DAT (Li et. al., 2004). Removal of the three hDAT glycosylation sites partially impaired DAT trafficking to the plasma membrane, suggesting that glycosylation increases DAT surface expression but is not essential for trafficking to the cell surface (Torres et al., 2003). Confocal microscopy studies demonstrated that the immunofluorescence of the single mutant N181Q was mostly on the cell surface, similar to the WT DAT. However, the immunofluorescence of the double (N181Q, N188Q) and triple mutants

(N181Q, N188Q, N205Q) was weaker at the cell surface, with a significant amount in the cytosol. Reversible biotinylation experiments to assess DAT endocytosis showed that the double and triple mutants were internalized at a faster initial rate, with a significantly reduced surface DAT half-life (Li et al., 2004). Despite several studies, the exact role of N-glycosylation in DAT function and cell surface expression remains unclear. It has been shown that N-glycosylation of SERT is important for homo-oligomerization and protein interactions that regulate serotonin uptake (Ozaslan et al., 2003). Similar to SERT, changes in the DAT N-glycosylation pattern may affect DAT-protein interactions leading to differences in transporter function and trafficking.

Analysis of the DAT primary amino acid sequence reveals that the protein has several consensus sites for phosphorylation by protein kinase A (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin dependent protein kinase (CaM kinase). To date PKC is the only kinase that has been well characterized as contributing to DAT phosphorylation. Several studies have shown that activation of PKC results in reduced DAT activity and surface expression. Activation of PKC by the phorbol ester 4-α-phorbol 12-myristate 13-acetate (PMA) decreased the DAT uptake activity in Sf9 cells, evident by the decreased V_{max} value. The PMA-mediated reduction in dopamine uptake was antagonized by staurosporine, a PKC inhibitor. Confocal microscopy studies revealed that sequestration of the DAT from the cell surface was responsible for the decreased V_{max} of dopamine transport. However, the DAT surface density in PMA-treated cells remained unchanged. Treating the cells with a PKC inhibitor promoted an increase in

dopamine uptake that was a result of recruitment of the internalized or intracellular DAT back to the cell surface. The data suggested that rapid shuttling of the DAT protein between the cell surface and intracellular compartments was responsible for the changes in dopamine transport activity in response to PKC activation. Moreover, the differential regulation of the DAT activity by a PKC-dependent mechanism was not a result of modifications in the DAT catalytic activity (Pristupa et al., 1998). Another study in PC12 cells stably transfected with hDAT demonstrated that the transporter undergoes endosomal recycling in response to PKC activation (Melikian and Buckley, 1999). DAT is internalized through a clathrin-mediated and dynamin-dependent mechanism in MDCK cells, evidenced by the complete blockade of PMA-mediated translocation of DAT with a dominant negative mutant of dynamin 1. However, this study showed that PKC activation results in complete lysosomal degradation of the transporter protein, inconsistent with the previous studies (Daniels and Amara, 1999). Truncation of the first 22 N-terminal amino acids, including several serine residues, nearly abolished detectable phosphorylation of hDAT without affecting functional regulation by PKC, suggesting that phosphorylation of these residues is not essential for PKC-mediated transporter internalization (Granas et al., 2003). The dopamine uptake activity, inhibitor binding and oligomerization of this truncated DAT were also not significantly different from the full-length DAT (Hastrup et al., 2001). DAT has a dileucine motif in the third intracellular loop and two tyrosine-based motifs, one in the second intracellular loop and one in the C-terminus. These motifs play key roles in the intracellular trafficking of

membrane proteins by mediating critical protein-protein interactions. Mutation of the dileucine motif and the two tyrosine-based trafficking motifs in hDAT did not significantly affect the PKC-mediated internalization, suggesting that PKC-mediated internalization does not involve these trafficking motifs (Granas et al., 2003). A recent study showed that the PKC-regulated endocytic signal resides in the C-terminal region (amino acids 587-591; FREKL) of the DAT (Holton et al., 2005). Mitogen-activated protein kinase (MAPK) has also been shown to regulate the DAT uptake activity and trafficking. Striatal synaptosomes and epitope tagged hDAT HEK 293 cells exhibited decreased V_{max} values without any change in K_m values when incubated with MAPK kinase (MEK) inhibitors. Biotinylation and confocal studies demonstrated that the decrease in V_{max} was a result of the internalization of surface DAT by clathrin-mediated endocytosis (Moron et al., 2003).

Substrates such as amphetamine and methamphetamine can also increase DAT phosphorylation in the absence of exogenous kinase or phosphatase. Cocaine and the PKC inhibitor bisindoylmaleimide were able to inhibit methamphetamine-induced DAT phosphorylation (Cervinski et al., 2005). Incubation with cocaine, mazindol or methylphenidate did not affect basal or PMA-stimulated DAT phosphorylation, but GBR12909 (another DAT blocker) strongly suppressed PMA-stimulated phosphorylation (Gorentla and Vaughan, 2005). Thus, DAT ligands can have different effects on DAT phosphorylation.

The only process identified to date as requiring DAT phosphorylation is substrate-induced dopamine efflux (Khoshbouei et al., 2004; Johnson et al.,

2005b). In HEK 293 cells stably expressing an N-terminal truncated DAT (lacking the first 22 amino acids) amphetamine-induced dopamine efflux was significantly reduced compared to the full-length DAT. To investigate the role of N-terminal phosphorylation of the DAT in dopamine efflux, all five N-terminal serine residues were simultaneously mutated to alanines. Surprisingly, this construct significantly impaired amphetamine-induced dopamine efflux, similar to the N-terminal truncated DAT. In contrast, simultaneous mutation of these same five serines to aspartate to simulate phosphorylation resulted in normal amphetamine-induced dopamine efflux. However, the V_{max} for dopamine uptake remained unaltered in all the mutants. The study suggests that the N-terminal phosphorylation of the DAT is essential for amphetamine-induced dopamine efflux but does not affect the inward transport of substrate (Khoshbouei et al., 2004). These results could lead to the design and synthesis of new therapeutic agents, such as drugs that inhibit amphetamine-induced dopamine efflux without affecting dopamine uptake.

Protein Interactions

Compelling evidence has shown that the DAT undergoes regulated trafficking, indicating the possible involvement of transporter-interacting proteins. Moreover, several proteins have been implicated in DAT-protein interactions that influence the cell surface expression and/or function of the DAT (reviewed in Torres, 2006). Synuclein, a protein that is abundantly expressed in different brain regions and highly enriched in presynaptic terminals, has been shown to

interact with the hDAT. The direct protein-protein interaction between hDAT and α-synuclein was found to involve the C-terminal region of hDAT (last 15 amino acids) and the non-Aβ component (NAC) domain of α-synuclein. Co-expression of this protein with hDAT enhanced the dopamine uptake activity and membrane clustering of the transporter (Lee et al., 2001). PICK1, a kinase associated protein widely expressed in many tissues including the brain, is a member of a family of PDZ domain containing proteins that play an important role in the targeting and clustering of several receptors and ion channels. This protein has been shown to interact with the C-terminal region (last 3 amino acids) of the DAT. Overexpression of PICK1 in mammalian cells with hDAT and in cultured neurons enhanced the DAT uptake activity due to an increase in the number of functional transporters at the cell membrane. Deletion of the PDZ-binding site in DAT impaired the targeting of the mutated transporter to neuronal processes in cultured neurons (Torres et al., 2001). Hic-5, a member of the focal adhesion family of adaptor proteins is highly expressed in several tissues including the brain. The amino-proximal portion of the intracellular carboxyl terminus of DAT has been shown to interact *in vitro* and *in vivo* with the LIM domain of Hic-5. Confocal studies demonstrated that Hic-5 colocalizes with DAT on the cell membrane. Hic-5 overexpression downregulates DAT uptake activity by decreasing DAT surface expression (Carneiro et al., 2002). It has been shown that syntaxin 1A and the receptor for activated C kinase (RACK1) interact with the DAT N-terminal region. Syntaxin 1A is a major component of the soluble *N*-ethylmaleimide-sensitive factor attachement protein receptor (SNARE) complex

that regulates neuronal exocytosis; RACK1 is a protein that regulates phosphorylation (Lee et al., 2004). The role of these protein interactions in regulation of DAT activity and trafficking has not yet been demonstrated. Syntaxin 1A has also been shown to interact with the related GABA transporter (GAT1) and the NET resulting in altered surface expression and transport activity (Horton and Quick 2001; Sung et al., 2003). Because DAT is structurally similar to these transporters, it is likely that syntaxin also regulates DAT uptake activity and trafficking. Parkin, a protein expressed exclusively in dopaminergic neurons is associated with Parkinson's disease (PD). Parkin contains a protein-ubiquitin E3 ligase that ubiquitinates many substrate proteins to enhance their degradation. Parkin, but not its PD-causing mutants, increased dopamine uptake at DAT by increasing its cell surface expression. Parkin enhanced the ubiquitination and degradation of misfolded DAT, so as to prevent it from interfering with the oligomerization and cell surface expression of native DAT. Thus, it indirectly increased the cell surface expression of the functional DAT (Jiang et al., 2004).

Characterizing the DUIP fluctuation phenomenon of cocaine and other DAT ligands and identifying the factor/s responsible for such potency fluctuations can help understand better the regulation of DAT activity. Specific Aim 1 is to study whether DAT ligands that inhibit DA uptake show DUIP fluctuations at WT DAT as a function of cell age. It also involves investigation of the physiological relevance of the DUIP fluctuation phenomenon. Specific Aim 2 is to investigate

the source of the DUIP fluctuation by assessing the role of the N- or C- terminus of the DAT.

III. MATERIALS AND METHODS

A. Materials and Equipment

1. Facilities

Laboratories – Mellon Hall of Science, Rooms 456, 414 and 214

2. Cell Lines

Chinese Hamster Ovary (CHO) cells stably transfected with wildtype DAT
Dr. Surratt's laboratory at Albert Einstein College of Medicine, NY

COS-7 (African green monkey kidney) cells
American Type Culture Collection (ATCC), Manassas, VA

N2A (murine neuroblastoma) cells
Dr. Gnegy's laboratory at University of Michigan, MI

3. Chemicals and Drugs

[^{3}H]-Dopamine
Perkin Elmer, Foster City, CA

[^{3}H]-WIN 35, 428
Perkin Elmer, Foster City, CA

Acetic acid, glacial
Fisher Scientific, Pittsburgh, PA

Agarose
Invitrogen Corporation, Carlsbad, CA

Alexa-Fluor 488, goat anti-rat IgG (H+L)
Molecular Probes, Eugene, OR

Ampicillin sodium salt
Acros, NJ

Bio-Rad protein assay dye reagent concentrate
Bio-Rad Laboratories Inc., Hercules, CA

Bovine serum albumin
Sigma Chemical Co., St. Louis, MO

Calcium chloride
Sigma-Aldrich Co., St. Louis, MO
Compressed carbon dioxide
Air Products, Pittsburgh, PA

D-(+)-Glucose
Sigma-Aldrich Co., St. Louis, MO

dNTP mix
Stratagene, La Jolla, CA

DH5α cells
Invitrogen, Carlsbad, CA

Dimethylsulfoxide (DMSO)
Sigma Chemical Co., St. Louis, MO

Dulbecco's Modified Eagle Medium (DMEM)
Hyclone, Logan, UT

Dulbecco's Phosphate Buffered Saline (DPBS) Ca/Mg-free
Cambrex Bioscience Walkersville Inc., MD

EcoR1
Invitrogen, Carlsbad, CA

EDTA
Sigma-Aldrich Co., St. Louis, MO

Ethanol 200 proof
Pharmaco Products Inc., Brookfield, CT

Ethanol, HPLC grade
Acros, NJ

Ethidium bromide
Fisher Scientific, Pittsburgh, PA

F-12 Nutrient Mixture
Hyclone, Logan, UT

Fetal bovine serum
Hyclone, Logan, UT

G-418 sulfate
Clontech Laboratories Inc., CA

L-Glutamine
Invitrogen, Carlsbad, CA

Glycerol
Fisher Scientific, Pittsburgh, PA

Glycine
Sigma-Aldrich Co., St. Louis, MO

Goat serum
Biomeda Corporation, Foster City, CA

GVA mounting solution
Zymed, San Francisco, CA

HEPES free acid
MP Biomedicals Inc., Solon, OH

HBSS/modified
Hyclone, Logan, UT

Isopropanol (DNase, RNase and protease free)
Fisher Scientific, Pittsburgh, PA

Kpn1
Invitrogen, Carlsbad, CA

LB agar
Fisher Scientific, Pittsburgh, PA

LB broth
Fisher Scientific, Pittsburgh, PA

MAB369 Rat anti-Dopamine Transporter
Chemicon International, Temecula, CA

Methanol, HPLC grade
Fisher Scientific, Pittsburgh, PA

Opti-MEM I
Invitrogen, Carlsbad, CA

Paraformaldehyde
Sigma Chemicals Co., St. Louis, MO
Penicillin-Streptomycin
Gibco-BRL, Grand Island, NY

PolyFect Transfection Reagent
Qiagen Inc., Valencia, CA

Polymerase, Pfu Turbo
Stratagene, La Jolla, CA

Potassium chloride
Sigma-Aldrich Co., St. Louis, MO

Potassium phosphate, monobasic
Sigma Chemical Co., St. Louis, MO

Primers
Sigma Genosys, The Woodlands, TX

Rhodamine phalloidin
Molecular Probes, Eugene, OR

RNase A (DNase free)
Fisher Scientific, Pittsburgh, PA

SOC media
Invitrogen, Carlsbad, CA

ScintiSafe scintillation fluid
Fisher Scientific, Pittsburgh, PA

Sodium Chloride
Sigma-Aldrich Co., St. Louis, MO

Sodium Hydroxide
Fisher Scientific, Pittsburgh, PA

Sodium Hydroxide 2N solution
Fisher Scientific, Pittsburgh, PA

Sodium Hydroxide 12N solution
Fisher Scientific, Pittsburgh, PA

Sodium lauryl sulfate
Sigma Chemical Co., St. Louis, MO
T4 DNA ligase
Invitrogen, Carlsbad, CA

Tris-EDTA buffer (DNase, RNase and protease free)
Acros, NJ

Tris free base
Fisher Scientific, Pittsburgh, PA

Tris-HCl salt
Sigma Chemical Co., St. Louis, MO

Triton X-100
Acros, NJ

Trypsin-EDTA 10X
Gibco-BRL, Grand Island, NY

Xba1
Gibco-BRL, Grand Island, NY

4. Kits

Gel extraction kit
Qiagen Inc., Valencia, CA

PCR purification kit
Qiagen Inc., Valencia, CA

Plasmid miniprep kit
Stratagene, La Jolla, CA

Plasmid purification kit (maxiprep)
Qiagen Inc., Valencia, CA

5. Other Materials

Cell culture flasks, 75 cm^2
Corning Inc., NY

Cell culture grade water
Hyclone, Logan, UT
Cell scraper, disposable
Fisher Scientific, Pittsburgh, PA

Centrifuge tubes, 15 ml
Corning Inc., NY

Cryogenic vials, 1.5 ml
Corning Inc., NY

Culture tubes, disposable
Fisher Scientific, Pittsburgh, PA

Falcon tubes, 14 ml
Fisher Scientific, Pittsburgh, PA

Falcon tubes, 50 ml
Fisher Scientific, Pittsburgh, PA

Filter unit, sterile
Millipore Corporation, MA

Glass microscope coverslips (# 1)
Fisher Scientific, Pittsburgh, PA

Glass microscope slides
Fisher Scientific, Pittsburgh, PA

Microcentrifuge eppendorf tubes, 1.5 ml
Fisher Scientific, Pittsburgh, PA

Parafilm
Fisher Scientific, Pittsburgh, PA

Pasteur pipettes, disposable
Fisher Scientific, Pittsburgh, PA

Pipette tips, disposable Redi-Tips TM (1, 10, 200, 1000 µl)
Fisher Scientific, Pittsburgh, PA

Polaroid film
Polaroid Corporation, Cambridge, MA

Polypropylene tubes
Fisher Scientific, Pittsburgh, PA

Respirator
Fisher Scientific, Pittsburgh, PA

Scintillation vials
Fisher Scientific, Pittsburgh, PA

Serological pipettes, sterile disposable (5, 10, 25 ml)
Fisher Scientific, Pittsburgh, PA

Syringes 10 ml, sterile
Fisher Scientific, Pittsburgh, PA

Thermowell PCR tubes
Corning Incorporated, NY

Tissue culture plates, sterile (6-well)
Fisher Scientific, Pittsburgh, PA

6. Equipment

Analytical balance
Mettler Toledo Inc., OH

Bottletop dispenser
Brinkmann Instruments Inc., NY

Cell culture incubator
Forma Scientific, MA

Centrifuge Model 228
Fisher Scientific, Pittsburgh, PA

Centrifuge 5415 C
Eppendorf Scientific Inc., NY

Centrifuge 5810 R
Eppendorf Scientific Inc., NY

Confocal laser microscope Leica TCS-SP2
Leica Microsystems Inc., PA

Dispenser eppendorf (50 ml)
Brinkmann Instruments Inc., NY

Electrophoresis Power Supply
Life Technologies Inc., Gaithersburg, MD

Horizontal Gel Electrophoresis System
Life Technologies Inc., Gaithersburg, MD

Inverted microscope Olympus CK40
Fisher Scientific, Pittsburgh, PA

Isotemp Incubator
Fisher Scientific, Pittsburgh, PA

Lab freezers and refrigerators
Forma Scientific, MA

Liquid Nitrogen tank

Liquid Scintillation Analyzer
Packard Instruments Co., CT

Millipore Milli-Q and Elix
Millipore Corporation, MA

Mixer (Style: 37600)
Thermolyne Corporation, IA

NapFLOW Laminar air flow unit
Fisher Scientific, Pittsburgh, PA

ORBIT Shaker
Lab-line Instruments Inc., IL

PCR Mastercycler
Eppendorf Scientific Inc., NY

pH meter AB15
Fisher Scientific, Pittsburgh, PA

Pipet-aid
Drummond Scientific Co., Broomall, PA

Pipetman (P-2, P-10, P-20, P-100, P-200, P-1000)
Mettler Toledo Company, Woburn, MA

Polaroid Gelcam
Polaroid Corporation, Cambridge, MA
Stir plate Nuova II
Thermolyne Corporation, IA

Universal Vacuum System UVS 400
Savant Instruments Inc., NY

UV Transilluminator M-26
UVP Inc., Upland, CA

UV-Visible Spectrophotometer DU 530
Beckman Instruments Inc., Fullerton, CA

Vacuum pressure pump
Barnant Co., IL

Vertex-2 Genie
Scientific Industries Inc., NY

Water bath 180 series
Precision Scientific, Winchester, VA

Water bath Iso TEMP 205
Fisher Scientific, Pittsburgh, PA

Weighing scale
Denver Instruments Co., CO

7. Computer Software

Adobe Acrobat Reader 7.0
Adobe Systems Inc.

Adobe Acrobat Writer
Adobe Systems Inc.

ChemDraw
CambridgeSoft Corporation, Cambridge, MA

GraphPad Prism 3.0
GraphPad Software, San Diego, CA

Microsoft Office Word & Excel 2003
Microsoft Corporation

B. Methodology and Procedures

1. Cell Culture

CHO-K1 cells stably transfected with WT DAT were grown in F-12 media supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin ,100 units/ml streptomycin and 100µg/ml G-418. COS-7 cells were cultured in "complete" DMEM media (supplemented with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin and 20 mM L-glutamine). N2A cells were grown in "complete" OPTI-MEM I media (supplemented with 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin). All cell types were grown as monolayers in 75 cm^2 flasks at 37ºC and 5% CO_2 and subcultured twice weekly or every 3 days. For subculturing the WT DAT CHO and COS-7 cells, the exhausted media in the flask was aspirated and the confluent adherent cells were washed once with 10 ml Hanks buffered salt solution (HBSS). To detach the cells from the flask, 3ml trypsin-EDTA was added and swirled to cover the cell monolayer completely. Two ml of the trypsin-EDTA was aspirated and the flask was returned to the incubator. The cells were checked every 2 minutes for detaching from the flask. The detached cells were resuspended in 9 ml complete media to inactivate the trypsin. Two ml of the cell suspension was transferred into a new labeled flask containing 18 ml of complete media. The flask was capped and the cells evenly suspended by gently swirling the flask. The flask was placed in the incubator and the cells were allowed to grow till the next subculture. For subculturing the N2A cells, the exhausted media in the flask was removed and 3 ml of trypsin-EDTA was added. Two ml of the trypsin-EDTA was

removed from the flask and the cells were incubated with the remaining 1 ml trypsin-EDTA at room temperature (inside the sterile laminar air flow unit) for 1-2 minutes until the cells detached from the flask. The cells were resuspended in 9 ml of complete media to inactivate the trypsin. The cell suspension was transferred into a sterile 15 ml tube and centrifuged for 4 minutes. The supernatant was decanted and cell pellet was resuspended in 10 ml complete media. Two ml of the cell suspension was transferred into a new labeled flask containing 18 ml of complete media. The flask was capped and the cells evenly suspended by gently swirling the flask. The flask was moved to the incubator and the cells were allowed to grow till the next subculture.

2. Polymerase Chain Reaction and Plasmid Preparation

The N- and C-terminus fragments of rat WT DAT were generated and amplified by Polymerase Chain Reaction. Primers were designed for the N- and C-terminus fragments with Kpn1 and EcoR1 restriction enzyme sites on the 5' ends of the sense and antisense primers respectively. The sequences of primers designed for the N-terminus (first 68 nucleotides) of WT DAT were: 5'-ATATATGGTACCATGAGTAAGAGCAAATGCTCCGTGGGAC-3' and 5'-ATATATGAATTCATCAATTTTCTTGCTCCAGGTCTCCCG-3'. The sequences of the primers designed for the C-terminus fragment (last 43 nucleotides) of WT DAT were: 5'-ATATATGGTACCTACAAGTTCTGCAGCCTGCCGGGGTC-3' and 5'-ATATATGAATTCCAGCAACAGCCAGTGACGCAGCG-3'. The primers were synthesized by Sigma Genosys Inc., U.S.A. WT DAT in Bluescript vector was

used as the template. The reaction tubes were prepared with the calculated quantities of template, primers, dNTP mix and Pfu Turbo DNA Polymerase. The reaction was set for 25 cycles with denaturation of the double stranded template DNA at 95ºC for 45 seconds, annealing of the primers to the single stranded DNA at 55ºC for 45 seconds and extension of the new DNA strand at 72ºC for 2 minutes. Agarose gel electrophoresis was employed to confirm the right sizes of the PCR products. The gel purified PCR products were digested with the restriction enzymes and ligated into pc3DNAGFP vector such that the DAT N- and C- terminus peptide fragments would be expressed with GFP tag attached to the C-terminus of the peptide when transfected into mammalian cells. The ligation product was transformed into DH5α cells by heat shock method and the cells were plated on ampicillin supplemented agar petri plates and incubated at 37ºC for 16 hours. The bacterial colonies were used to inoculate 5 ml of LB broth and the bacteria were allowed to grow for 16 hours. The supercoiled DNA was extracted using the Stratagene mini-prep kit and after confirming the right DNA sequence (sequencing facility, University of Pittsburgh) and size (by digesting with restriction enzymes and agarose gel electrophoresis), 200 ml LB broth was inoculated to produce large amounts of the plasmid of interest. The DNA concentration was estimated by measuring the absorbance at 260 nm and purity was determined by the ratio of the absorbance values at 260 and 280 nm. Plasmid DNA with a ratio of greater than or equal to 1.7 was considered pure enough for performing transient transfections. The pcDNA3 plasmids encoding

human DAT (hDAT) and del-20 hDAT (hDAT lacking the first 20 amino acids) were kindly provided by Dr. Gonzalo E. Torres, University of Pittsburgh.

3. Cell Transfections

Stably transfected WT DAT CHO cell line employed in the study was developed in Dr. Surratt's laboratory at Albert Einstein College of Medicine, NY. Lipofectamine transfection method and G-418 mediated stable transfectant selection was employed for preparing the stable cell line.

Polyfect method with modification of the manufacturer's protocol was employed for transiently transfecting COS-7 cells. The day before transfection, 4 x 10^5 cells were seeded in 6-well plates and incubated at 37ºC and 5% CO_2 such that the cells would be 50-80% confluent on the day of transfection. Prior to transfection, the growth media in the plates was gently aspirated, the cell monolayers were washed with 1 ml phosphate buffered saline (PBS), 1.5 ml fresh complete media was added to the cells in each well, and the cells were maintained at 37ºC and 5% CO_2 until the addition of transfection complexes. For preparing the transfection mixture for the cells in one well, 1.5 µg DNA was added to 100 µl media (containing no serum, proteins, or antibiotics) in a sterile tube and mixed by vortexing for few seconds. Ten µl Polyfect reagent was added to the DNA solution, mixed by vortexing for 10 seconds and incubated for 9 minutes at room temperature to allow complex formation. Six hundred µl complete media was added to the reaction tube containing transfection complexes and mixed by pipetting up and down twice. The total volume of the

transfection mix was transferred immediately to the cells in a single well and the plate was gently swirled to ensure uniform distribution of the complexes. The cells were then incubated at 37ºC and 5% CO_2 and pharmacological assays were performed 24 - 48 hours after transfection. When performing transient transfections of cells in more than one well, all the volumes were scaled up appropriately. In experiments manipulating the DAT expression levels, suboptimal amounts of plasmid were employed. Vector plasmid was employed to ensure that each well received the same amount of total DNA. In experiments requiring co-transfection of different plasmids, the required amounts of each plasmid were calculated and vector plasmid was employed to ensure that each treatment supplied the same amount of total DNA to the cells.

Transient transfections with N2A cells were conducted via the modified calcium phosphate method (Graham and van der Eb, 1973). The day before transfection, cells were seeded in 6-well plates and incubated at 37ºC and 5% CO_2 such that the cells would be 30-40% confluent on the day of transfection. Four hours prior to transfection, the media in the wells was replaced with 2 ml fresh media. All the reagents were filter sterilized. The transfection procedure involved preparing 2 tubes, with the contents of one tube added slowly to the other. Preparing the first tube involved addition of Millipore water, plasmid DNA, 10x Tris-EDTA (TE) buffer pH 8.0 and 0.5 M $CaCl_2$ in the order listed and mixing by gently vortexing. The second tube contained 2x HEPES – buffered saline (HBS) solution. The contents of first tube were added to the second tube dropwise with continuous gentle vortexing and incubated at room temperature.

Soon after the DNA complexes started forming, the mixture was pipetted up and down thrice to ensure a uniform suspension and to reduce the particle size of the complexes. Two hundred µl of the above mixture was added to each well and the plate was gently swirled to ensure uniform distribution. The following day, the transfection mixture in each well was replaced with 2 ml fresh media. The cells were used for the pharmacological assays 48 hours later. In experiments manipulating the DAT expression levels, suboptimal amounts of plasmid were employed and same amount of total DNA was added to the cells by employing vector plasmid. In experiments requiring co-transfection of different plasmids, the required amounts of each plasmid were calculated and vector plasmid was employed to ensure that each treatment supplied the same amount of total DNA to the cells.

4. [^{3}H]-Dopamine Uptake Assays

All [^{3}H]-dopamine uptake assays were performed with cells grown in 6-well plates. For performing simple [^{3}H]-dopamine uptake assays, cell monolayers were washed 2 x 1 ml with "KRH buffer" (25 mM HEPES, pH 7.3, 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose) supplemented with 50 mM ascorbic acid (KRH/AA), incubated with 750 µl KRH/AA buffer (total uptake) or 10 µM mazindol (non-specific uptake) for 10 minutes, followed by incubation with10 nM [^{3}H]-dopamine for an additional 5 minutes. Uptake was quenched by washing the cell monolayers 2 x 1 ml with KRH/AA buffer. Cell monolayers were solubilized by incubating with 1 ml of 1%

SDS at room temperature for 1 hour with gentle shaking. The cell lysates were transferred into scintillation vials containing 5 ml of ScintiSafe fluid and the incorporated radioligand was determined by employing a liquid scintillation counter. The specific uptake was calculated as the difference between total and non-specific uptake, i.e. uptake in the presence and absence of 10 μM mazindol.

For performing [^{3}H]-dopamine uptake inhibition assays, cell monolayers were washed 2 x 1 ml with KRH/AA buffer, incubated with 750 μl DAT blocker or substrate for 10 minutes, followed by incubation with10 nM [^{3}H]-dopamine for an additional 5 minutes. The uptake inhibition assays included nonradioactive DAT inhibitors or substrates at the following concentration ranges: cocaine, 1nM – 100 μM; benztropine, 1 nM – 30 μM; mazindol, 0.1 nM – 10 μM; methylphenidate 3 nM – 30 μM; (+)-amphetamine, 1 nM – 10 μM. Uptake was quenched by washing the cell monolayer 2 x 1 ml with KRH/AA buffer. Cell monolayers were solubilized by incubating with 1 ml of 1% SDS at room temperature for 1 hour with gentle shaking. The cell lysates were transferred into scintillation vials containing 5 ml of ScintiSafe fluid and the incorporated radioligand was determined by employing a liquid scintillation counter. Non-specific uptake was determined by using 10 μM mazindol, or 30 μM cocaine if mazindol was the drug being assessed. The specific uptake was calculated as the difference between total and non-specific uptake. The IC_{50} values for [^{3}H]-dopamine uptake inhibition were determined with GraphPad Prism 3.0.

5. Ligand Binding Assays

All ligand binding assays were performed with cells grown in 6-well plates. All [^{3}H]-WIN 35,428 binding inhibition assays were performed exactly as described above for the dopamine uptake inhibition assays except that 1 nM [^{3}H]-WIN 35,428 was used in place of [^{3}H]-dopamine, and the cells were incubated with a mixture of radioligand and the nonradioactive competitor for 15 minutes. One nM final concentration of [^{3}H]-WIN 35,428 was achieved by adding 10 nM solution to each dilution of the nonradioactive competitor. The concentration ranges of the different nonradioactive DAT inhibitors or substrates employed in the binding assays were as indicated above for the dopamine uptake inhibition assays. Non-specific uptake was determined by using 10 μM mazindol or 30 μM cocaine if mazindol was the drug being assessed. [^{3}H]-WIN 35,428 saturation binding assays were performed by incubating the cells with a mixture of nonradioactive WIN 35,428 (1nM – 10 μM) and 1 nM [^{3}H]-WIN 35,428 for 15 minutes. Data were analyzed with GraphPad Prism 3.0 to obtain K_d, K_i and B_{max} values.

6. Immunocytochemistry and Confocal Microscopy

Immunocytochemistry and confocal microscopy was performed with COS-7 cells. Cells were seeded on methanol treated coverslips in 6-well plates such that they would be 40-80% confluent the next day. Polyfect reagent was used to perform the transient transfections with the cDNA plasmids. Forty-eight hours after transfection, the cells were washed thrice with PBS and fixed using 4%

paraformaldehyde solution in PBS at room temperature for 15 minutes. The fixed cells were washed three times with PBS and incubated with blocking-permeabilizing solution (5% goat serum, 0.5% BSA, 0.1% Triton X-100 in PBS solution) for 45 minutes. The cells were next washed thrice with BSA buffer (0.5% BSA solution in PBS) and incubated with rat monoclonal anti-DAT antibody at 1:1000 dilution for 1 hour. After washing five times with BSA buffer to remove the excess anti-DAT antibody, the cells were incubated with secondary antibody (goat anti-rat Alexa Fluor 488) at 1:500 dilution and rhodamine phalloidin at 1:250 dilution for 1 hour. When transfections were performed with plasmids coding GFP, the antibody incubation steps were eliminated and cells were incubated with rhodamine phalloidin at 1:250 dilution for 1 hour. After three washes in BSA buffer and three washes in PBS, the coverslips were slightly air-dried and mounted on slides using GVA mounting solution. The slides were left in dark at 4 ºC overnight to dry completely. All the fluorophores were visualized using a Leica TCS-SP2 confocal laser microscope with an oil immersion 100x objective. Alexa 488 and GFP were excited at 488 nm with an argon/kypton laser and emission photons from 500 – 600 nm were accumulated by the photomultiplier tube. Rhodamine phalloidin was excited at 543 nm with a helium/neon laser and emission photons from 550 to 650 nm were accumulated. The fluorophores were detected separately and overlay images were generated automatically by the imaging software. In experiments where quantification of the images was performed, 10 individual cells were selected randomly by screening the whole slide. All scan related and in-line parameters except Z-level

were fixed when acquiring images for each fluorophore. Images were quantified using the histogram quantification tool of the Leica Confocal Software. The mean intensity values for ten different regions on the cell membrane having the same number of pixels and area were obtained. The average intensity of the ten different regions on the cell membrane was calculated and the mean intensity for each fluorophore was determined by averaging the mean intensities of ten different cells. For counting the number of cells transfected, ten different fields were selected randomly by screening the whole slide using 20x objective.

7. Protein Assays

Protein assays were performed on CHO cells stably transfected with WT DAT, and COS-7 and N2A cells transiently transfected with WT DAT. For all the experiments that required protein content estimation, two wells were reserved for determining the protein content. The protein assays were performed as follows: the adherent cell monolayers were washed 2 x 1 ml with PBS, the cells were lysed with 0.5 ml 0.2 N sodium hydroxide solution, gently scraped with a cell scraper and incubated at 4ºC with gentle shaking for 1 hour. The cell lysates were transferred into Eppendorf tubes and stored at -20ºC until the protein content was estimated. All protein assays were performed using the Bradford method. Bovine serum albumin (BSA; 1 mg/ml stock solution) was used as the protein standard for generating the protein standard curve. Six different known concentrations of protein were prepared by diluting 1, 2, 3, 4, 5 and 6 µl of BSA stock solution to 800 µl with sterile water to achieve final concentrations of 1, 2,

3, 4, 5 and 6 µg/ml respectively after the final addition of 200 µl BioRad reagent. Tubes for the test samples were also prepared simultaneously by adding 20 µl test sample to sterile water to obtain a final volume of 800 µl. Two hundred µl BioRad reagent was then added to all the known and test sample tubes. The tubes were vortexed for 10 seconds and incubated at room temperature for 10 minutes. The absorbance values were determined at 595 nm using UV-Visible spectrophotometer. Protein standard curve was obtained by plotting the absorbance values of the known samples against the amount of protein in each sample using Microsoft Excel. The amount of protein in each of the test samples was calculated from the standard curve.

8. Statistics

Statistical significance was calculated using GraphPad Prism 3.0. Statistical significance among three groups was determined using one-way analysis of variance with Newman-Keuls post hoc test. Two-group comparisions were made using a two-tail Student's *t* test.

IV. RESULTS

A. Effects of cell age and DAT cell surface expression on dopamine uptake inhibition potencies of classical DAT ligands.

It was empirically observed for years that the DUIP of cocaine at WT DAT was not static and changes in cocaine DUIP occurred within the same cell line when assays were performed employing different passages of cells. In an attempt to address this issue, the present study investigated the effect of cell state on DUIP fluctuation of cocaine and the trend this fluctuation follows with increasing cell passage number. Different passages of stably transfected WT DAT CHO cells were employed and passages 9 – 20, 25 – 36 and 40 – 54 were classified as “low”, “medium” and “high” respectively. All uptake and binding assays were performed in parallel and under identical conditions. A decrease in the DUIP of cocaine at WT DAT was observed with increasing cell passage number (Figure 5 and Table 1). The IC_{50} value for inhibition of [^{3}H]-dopamine uptake at low passage was about 2-fold less when compared to high passage (Table 1). On the other hand, the apparent binding affinity of cocaine did not vary with cell passage, evidenced by the unchanging K_i value from inhibition of [^{3}H]-WIN 35,428 binding assays (Figure 5 and Table 1). The study was further expanded to test if this trend was exhibited by other classical DAT blockers. Mazindol, methylphenidate and benztropine were the other DAT inhibitors tested and a trend similar to cocaine for the DUIP was observed for these drugs with cell passage (Table 1). The study also investigated if this property was exhibited by DAT substrates such as (+)-amphetamine. Interestingly, no significant fluctuation in DUIP was observed for amphetamine (Figure 6 and Table 1).

Similar to that of cocaine, the apparent binding affinities of the other DAT blockers and amphetamine did not vary with cell passage (Table 1).

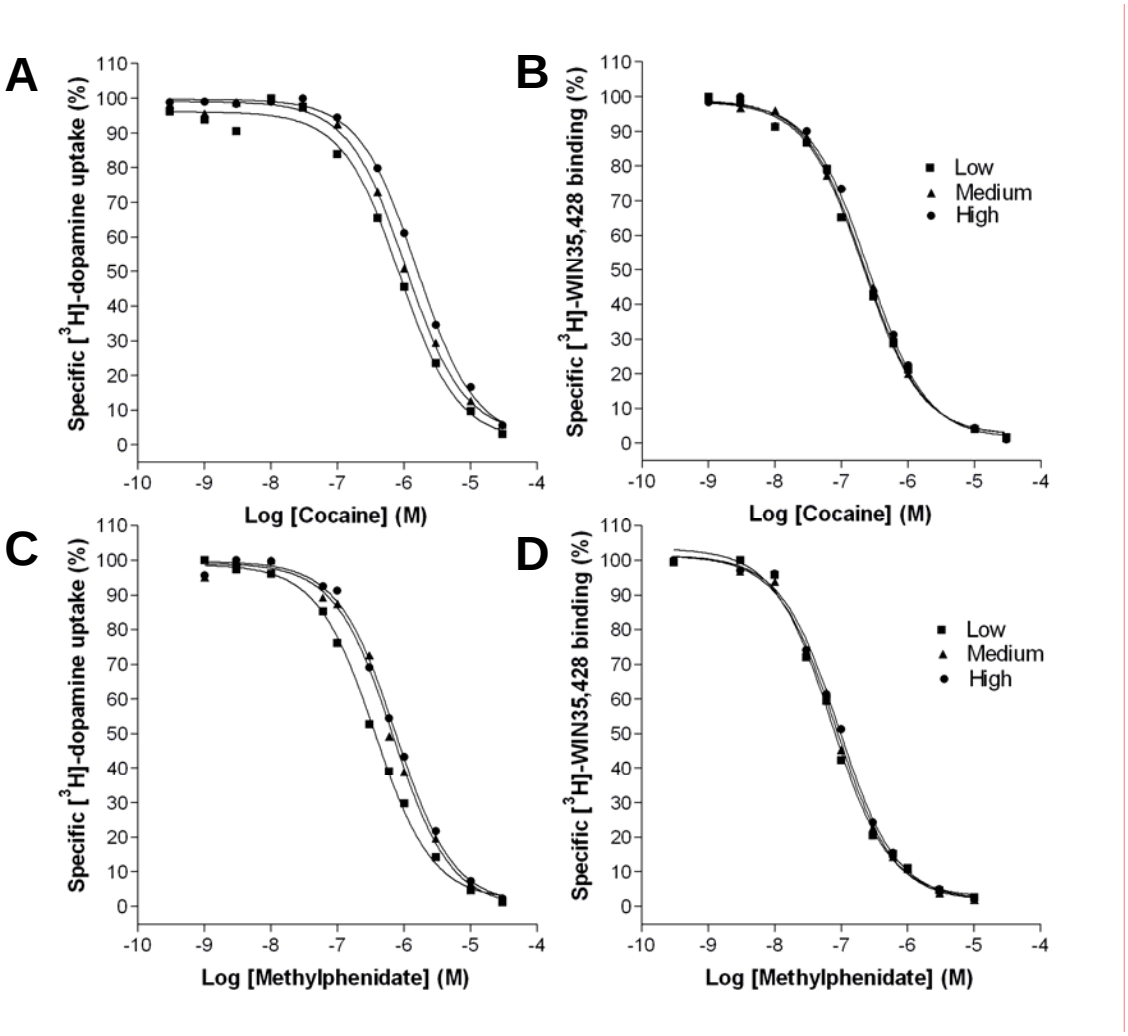


Figure 5. Cocaine and mazindol (DAT blockers) inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN 35,428 binding at WT DAT CHO cells.

Cocaine inhibition of [^{3}H]-dopamine uptake (Panel A), cocaine inhibition of [^{3}H]-WIN 35,428 binding (Panel B), methylphenidate inhibition of [^{3}H]-dopamine uptake (Panel C) and methylphenidate inhibition of [^{3}H]-WIN 35,428 binding (Panel D) at different passages of WT DAT CHO cells. Passages 9 – 20, 25 – 36 and 40 – 54 were classified as "low", "medium" and "high", respectively. Uptake and binding experiments for each drug at different passages were performed in parallel and under identical conditions. The data are representative of at least 3 independent experiments.

The ratios of dopamine uptake inhibition potency-to-binding affinity for the different DAT ligands at the different passages of WT DAT CHO cells were calculated. (Table 2; K_i values (not shown) and IC_{50} values for [^{3}H]-dopamine uptake inhibition in Table 1 were essentially identical, allowing direct comparison of uptake and binding inhibition constants).

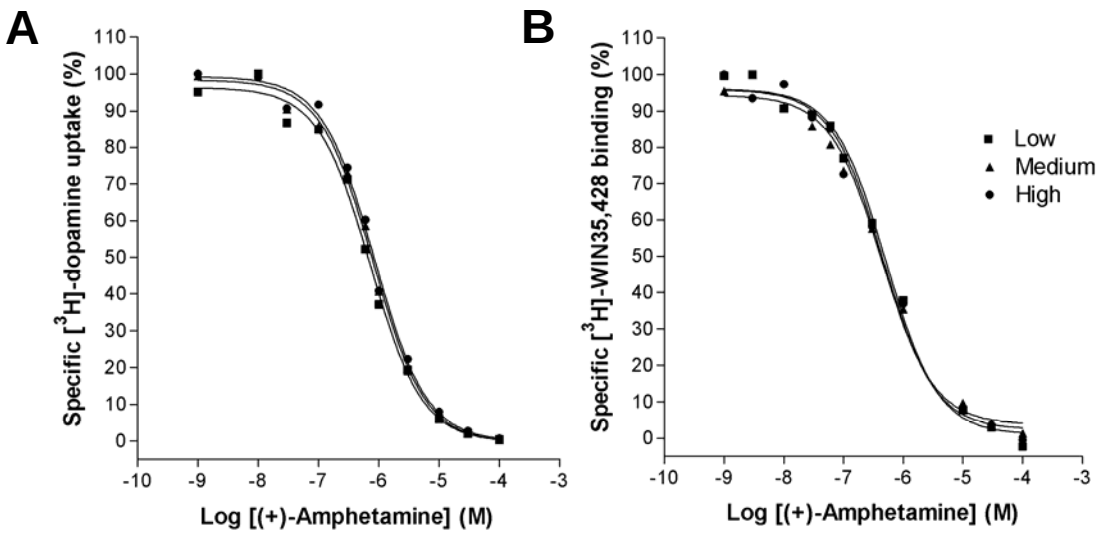


Figure 6. (+)-Amphetamine (DAT substrate) inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN 35,428 binding at WT DAT CHO cells.

Amphetamine inhibition of [^{3}H]-dopamine uptake (Panel A) and amphetamine inhibition of [^{3}H]-WIN 35,428 binding (Panel B) under identical conditions at different passages of WT DAT CHO cells. Passages 9 – 20, 25 – 36 and 40 – 54 were classified as "low", "medium" and "high", respectively. The data are representative of at least 3 independent experiments.

Table 1. Dopamine uptake inhibition potencies and binding affinities of DAT ligands at WT DAT CHO cells as a function of cell passage number.

IC_{50} and K_i values were derived from experiments incubating the stably-transfected cells with nonradioactive DAT inhibitors in the presence of [^{3}H]-dopamine and [^{3}H]-WIN 35,428, respectively, at 22ºC in KRH buffer. Passages 9 -20, 25 - 36 and 40 - 54 were classified as "low", "medium" and "high", respectively. Mean ± SEM for at least 3 independent experiments.

	Cell Passage		
	Low	**Medium**	**High**
[^{3}H]-DA uptake inhibition		**IC_{50} (nM)**	
Cocaine	719 ± 94	1055 ± 52	1503 ± 127[a]
Mazindol	44 ± 4	74 ± 5	93 ± 17[a]
Methylphenidate	352 ± 17	640 ± 22	662 ± 45[a]
Benztropine	236 ± 40	395 ± 45	413 ± 27[a]
(+)-Amphetamine	671 ± 88	756 ± 86	906 ± 39
[^{3}H]-WIN 35,428 inhibition		**K_i (nM)**	
Cocaine	179 ± 22	197 ± 22	251 ± 28
Mazindol	15 ± 2	19 ± 2	20 ± 2
Methylphenidate	74 ± 7	72 ± 5	85 ± 6
Benztropine	78 ± 6	85 ± 9	92 ± 10
(+)-Amphetamine	524 ± 31	478 ± 52	557 ± 78
[^{3}H]-WIN 35,428 binding			
K_d (nM)	17 ± 1	17 ± 2	18 ± 1
B_{max} (pmol/mg)	9 ± 2	11 ± 2	19 ± 3[a]

[a]$P < 0.05$ versus low passage cells for that assay (one-way ANOVA, Newman-Keuls post hoc test)

[^{3}H]-WIN 35,428 saturation binding assays demonstrated that there was an increase in the surface expression of WT DAT in CHO cells with increasing cell passage number, evidenced by the increased B_{max} value for [^{3}H]-WIN 35,428 binding at high passage cells. However, the binding affinity of [^{3}H]-WIN 35,428 remained unchanged at the different cell passages (Table 1).

Table 2. **Ratios of dopamine uptake inhibition potency-to-binding affinity for DAT ligands at WT DAT CHO cells as a function of cell passage number.**

Ratios were calculated using the IC_{50} values for inhibition of [^{3}H]-dopamine uptake and K_i values for inhibition of [^{3}H]-WIN 35,428 binding in Table 1. K_i values (not shown) and IC_{50} values for [^{3}H]-dopamine uptake inhibition were essentially identical, allowing direct comparison of uptake and binding inhibition constants.

	DUIP:Affinity Ratio		
	Cell Passage		
	Low	**Medium**	**High**
Cocaine	4.0	5.3	5.9
Mazindol	2.9	3.8	4.7
Methylphenidate	4.8	8.8	7.8
Benztropine	3.0	4.6	4.5
(+)-Amphetamine	1.3	1.6	1.6

To address whether the decrease in DUIP of cocaine at high passage WT DAT CHO cells was simply a result of increase in DAT levels, assays were performed manipulating the cell density (% confluence) of WT DAT CHO cells. The monolayer was defined as 100% confluent when all cells appeared to contact neighboring cells so as not to leave open spaces on the culture plate. Cocaine inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN 35,428 binding assays

performed on 20%, 100% and 150% confluent monolayers suggested that cell density had no effect on either cocaine DUIP or binding affinity (Reference: Structural determinants for substrate and inhibitor recognition by the dopamine transporter, Dissertation of Okechukwu T. Ukairo, Duquesne University). To further investigate the DUIP shift of cocaine, naive COS-7 cells were transiently transfected with varying amounts of a cDNA plasmid encoding the WT DAT to achieve different DAT expression levels with respect to individual cells. The amounts of plasmid employed in the transfections were 25, 50 and 100% of what is optimal. [^{3}H]-WIN 35,428 saturation binding analysis confirmed that B_{max} values shifted in accordance with manipulation of plasmid level (Table 3). B_{max} values reflect DAT expression at the cell surface as it has been shown that WIN 35,428 does not cross the plasma membrane of the intact cell appreciably (Chen et al., 2004). The [^{3}H]-dopamine uptake assays demonstrated that the DUIP of cocaine differed significantly between cells transfected with 25% and 100% of optimal plasmid levels while WIN 35,428 binding affinity remained unaffected (Table 3). Confocal microscopic analysis further confirmed the changes in COS-7 cell surface DAT expression observed in the binding experiments (Figure 7).

Table 3. Dopamine uptake inhibition potency of cocaine and binding affinity of WIN 35,428 at COS-7 cells as a function of amount of WT DAT plasmid introduced.

Naive COS-7 cells were transiently transfected with 375 (25%), 750 (50%) or 1500 (100%) ng of WT DAT cDNA plasmid to manipulate the surface expression of DAT. [^{3}H]-WIN 35,428 saturation binding assays were performed parallel to [^{3}H]-dopamine uptake inhibition assays and under identical conditions. Mean ± SEM for at least 3 independent experiments.

	WT DAT Plasmid (% of Optimal)		
	25	**50**	**100**
[^{3}H]-DA uptake inhibition		**IC_{50} (nM)**	
Cocaine	228 ± 7	353 ± 20	558 ± 75[a]
[^{3}H]-WIN 35,428 binding			
K_d (nM)	12 ± 2	15 ± 2	14 ± 2
B_{max} (pmol/mg)	0.8 ± 0.4	2.0 ± 0.5	4.0 ± 0.2[a]

[a]$P < 0.05$ versus low passage cells for that assay (one-way ANOVA, Newman-Keuls post hoc test)

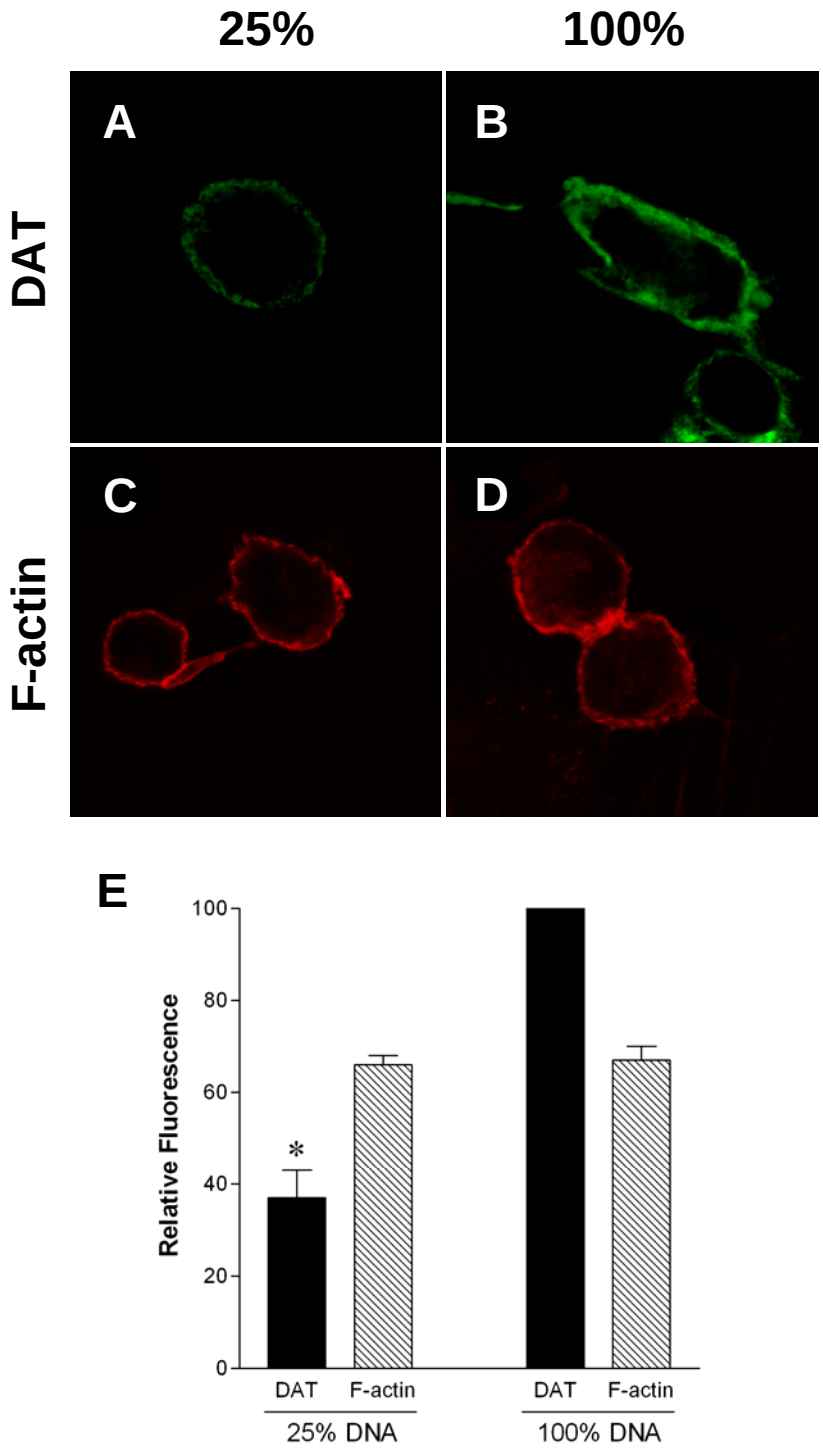


Figure 7. Confocal microscopy of transiently transfected COS-7 cells.

COS-7 cells were transiently transfected with 25% or 100% of optimal WT DAT cDNA plasmid levels. The expressed DAT protein was visualized with Alexa Fluor 488 (green signal; Panels A and B) and cortical F-actin, a marker at the plasma membrane, was visualized with rhodamine phalloidin (red signal; Panels C and D). Shown are representative confocal images of three independent experiments. Cell membrane signal intensities were quantitated for each of the four scenarios represented by Panels A – D (Panel E). Scale bar = 8.00 µm for all the images. The data in Panel E represent an average of three independent experiments. *P<0.05 relative to DAT with 100% DNA.

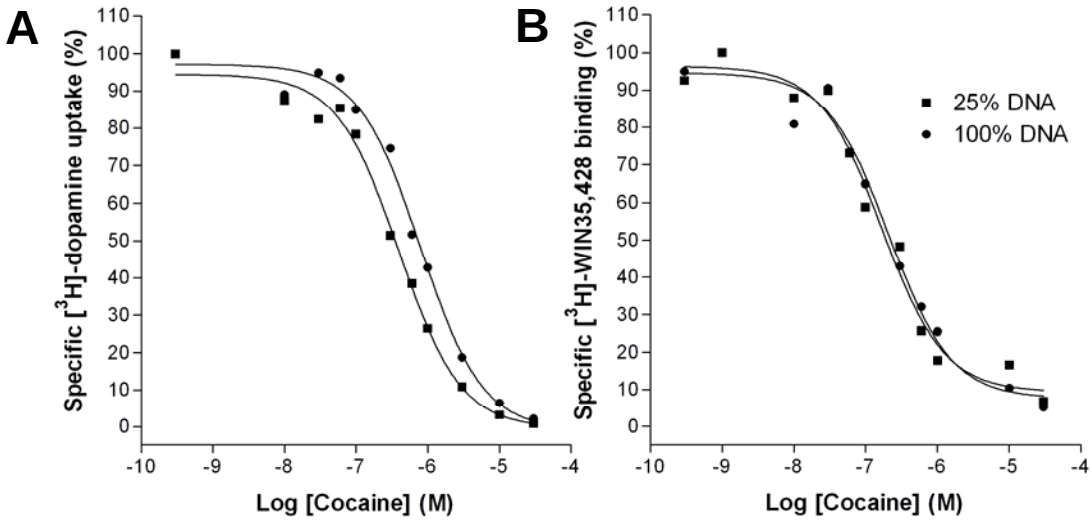


Figure 8. Cocaine inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN 35,428 binding at N2A cells expressing varying levels of WT DAT.

Cocaine inhibition of [^{3}H]-dopamine uptake (Panel A) and cocaine inhibition of [^{3}H]-WIN 35, 428 binding (Panel B) under identical conditions at N2A neuroblastoma cells transiently transfected with 375 (25%) or 1500 (100%) ng of WT DAT cDNA plasmid. The data are representative of at least 3 independent experiments.

All the above experiments studying the DUIP fluctuations of DAT ligands were performed in non-neuronal cell systems (CHO and COS-7). DAT is a protein expressed *in vivo* in neuronal cells. Therefore, to address the physiological significance of the cocaine DUIP fluctuation phenomenon, a neuronal cell line was tested. Naive N2A neuroblastoma cells were transiently transfected with varying amounts of WT DAT cDNA plasmid. The amounts of plasmid employed in the transfections were 25% and 100% of what is optimal. Similar to the COS-7 cell experiment, cocaine DUIP was significantly altered by the plasmid titration in N2A cells. The binding affinities for cocaine and WIN 35,428 were unaffected (Figure 8 and Table 4). Again, B_{max} values from parallel

WIN 35,428 saturation binding analysis indicated that DAT expression was predictably shifted by the manipulation of plasmid level (Table 4).

Table 4. Dopamine uptake inhibition potency and binding affinity of cocaine at N2A neuroblastoma cells as a function of amount of WT DAT plasmid introduced.

Naive N2A cells were transiently transfected with 375 (25%) or 1500 (100%) ng of WT DAT plasmid to manipulate the surface expression of DAT. [^{3}H]-WIN 35,428 binding assays were performed parallel to the [^{3}H]-dopamine uptake inhibition assays and under identical conditions. Mean ± SEM for at least 3 independent experiments.

	WT DAT Plasmid (% of Optimal)	
	25	**100**
	IC_{50} (nM)	
[^{3}H]-DA uptake inhibition		
Cocaine	476 ± 46	789 ± 64[a]
	K_i (nM)	
[^{3}H]-WIN 35,428 inhibition		
Cocaine	229 ± 34	196 ± 11
[^{3}H]-WIN 35,428 binding		
K_d (nM)	25 ± 2	18 ± 3
B_{max} (pmol/mg)	1.3 ± 0.4	2.8 ± 0.5[a]

[a]$P < 0.05$ versus low passage cells for that assay (two-tail Student's *t* test)

B. Assessing the role of N- and C-terminus of DAT in the DUIP fluctuation of cocaine.

Several proteins directly interact with and play an important role in the trafficking and functioning of the DAT (reviewed in Torres, 2006). Differences in the DAT brought about by protein interactions might be responsible for the formation of two or more distinct conformations of the transporter and cocaine may bind to these multiple DAT conformations with different affinities. To investigate the role of protein interactions of DAT in the DUIP fluctuation, N- and C-terminal fragments of WT DAT were overexpressed in two cell systems that showed the DUIP fluctuation, COS-7 and N2A. The N- or C-terminal fragments of DAT may act as "decoys" by intercepting the DAT modulators and altering the dopamine uptake and DUIPs of the DAT blockers. Studies in COS-7 cells were carried out by co-expressing WT DAT and the N- or C-terminal fragments of WT DAT. [^{3}H]-dopamine uptake assays demonstrated that the N- and C-terminal fragments of DAT did not exhibit any significant effect on the net dopamine uptake at the DAT (Figure 9). Cocaine inhibition of [^{3}H]-dopamine uptake and cocaine inhibition of [^{3}H]-WIN 35,428 binding assays demonstrated that overexpression of the N- or C-terminal fragments of the DAT neither affected the DUIP nor the binding affinity of cocaine at the DAT in a significant manner (Figure 10 and Table 5). The overexpression of the DAT terminal fragments in COS-7 cells was confirmed by confocal microscopy studies (Figure 11). Studies in N2A neuroblastoma cell system were carried out by intentionally manipulating the DAT cell surface expression and co-expressing the N- or C-terminal fragments of the DAT with the WT DAT. Cocaine inhibition of [^{3}H]-dopamine

uptake assays demonstrated that the N- and C-terminal decoys employed in the N2A neuroblastoma cell system do not show any significant change in the DUIP of cocaine at the different DAT expression levels (Figure 13 and Table 6).

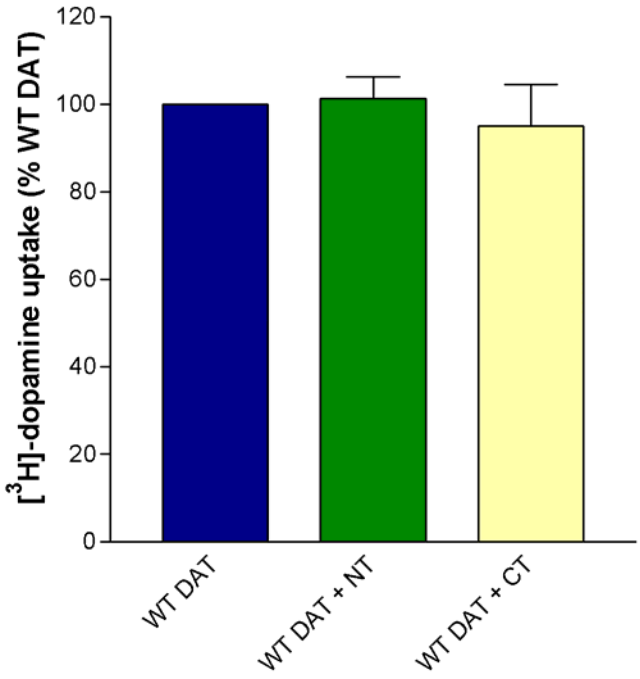


Figure 9. [^{3}H]-Dopamine uptake at DAT in the presence of N- or C-terminal fragments of DAT.

Specific [^{3}H]-dopamine uptake as a percentage of WT DAT was measured in COS-7 cells transiently co-transfected with WT DAT and control vector (blue) or N- (green) or C- (yellow) terminal fragments of WT DAT. The N- and C-terminal fragments of DAT are abbreviated as NT and CT respectively. The data represent an average of three independent experiments.

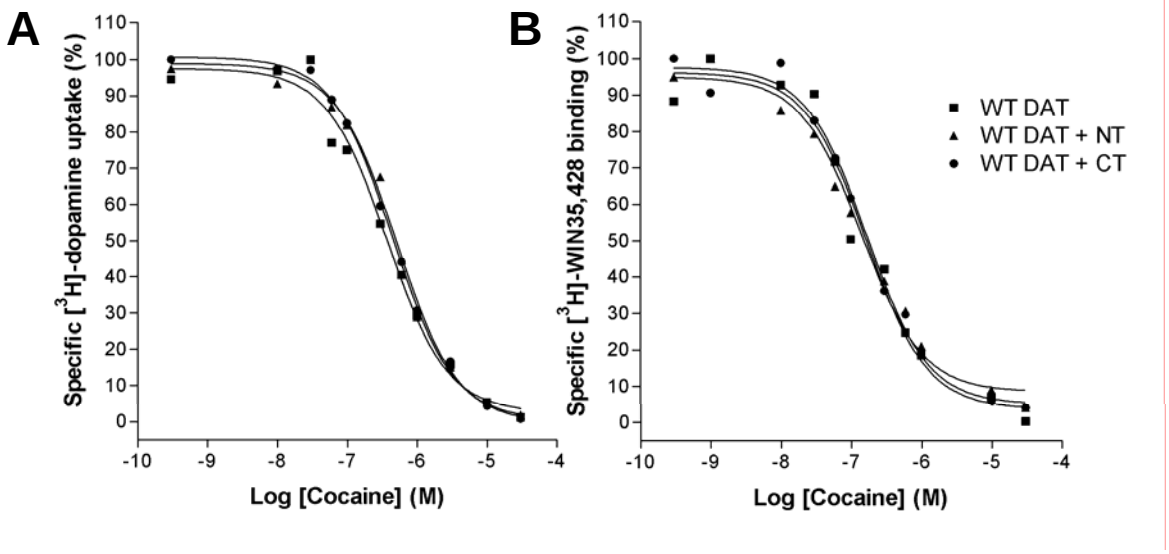


Figure 10. Cocaine inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN 35,428 binding at DAT in the presence of N- or C-terminal fragments of DAT.

Cocaine inhibition of [^{3}H]-dopamine uptake (Panel A) and cocaine inhibition of [^{3}H]-WIN 35, 428 binding (Panel B) performed in parallel and under identical conditions in COS-7 cells transiently co-transfected with 750 ng WT DAT and 750 ng control vector (squares) or N- (triangles) or C- (circles) terminal fragments of WT DAT. The N- and C- terminal fragments of DAT are abbreviated as NT and CT respectively. The data are representative of at least three independent experiments.

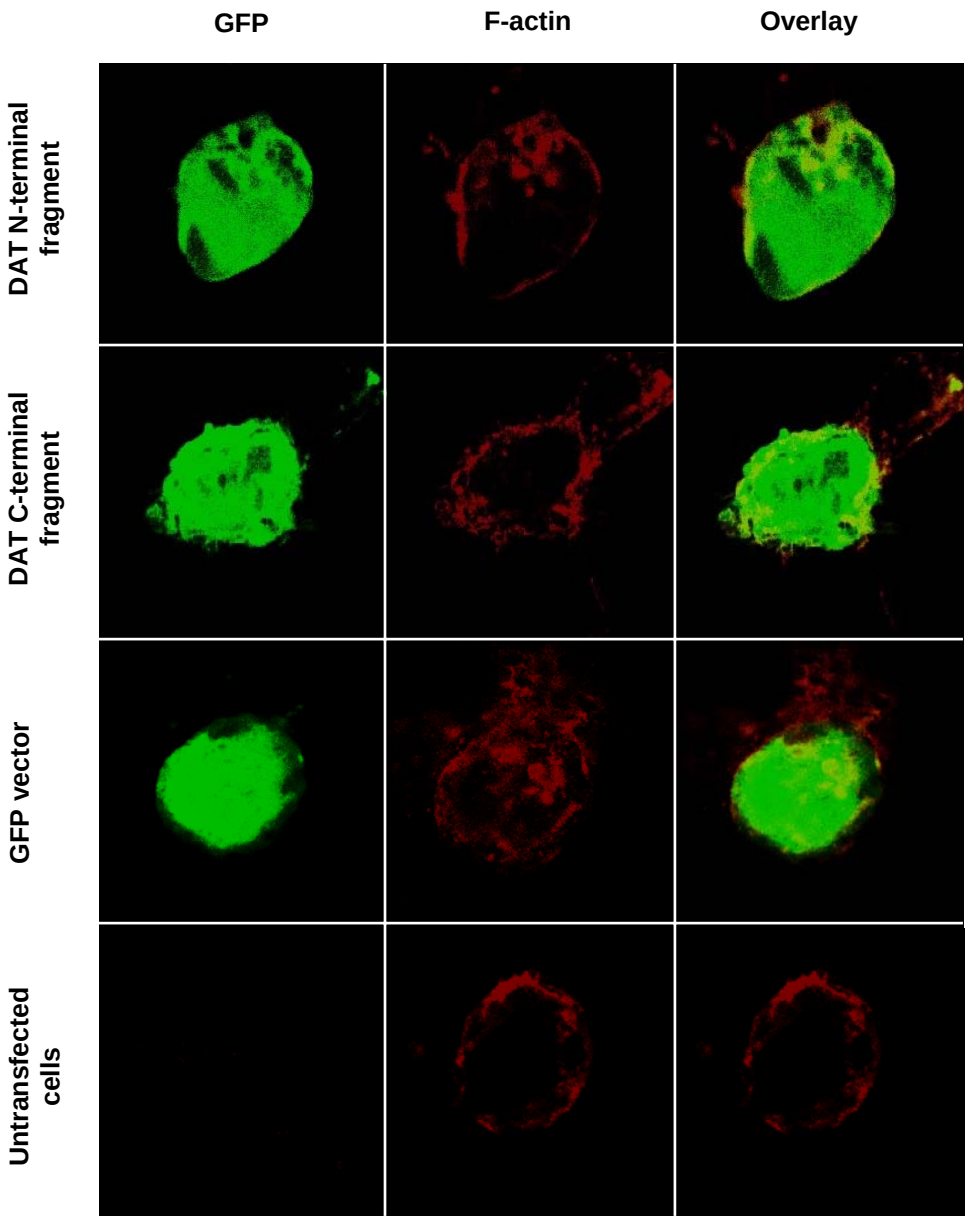
Table 5. Effect of overexpression of N- or C-terminal fragments of DAT on the DUIP and binding affinity of cocaine at DAT.

Cocaine inhibition of [^{3}H]-dopamine uptake and cocaine inhibition of [^{3}H]-WIN 35,428 binding assays were performed in parallel and under identical conditions in COS-7 cells transiently co-transfected with WT DAT and control vector or N- or C-terminal fragments of WT DAT. Mean ± SEM for at least 3 independent experiments.

Cocaine	DAT	DAT+NT	DAT + CT
		IC_{50} (nM)	
[^{3}H]-Dopamine uptake inhibition	474 ± 54	504 ± 40	512 ± 36
		K_i (nM)	
[^{3}H]-WIN 35,428 inhibition	206 ± 29	209 ± 38	256 ± 74

Figure 11. Confocal microscopic localization of DAT N- and C-terminal fragments.

COS-7 cells were transiently co-transfected with WT DAT and control vector or N- or C-terminal fragment of DAT. The expressed N- or C-terminus fragment proteins were visualized with the attached GFP tag (green signal) and cortical F-actin, a marker at the plasma membrane, was visualized with rhodamine phalloidin (red signal). The fluorophores were detected separately and overlay images were generated. Scale bar = 8.00 μm for all the images. Shown are representative confocal images of three independent experiments.



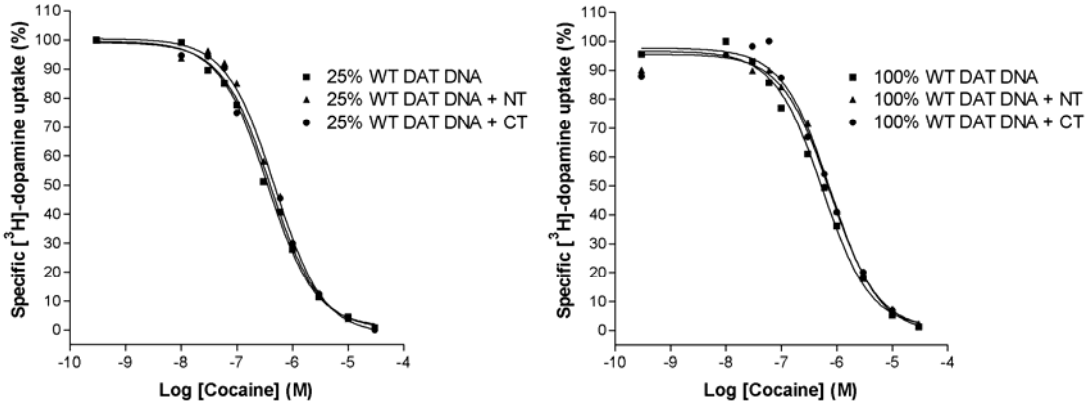


Figure 12. Cocaine inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN 35,428 binding at DAT in the presence of N- or C-terminal fragments of DAT.

Cocaine inhibition of [^{3}H]-dopamine uptake in N2A cells transiently co-transfected with 200 (25%, Panel A) or 1000 (100%, Panel B) ng WT DAT and 500 ng control vector (squares) or N- (triangles) or C- (circles) terminus fragments of WT DAT. All uptake experiments were performed under identical conditions. The data are representative of at least three independent experiments.

Table 6. Effect of overexpression of N- or C-terminal fragments of DAT on the DUIP and binding affinity of cocaine at DAT.

Cocaine inhibition of [^{3}H]-dopamine uptake in N2A cells transiently co-transfected with varying amounts of WT DAT and control vector or N- or C-terminal fragments of WT DAT. All the uptake experiments were performed under identical conditions and on the same day. Mean ± SEM for at least 3 independent experiments.

	DAT	DAT+NT	DAT + CT
		IC_{50} (nM)	
25% DAT DNA	336 ± 34	420 ± 36	326 ± 57
100% DAT DNA	593 ± 124	624 ± 76	682 ± 76

Another possibility for the DUIP fluctuation in the present study could be a change in the phophorylation state of the DAT. Studies have shown that the DAT is N-terminally phosphorylated (Foster et al., 2002, Granas et al., 2003) and phosphorylation can affect the cell surface trafficking of the transporter (Holton et al., 2005). To test if the N-terminal phosphorylation plays any role in the DUIP shift of cocaine, inhibition of [^{3}H]-dopamine uptake and inhibition of [^{3}H]-WIN 35,428 binding assays were performed in COS-7 cells expressing human DAT (hDAT) and hDAT lacking the first 20 amino acids (del-20 hDAT). No significant difference was observed in the DUIP of cocaine at hDAT compared to the N-terminal truncated hDAT (Figure 13 and Table 7). Studying the dopamine uptake at del-20 hDAT was another approach to assess the role of N-terminus of the DAT in the DUIP fluctuation.

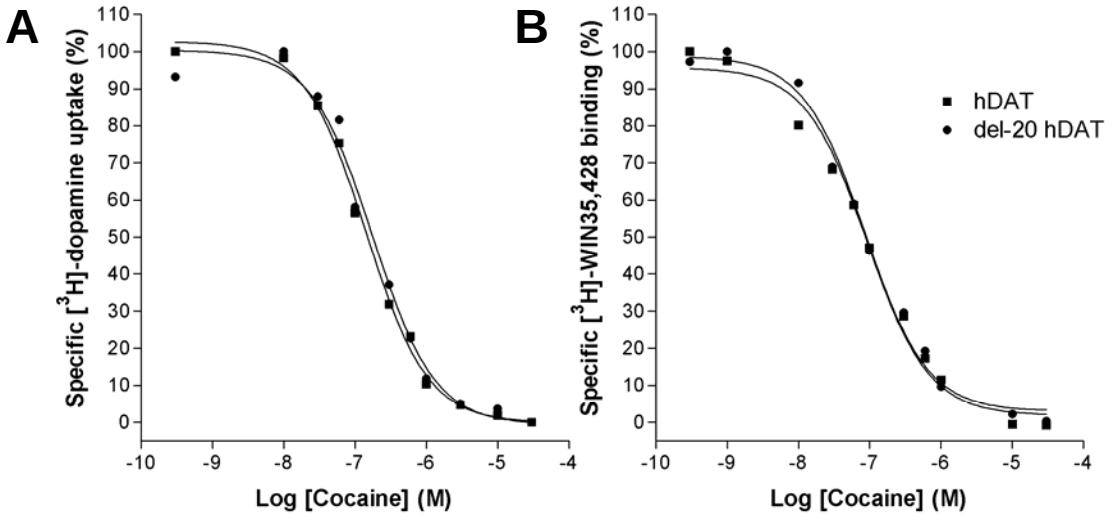


Figure 13. Cocaine inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN 35,428 binding at hDAT and del-20 hDAT.

Cocaine inhibition of [^{3}H]-dopamine uptake (Panel A) and [^{3}H]-WIN 35,428 binding (Panel B) under identical conditions at hDAT (squares) and del-20 hDAT (circles) transiently transfected in COS-7 cells. The data are representative of 3 independent experiments.

Table 7. Effect of N-terminal truncation on the DUIP and binding affinity of cocaine at hDAT.

Cocaine inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN 35,428 binding performed in parallel and under identical conditions at hDAT and del-20 hDAT transiently transfected in COS-7 cells. Mean ± SEM for at least 3 independent experiments.

Cocaine	hDAT	del-20 hDAT
	IC_{50} (nM)	
[^{3}H]-Dopamine uptake inhibition	214 ± 56	183 ± 30
	K_i (nM)	
[^{3}H]-WIN 35,428 inhibition	799 ± 139	666 ± 115

V. DISCUSSION

A number of studies have demonstrated that the DUIPs of the classical DAT blockers do not correlate well with their apparent binding affinities at the WT DAT. These DAT blockers are more potent in inhibiting [^{3}H]-WIN 35,428 binding than in inhibiting [^{3}H]-dopamine uptake (Pristupa et al., 1994; Eshleman et al., 1999; Wang et al., 2003). In the present study, the DUIPs of cocaine, mazindol, methylphenidate and benztropine followed a decreasing trend with increasing cell passage; statistically significant differences in the DUIPs were observed between the low and high passages while their binding affinities remained unchanged in WT DAT CHO cells. Neither the DUIP nor the binding affinity of amphetamine (DAT substrate) fluctuated at the different passages (Table 1). The loss of correlation between DUIP and binding affinity at DAT might be a unique property of DAT blockers that is not exhibited by DAT substrates. Besides being a substrate at the DAT, amphetamine has the ability to induce dopamine efflux via the DAT by reversal of the transporter (reviewed in Kahlig and Galli, 2003). It has been shown that amphetamine can cause dopamine efflux in dopamine preloaded COS-7 cells (transfected with WT DAT cDNA plasmid), and cocaine and other DAT blockers either inhibit or have no effect on the dopamine release in these cells (Eshleman et al., 1994; Pifl et al., 1995). Thus, DAT blockers differ from DAT substrates in several respects.

The ratios of dopamine uptake inhibition potency-to-binding affinity for DAT ligands further demonstrate the differences in the behavior of amphetamine and the tested DAT blockers at WT DAT CHO cells as a function of cell passage

number (Table 2). The dopamine uptake inhibition K_i value of amphetamine was not significantly different from its binding affinity K_i value at WT DAT, evidenced by the near unity (1.3 – 1.6) uptake inhibition potency:binding affinity ratios. On the other hand, the dopamine uptake inhibition K_i values of DAT blockers were significantly different from their binding affinity K_i values at WT DAT (uptake inhibition potency:binding affinity ratios ranged from 2.9 – 8.8 for different DAT blockers at the different passages). Moreover, these ratios were comparably different for DAT blockers at low versus high passage WT DAT CHO cells; amphetamine did not exhibit such differences. Thus, two inferences can be drawn from the above observations: a) amphetamine is equipotent in inhibiting dopamine uptake and WIN binding at WT DAT while DAT blockers are not b) the uptake inhibition profile of DAT blockers is comparably different at low and high passage WT DAT CHO cells, a phenomenon that does not occur with amphetamine. However, this differential behavior of DAT blockers and DAT substrates at DAT in terms of uptake inhibition potencies needs further investigation by employing other DAT substrates such as methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA).

Contrary to the studies that have shown that DAT expression in the brain decreases with aging (Bannon and Whitty, 1997; Salvatore et al., 2003), the saturation binding experiments in the present study demonstrated increasing DAT surface expression with increasing cell passage (Table 1). However, the binding affinity of [^{3}H]-WIN 35,428 remained unaffected, an observation in agreement with the previous reports demonstrating unchanging binding affinities

of DAT blockers despite changes in DAT surface expression (Shimizu and Prasad, 1991; Hebert et al., 1999). In another study, altering the cell density (% confluence) by over 7-fold to manipulate the total number of DAT molecules in each well of the culture dish neither affected the DUIP nor the binding affinity of cocaine (Reference: Structural determinants for substrate and inhibitor recognition by the dopamine transporter, Dissertation of Okechukwu T. Ukairo, Duquesne University). Cocaine treatment increases DAT surface expression (Little et al., 2002; Daws et al., 2002) and amphetamine exposure decreases DAT surface expression (Gulley et al., 2002; Saunders et al., 2000; Zahniser and Sorkin, 2004). These inhibitor- or substrate-mediated changes in DAT surface expression would be irrelevant here because the present study employed drug concentrations that were not high enough and drug incubations that were not long enough to cause such changes. Three μM amphetamine exposure increased surface DAT levels within 1 minute, followed by rapid reversion to normal levels (Johnson et al., 2005). This event may or may not be relevant to the present experiments, in which amphetamine incubations were performed for 10 minutes. Therefore, the potency fluctuation observed with the DAT blockers was not a direct consequence of changes in DAT surface expression. Some sort of cell age-related DAT modification might play a significant role in the DUIP fluctuations of the DAT blockers.

Considering the DUIP fluctuation of cocaine with cell passage/state, it is unlikely that a single cocaine binding site of the DAT dictates dopamine uptake inhibition. However, no solid evidence exists for two different binding sites for a

single DAT ligand on the same DAT protein. Previous studies provide evidence for the existence of DAT in more than one conformation or population. The DAT conformation or population responsible for high affinity binding of DAT blockers may be less responsible for dopamine uptake (Wang et al., 2003; Ukairo et al., 2005). Distribution of the DAT conformations can be altered by DAT mutation (Loland et al., 2002). Similarly, the distribution of the different DAT conformations or populations may be influenced by cell state, possibly by alterations in their trafficking to the cell surface. A plausible explanation for the loss of correlation between potency and apparent binding affinity of DAT blockers, and DUIP fluctuations with cell passage observed in the present study is the existence of two different DAT populations on the cell surface (two-population model; Wang et al., 2003). Of the two different populations, "Population 1" has a higher affinity for inhibitor binding but is less responsible for dopamine uptake. On the contrary, "Population 2" has a lower affinity for inhibitor binding but is largely responsible for dopamine uptake (Figure 14). The DAT blocker has to bind to Population 2 (lower DAT blocker affinity), a population underrepresented in binding experiments, to inhibit dopamine uptake. Binding experiments largely represent Population 1 (higher DAT blocker affinity). Hence, the [^{3}H]-dopamine uptake inhibition IC_{50} values are higher than the inhibition of [^{3}H]-WIN 35,428 binding K_i values at the DAT (Table 1). Extending this discussion to the cell passage-related DUIP fluctuation of DAT blockers, the surface expression of Population 2 increases with cell passage while surface expression of Population 1 remains unaltered; net surface DAT levels are

increasing. In addition to these surface expression changes, the DAT inhibitor affinity at Population 2 decreases further with cell age (certain intracellular factors may be involved); the net result being a shift in the DUIP of DAT blockers at higher passage without any change in their binding affinities. This model also resolves the observation that the binding affinity (K_d) of WIN 35,428 remains unaltered despite changes in surface expression in the saturation binding experiments (Table 1). Studies have consistently demonstrated unchanging binding affinity with increasing DAT surface expression (Shimizu and Prasad, 1991; Hebert et al., 1999). The differences in binding sites of DAT blockers and substrates and the fact that amphetamine also acts as a substrate at DAT might form the basis for the divergent behavior of DAT blockers and amphetamine with respect to potency fluctuation. Amphetamine-mediated reversal of the transporter, a characteristic property not exhibited by the DAT blockers, may have an influence on the number of transporter molecules available for dopamine uptake or on the DUIP of amphetamine. The fact that amphetamine (a DAT substrate) displaced [^{3}H]-WIN 35,428 (a DAT blocker) in binding assays needs to be considered before elucidating the divergent behavior of amphetamine from the DAT blockers tested.

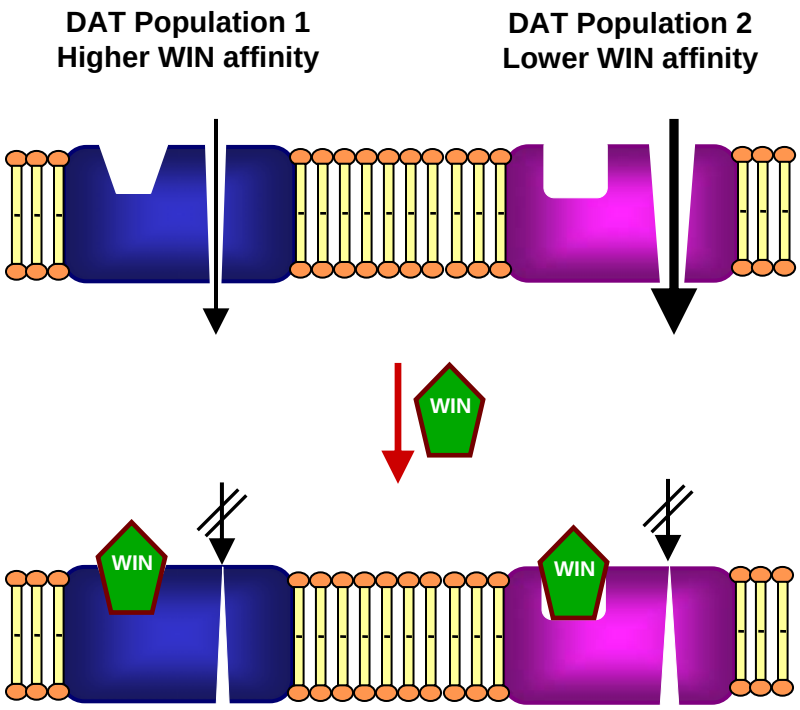


Figure 14. Two population model of the DAT.
Population 1 (blue) displays a higher affinity for WIN 35,428 (green) but is less responsible for dopamine uptake. Population 2 (pink) displays a lower affinity for WIN 35,428 but is largely responsible for the dopamine uptake. WIN 35,428 has to bind to Population 2 to inhibit dopamine uptake. Thus, uptake inhibition experiments principally represent Population 2. Binding experiments largely represent Population 1. Reproduced from Wang et al.,2003.

To mimic the cell passage-related changes in surface expression observed in WT DAT CHO cells, naive COS-7 cells were transfected with different amounts of a cDNA plasmid encoding the WT DAT. Thus, manipulation of DAT surface expression in individual cells was attempted. Indeed, a significant decrease in B_{max} was observed when a 4-fold lower amount of DNA was employed (Table 3). Interestingly, the DUIP shift for DAT blockers (similar to that observed with different passages of WT DAT CHO cells) correlated with

altered DAT surface expression in the COS-7 cells. Consistent with the previous results, the binding affinity remained static. Quantitative confocal microscopy further confirmed the changes in DAT surface expression (Figure 7). Similar experiments were performed in N2A neuroblastoma cells to test the physiological relevance of the DUIP shift. The DUIP fluctuation phenomenon extended to the neuronal cell line studied (Table 4).

As briefly mentioned earlier, intracellular events (e.g., altered posttranslational DAT modification pattern or DAT homo- or hetero-oligomerization) might be controlling the relative distribution of the DAT populations (responsible for the DUIP fluctuations of DAT blockers at DAT). Physiological changes in the cell cause variations in the glycosylation patterns of proteins (Lis and Sharon, 1993; Patel et al., 1994). N-glycosylation of the DAT increased the DUIPs of cocaine-like drugs without affecting their binding affinities (Li et al., 2004). Thus, a slightly different pattern of posttranslational modification of DAT can lead to the expression of more than one population of transporters in the cell. DAT homo-oligomerization state affects transporter function and DAT ligands can modify this state (Sorkina et al., 2003; Hastrup et al., 2003).

Several proteins have been implicated in DAT-protein interactions and these protein interactions influence the cell surface expression or function of the DAT (reviewed in Torres, 2006). Synuclein and PICK1 interact with the C-terminal region of the DAT resulting in increased DAT surface expression and increased dopamine uptake. Hic-5 interacts with the amino-proximal portion of the intracellular carboxyl terminus of DAT and overexpression of this protein

downregulates DAT uptake activity by decreasing the DAT surface expression. Syntaxin 1A and RACK1 interact with the N-terminal region of the DAT but the effects of these interactions on DAT surface expression and function have not yet been established. Syntaxin 1A interacts with the N-terminal region of the GAT1 and the NET, and modulates surface expression and transport activity (Deken et al., 2000; Horton and Quick, 2001; Sung et al., 2003; Hansra et al., 2004). Therefore, it is likely that syntaxin 1A also plays a significant role in regulating the activity and/or trafficking of the DAT (GAT1 and NET belong to the same family of transporters to which DAT belongs). Alterations brought about by protein interactions with the DAT may be responsible for the formation of different DAT populations and cocaine may bind to these dissimilar DAT populations with different affinities. In order to test whether intracellular factor interactions with the DAT N- or C-terminus was related to the DUIP fluctuations, GFP fusion polypeptides of the DAT N- or C-terminal fragments were coexpressed with WT DAT in COS-7 cells. The DAT fragments were intended to serve as “decoys” by intercepting the DAT modulators responsible for the DUIP shifts. Overexpression of the N- or C-terminal decoys did not affect the net dopamine uptake and DUIP of cocaine at DAT significantly (Figure 9 and Table 5). The confocal images provided evidence for overexpression of the decoys in the cells (Figure 11). A decoy comprised of the region of the DAT from the beginning of the N-terminus to the end of the second transmembrane domain that decreased the DAT uptake activity by some unknown mechanism was employed as a positive control (not shown) in all the experiments. Thus, the DAT N- or C-terminal interactions with

intracellular proteins do not appear to be responsible for the DUIP shift in COS-7 cells. The level of expression of a given DAT-modulating protein may depend on the cell type. To test the possibility of DAT termini-protein interactions, another cell system N2A neuroblastoma (that showed the DUIP fluctuation) was employed. This time the DAT surface expression was intentionally manipulated (four-fold) to begin with a cell system that already showed the DUIP fluctuation before assessing the effect of overexpression of the decoys. If protein interactions at the DAT termini are responsible for the fluctuation, the decoy overexpression would shift the DUIP of cocaine at one of the expression levels such that it equals the DUIP at the other expression level. No significant differences were observed in the DUIP of cocaine by overexpression of the decoys. Thus, DAT N- or C-terminal interactions with intracellular proteins do not appear to account for the DUIP shift in N2A cells. Another approach for future studies would be to overexpress a given DAT interacting factor in a DAT-bearing cell system to test for DUIP shifts.

Another possibility for the DUIP fluctuation in the present study could be a change in the phosphorylation state of the DAT. The N-terminal region of the DAT possesses multiple sites for phosphorylation (Foster et al., 2002; Granas et. al 2003; Lin et al., 2003). Deletion of the first 22 N-terminal residues, which included the terminal five serines, abolished detectable hDAT phosphorylation (Granas et al., 2003). Additionally, dopamine uptake in mouse striatum was significantly affected by a wide-spectrum inhibitor of protein kinases (Simon et al., 1997). A change in the phosphorylation state of the DAT may result in the

existence of cell surface DAT in two or more conformations or populations with different phosphorylation states which may affect the ability of cocaine to bind to the transporter. The hDAT mutant, del-20 hDAT, employed in the present study lacks the first 20 amino acids including the five N-terminal serines that have been demonstrated to be the most important residues in DAT phosphorylation. Removal of these serines did not affect the DUIP of cocaine (Table 7). Thus, the N-terminal phosphorylation of DAT, specifically involving the first five serines, is not associated with the DUIP shift. However, other serine and threonine residues are present in the DAT sequence; the role of phosphorylation in creating distinct DAT populations cannot be totally ruled out. Phosphorylation can occur at any of the serine or threonine residues in the DAT that are exposed to the intracellular compartment. To completely assess the role of phosphorylation state of DAT in DUIP fluctuations, DAT mutants with N-terminal truncations lacking the other N-terminal serines and threonines, C-terminal truncations and point mutants at the serines and threonines in the intracellular loops should be employed.

Further studies investigating the occurrence and regulation of the different DAT populations or conformations and their association with the DUIP shifts may help identify the factors influencing the DAT activity, which in turn may identify targets for anti-cocaine and anti-amphetamine therapeutics.

VI. REFERENCES

Bannon MJ (2005) The dopamine transporter: role in neurotoxicity and human disease. *Toxicol Appl Pharmacol* 204:355-360.

Bannon MJ and Whitty CJ (1997) Age-related and regional differences in dopamine transporter mRNA expression in human midbrain. *Neurology* 48:969-977.

Bergman J, Madras BK, Johnson SE, Spealman RD (1989) Effects of cocaine and related drugs in nonhuman primates.III.Self-administration by squirrel monkeys. *J Pharmacol Exp Ther* 251:150-155.

Boja JW, Markham L, Patel A, Uhl G and Kuhar MJ (1992) Expression of a single dopamine transporter cDNA can confer two cocaine binding sites. *Neuroreport* 3:247-248.

Boja JW, Rahman MA, Phillip A, Lewin AH, Carroll FI and Kuhar MJ (1991) Isothiocyanate derivatives of cocaine: irreversible inhibition of ligand binding at the dopamine transporter. *Mol Pharmacol* 39:339-345.

Carneiro AM. Ingram SL et al. (2002) The multiple LIM domain-containing adaptor protein Hic-5 synaptically colocalizes and interacts with the dopamine transporter. *J Neurosci* 22:7045-7054.

Carvelli L, McDonald PW, Blakely RD, Defelice LJ (2004) Dopamine transporters depolarize neurons by a channel mechanism. *Proc Acad Sci USA* 101:16046-16051.

Cervinski MA, Foster JD, Vaughan RA (2005) Psychoactive substrates stimulate dopamine transporter phosphorylation and down-regulation by cocaine-sensitive and protein kinase C-dependent mechanisms. *J Biol Chem* 280:40442-40449.

Chait LD, Uhlenhuth EH, Johanson CE (1987) Reinforcing and subjective effects of several anorectics in normal human volunteers. *J Pharmacol Exp Ther* 242:777-783.

Chen N, Justice JB (2000) Differential effects of structural modification of human dopamine transporter on the inward and outward transport of dopamine. *Brain Res Mol Brain Res* 75:208-215.

Chen N, Reith ME (2003) Na^+ and the substrate permeation pathway in dopamine transporters. *Eur J Pharmacol* 479:213-221.

Chen N, Sun L, Reith ME (2002) Cationic interactions at the human dopamine transporter reveal binding conformations for dopamine distinguishable from those for the cocaine analog 2 alpha-carbomethoxy-3 alpha-(4-fluorophenyl)tropane. *J Neurochem* 81:1383-1393.

Chen N, Zhen J and Reith, MEA (2004) Mutation of Trp84 and Asp313 of the dopamine transporter reveals similar mode of binding interaction for GBR12909 and benztropine as opposed to cocaine. *J Neurochem* 89:853-864.

Chen R, Tilley MR, Wei H, Zhou F, Zhou FM et al. (2006) Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter. *Proc Natl Acad Sci USA* 103:9333-9338.

Chi L, Reith ME (2003) Substrate-induced trafficking of the dopamine transporter in heterologously expressing cells and in rat striatal synaptosomal preparations. *J Pharmacol Exp Ther* 307:729-736.

Daniels GM and Amara SG (1999) Regulated trafficking of the human dopamine transporter. Clathrin-mediated internalization and lysosomal degradation in response to phorbol esters. *J Biol Chem* 274:35794-35801.

Daws LC, Callaghan PD, Moron JA, Kahlig KM, Shippenberg TS, Javitch JA and Galli A (2002) Cocaine increases dopamine uptake and cell surface expression of dopamine transporters. *Biochem Biophys Res Commun* 290:1545-1550.

Deken SL, Beckman ML, Boos L, Quick MW (2000) Transport rates of GABA transporters: regulation by the N-terminal domain and syntaxin 1A. *Nat NeuroSci* 3:998-1003.

Duncan RR, Greaves J, Weigand UK, Matskevich I, Bodammer G, Apps DK, Shipston MJ, Chow RH (2003) Functional and spatial segregation of secretory vesicle pools according according to vesicle age. *Nature* 422:176-180.

Edvardsen O, Dahl SG (1994) A putative model of the dopamine transporter. *Brain Res Mol Brain Res* 27:265-274.

Eshleman AJ, Carmolli M, Cumbay M, Martens CR, Neve KA and Janowsky A (1999) Characteristics of drug interactions with recombinant biogenic amine transporters expressed in the same cell type. *J Pharmacol Exp Ther* 289:877-885.

Eshleman AJ, Henningsen RA, Neve KA, Janowsky A (1994) Release of dopamine via the human transporter. *Mol Pharmacol* 45:312-316.

Foster JD, Pananusorn B and Vaughan RA (2002) Dopamine transporters are phosphorylated on N-terminal serines in rat striatum. *J Biol Chem* 277:25178-25186.

Giros B, Caron MG (1993) Molecular characterization of the dopamine transporter. *Trends Pharmacol Sci* 14:43-49.

Giros B, el Mestikawy S, Godinot N, Zheng K, Han H, Yang-Feng T, Caron MG (1992) Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. *Mol Pharmacol* 42:383-390.

Giros B, Jaber M, Jones SR, Wightman RM and Caron MG (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* 379:606-612.

Gorentla BK, Vaughan RA (2005) Differential effects of dopamine and psychoactive drugs on dopamine transporter phosphorylation and regulation. *Neuropharmacology* 49:759-68.

Gracz LM and Madras BK (1995) [3H]WIN 35,428 ([3H]CFT) binds to multiple charge-states of the solubilized dopamine transporter in primate striatum. *J Pharmacol Exp Ther* 273:1224-1234.

Graham FL and van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.

Granas C, Ferrer J, Loland CJ, Javitch JA and Gether U (2003) N-terminal truncation of the dopamine transporter abolishes phorbol ester- and substance P receptor-stimulated phosphorylation without impairing transporter internalization. *J Biol Chem* 278:4990-5000.

Gulley JM, Doolen S, Zahniser NR (2002) Brief, repeated exposure to substrates down-regulates dopamine transporter function in Xenopus oocytes in vitro and rat striatum in vivo. *J Neurochem* 83:400-411.

Hansra N, Arya S, Quick MW (2004) Intracellular domains of a rat brain GABA transporter that govern transport. *J Neurosci* 24:4082-4087.

Hastrup H, Karlin A, Javitch JA (2001) Symmetrical dimer of the human dopamine transporter revealed by cross-linking Cys-306 at the extracellular end of the sixth transmembrane segment. *Proc Natl Acad Sci USA* 98:10055-10060.

Hastrup H, Sen N and Javitch JA (2003) The human dopamine transporter forms a tetramer in the plasma membrane: cross-linking of a cysteine in the fourth transmembrane segment is sensitive to cocaine analogs. *J Biol Chem* 278:45045-45048.

Hebert MA, Gerhardt GA (1999) Age-related changes in the capacity, rate, and modulation of dopamine uptake within the striatum and nucleus accumbens of Fischer 344 rats: an in vivo electrochemical study. *J Pharmacol Exp Ther* 288:879–87.

Hebert MA, Larson GA, Zahniser NR, Gerhardt GA (1999) Age-related reduction in [3H]WIN 35,428 binding to the dopamine transporter in nigrostriatal and mesolimbic brain regions of the Fischer 344 rat. *J Pharmacol Exp Ther* 288:1334–9.

Hersch SM, Yi H, Heilman CJ, Edwards RH, Levey AI (1997) Subcellular localization and molecular topology of the dopamine transporter in the striatum and substantia nigra. *J Comp Neurol* 388:211-227.

Holton KL, Loder MK and Melikian HE (2005) Nonclassical, distinct endocytic signals dictate constitutive and PKC-regulated neurotransmitter transporter internalization. *Nat Neurosci* 8:881-888.

Horton N and Quick MW (2001) Syntaxin 1A up-regulates GABA transporter expression by subcellular redistribution. *Mol Membr Biol* 18:39-44.

Itokawa M, Lin Z, Cai NS, Wu C, Kitayama S, Wang JB, Uhl GR (2000) Dopamine transporter transmembrane domain polar mutants: ΔG and ΔΔG values implicate regions important for transporter functions. *Mol Pharmacol* 57:1093-1103.

Itokawa M, Lin Z, Uhl GR (2002) Dopamine efflux via wild-type and mutant dopamine transporters: alanine substitution for proline-572 enhances efflux and reduces dependence on extracellular dopamine, sodium and chloride concentrations. *Brain Res Mol Brain Res* 108:71-80.

Ingram SL, Prasad BM, Amara SG (2002) Dopamine transporter-mediated conductances increase excitability of midbrain dopamine neurons. *Nat Neurosci* 5:971-978.

Jiang H, Jiang Q, Feng J (2004) Parkin increases dopamine uptake by enhancing the cell surface expression of dopamine transporter. *J Biol Chem* 279:54380-54386.

Johnson LA, Furman CA, Zhang M, Guptaroy B and Gnegy ME (2005a) Rapid delivery of the dopamine transporter to the plasmalemmal membrane upon amphetamine stimulation. *Neuropharmacology* 49:750-758.

Johnson LA, Guptaroy B, Lund D, Shamban S, Gnegy ME (2005b) Regulation of amphetamine-stimulated dopamine efflux by protein kinase C beta. *J Biol Chem* 280: 10914-10919.

Kahlig KM, Binda F, Khoshbouei H, Blakely RD, McMahon DG, Javitch JA, Galli A (2005) Amphetamine induces dopamine efflux through a dopamine transporter channel. *Proc Natl Acad Sci USA* 102:3495-3500.

Kahlig KM and Galli A (2003) Regulation of dopamine transporter function and plasma membrane expression by dopamine, amphetamine and cocaine. *Eur J Pharmacol* 479:153-158.

Khoshbouei H, Sen N, Guptaroy B, Johnson L, Lund D, Gnegy ME, Galli A, Javitch JA (2004) N-terminal phosphorylation of the dopamine transporter is required for amphetamine-induced efflux. *PLoS Biol* 2:E78.

Kilic F and Rudnick G (2000) Oligomerization of serotonin transporter and its functional consequences. *Proc Natl Sci USA* 97:3106-3111.

Kilty JE, Lorang D, Amara SG (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science* 254:578-579.

Kocabas AM, Rudnick G, Kilic F (2003) Functional consequences of homo- but not hetero-oligomerization between transporters for the biogenic amine neurotransmitters. *J Neurochem* 85:1513-1520.

Kuhar MJ (1992) Molecular pharmacology of cocaine: a dopamine hypothesis and its implications. *Ciba Found Symp* 166:81-89.

Lee SH, Chang MY, Lee KH, Park BS, Lee YS, Chin HR (2000) Importance of valine at position 152 for the substrate transport and 2 beta-carbomethoxy-3-beta-(4-fluorophenyl)tropane binding of dopamine transporter. *Mol Pharmacol* 57:883-889.

Lee FJ, Liu F, Pristupa ZB, Niznik HB (2001) Direct binding and functional coupling of α-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *FASEB J.* 15:916-926.

Lee KH, Kim MY, Kim DH, Lee YS (2004) Syntaxin 1A and receptor for activated C kinase interact with the N-terminal region of human dopamine transporter. *Neurochem Res* 29:1405-1409.

Lew R, Vaughan R, Simantov R, Wilson A, Kuhar MJ (1991) Dopamine transporters in the nucleus accumbens and the striatum have different apparent molecular weights. *Synapse* 8:152-153.

Li LB, Chen N, Ramamoorthy S, Chi L, Cui XN, Wang LC and Reith MEA (2004) The role of *N*-glycosylation in function and surface trafficking of the human dopamine transporter. *J Biol Chem* 279:21012-21020.

Lin Z, Uhl GR (2002) Dopamine transporter mutants with cocaine resistance and normal dopamine uptake provide targets for cocaine antagonism. *Mol Pharmacol* 61:885-891.

Lin Z, Uhl GR (2003) Human dopamine transporter gene variation: effects of protein coding variants V55A and V382A on expression and uptake activities. *Pharmacogenomics* J 3:159-168.

Lin Z, Wang W, Kopajtic T, Revay RS, Uhl GR (1999) Dopamine transporter transmembrane phenylalanine mutations can selectively influence dopamine uptake and cocaine analog recognition. *Mol Pharmacol* 56:434-447.

Lin Z, Itokawa M, Uhl GR (2000a) Dopamine transporter proline mutations influence dopamine uptake, cocaine analog recognition, and expression. *FASEB J* 14:715-728.

Lin Z, Wang W, Uhl GR (2000b) Dopamine transporter tryptophan mutants highlight candidate dopamine and cocaine selective domains. *Mol Pharmacol* 58:1581-1592.

Lin Z, Zhang PW, Zhu X, Melgari JM, Huff R, Spieldoch RL, Uhl GR (2003) Phosphatidylinositol 3-kinase, protein kinase C, and MEK1/2 kinase regulation of dopamine transporters (DAT) requires N-terminal DAT phosphoacceptor sites. *J Biol Chem* 278:20162-20170.

Lis H and Sharon N (1993) Protein glycosylation. Structural and functional aspects. *Eur J Biochem* 218:1-27.

Little KY, Elmer LW, Zhong H, Scheys JO and Zhang L (2002) Cocaine induction of dopamine transporter trafficking to the plasma membrane. *Mol Pharmacol* 61:436-445.

Loland CJ, Norregaard L, Litman T and Gether U (2002) Generation of an activating Zn^{2+} switch in the dopamine transporter: mutation of an intracellular tyrosine constitutively alters the conformational equilibrium of the transporter cycle. *Proc Natl Acad Sci USA* 99:1683-1688.

Madras BK, Spealman RD, Fahey MA, Neumeyer JL, Saha JK and Milius RA (1989) Cocaine receptors labeled by [3H]2 beta-carbomethoxy-3-beta-(4-fluorophenyl)tropane. *Mol Pharmacol* 36:518-524.

Marsden CA (2006) Dopamine: the rewarding years. *Br J Phrarmacol* 147:S136-S144.

Melikian HE and Buckley KM (1999) Membrane trafficking regulates the activity of the human dopamine transporter. *J Neurosci* 19:7699-7710.

Moron JA, Zakharova I, Ferrer JV et al. (2003) Mitogen-activated protein kinase regulates dopamine transporter surface expression and dopamine transport capacity. *J Neurosci* 23:8480-8488.

Newman AH, Kulkarni S (2002) Probes for the dopamine transporter: new leads towards a cocaine-abuse therapeutic-a focus on analogues of benztropine and rimcazole. *Med Res Rev* 22:429-464.

Nirenberg MJ, Chan J, Vaughan RA, Uhl GR, Kuhar MJ, Pickel VM (1997) Immunogold localization of the dopamine transporter: an ultrastructural study of the rat ventral tegmental area. *J Neurosci* 17:5255-5262.

Nirenberg MJ, Vaughan RA, Uhl GR, Kuhar MJ, Pickel VM (1996) The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. *J Neurosci* 16:436-477.

Ozaslan D, Wang S, Ahmed BA, Kocabas AM, McCastlain JC, Bene A, Kilic F (2003) Glycosyl modification facilitates homo- and hetero-oligomerization of the serotonin transporter. A specific role for sialic acid residues. *J Biol Chem* 278:43991-44000.

Pifl C, Drobny H, Reither H, Hornykiewicz O, Singer EA (1995) Mechanism of the dopamine-releasing actions of amphetamine and cocaine: plasmalemmal dopamine transporter versus vesicular monoamine transporter. *Mol Pharmacol* 47:368-373.

Patel A, Uhl GR, Kuhar MJ (1993) Species differences in dopamine transporters: postmortem changes and glycosylation differences. *J Neurochem* 61:496-500.

Patel AP, Cerruti C, Vaughan RA, Kuhar MJ (1994) Developmentally regulated glycosylation of dopamine transporter. *Brain Res Dev Brain Res* 83:53-58.

Pieribone VA, Shupliakov O, Brodin L, Hilfiker-Rothenfluh S, Czernik AJ, Greengard P (1995) Distinct pools of synaptic vesicles in neurotransmitter release. *Nature* 375:493-497.

Pristupa ZB, McConkey F, Liu F, Man HY, Lee FJ, Wang YT, Niznik HB (1998) Protein kinase-mediated bidirectional trafficking and functional regulation of the human dopamine transporter. *Synapse* 30:79-87.

Pristupa ZB, Wilson JM, Hoffman BJ, Kish SJ and Niznik HB (1994) Pharmacological heterogeneity of the cloned and native human dopamine transporter: Dissociation of [^{3}H]WIN 35,428 and [^{3}H]GBR 12,935 binding. *Mol Pharmacol* 45:125-135.

Reith ME and Coffey LL (1994) Structure-activity relationships for cocaine congeners in inhibiting dopamine uptake into rat brain synaptic vesicles and bovine chromaffin granule ghosts. *J Pharmacol Exp Ther* 271:1444-1452.

Reith ME, Xu C, Chen NH (1997) Pharmacology and regulation of the neuronal dopamine transporter. *Eur J Pharmacol* 324:1-10.

Rocha BA, Fumagalli F et al. (1998) Cocaine self-administration in dopamine transporter knockout mice. *Nature Neurosci* 1:132-133.

Rothman RB, Cadet JL, Akunne HC, Silverthorn ML, Baumann MH, Carroll FI, Rice KC, de Costa BR, Partilla JS and Wang JB (1994) Studies of the biogenic amine transporters. IV. Demonstration of a multiplicity of binding sites in rat caudate membranes for the cocaine analog [125I]RTI-55. *J Pharmacol Exp Ther* 270:296-309.

Rudnick G. (1997) Mechanisms of biogenic amine neurotransmitter transporters. In: Neurotransmitter Transporters: Structure, Function, and Regulation, M.E.A. Reith, ed., Humana Press, Totowa, NJ, 73-100.

Salvatore MF, Apparsundaram S and Gerhardt GA (2003) Decreased plasma membrane expression of striatal dopamine transporter in aging. *Neurobiol Aging* 24:1147-1154.

Saunders C, Ferrer JV, Shi L, Chen J, Merrill G, Lamb ME, Leeb-Lundberg LMF, Carvelli L, Javitch JA and Galli A (2000) Amphetamine-induced loss of human dopamine transporter activity: An internalization-dependent and cocaine-sensitive mechanism. *Proc Natl Acad Sci USA* 97:6850-6855.

Schmid JA, Scholze P, Kudlaceko O, Freissmuth M, Singer EA, Sitte HH (2001) Oligomerization of the human serotonin transporter and of the rat GABA transporter 1 visualized by fluorescence resonance energy transfer microscopy in living cells. *J Biol Chem* 276:3805-3810.

Schultz W (2002) Getting formal with dopamine and reward. *Neuron* 36:241-263

Shimizu I and Prasad C (1991) Relationship between [3H]mazindol binding to dopamine uptake sites and [3H]dopamine uptake in rat striatum during aging. *J Neurochem* 56:575-579.

Simon JR, Bare DJ, Ghetti B, Richter JA (1997) A possible role for tyrosine kinases in the regulation of the neuronal dopamine transporter in mouse striatum. *Neurosci Lett* 224:201-205.

Sonders MS, Zhu SJ, Zahniser NR, Kavanaugh MP, Amara SG (1997) Multiple ionic conductances of the human dopamine transporter: the actions of dopamine and psychostimulants. *J Neurosci* 17:960-974.

Sorkina T, Doolen S, Galperin E, Zahniser NR and Sorkin A (2003) Oligomerization of dopamine transporters visualized in living cells by fluorescence resonance energy transfer microscopy. *J Biol Chem* 278:28274-28283.

Sung U, Apparsundaram S, Galli A, Kahlig KM, Savchenko V, Schroeter S, Quick MW, Blakely RD (2003) A regulated interaction of syntaxin 1A with the antidepressant-sensitive norepinephrine transporter establishes catecholamine clearance capacity. *J Neurosci* 23:1697-1709.

Surratt CK, Wang JB, Yuhasz S, Amzel M, Kwon HM, Handler JS, Uhl GR (1993) Sodium- and chloride-dependent transporters in brain, kidney, and gut: lessons from complementary DNA cloning and structure-function studies. *Current opin Nephrol Hypertens* 2:744-760.

Syringas M, Janin F, Mezghanni S, Giros B, Costentin J, Bonnet JJ (2000) Structural domains of chimeric dopamine-noradrenaline human transporters involved in the Na(+)- and Cl(-)-dependence of dopamine transport. *Mol Pharmacol* 58:1404-1411.

Torres GE (2006) The dopamine transporter proteome. *J Neurochem* 97:3-10.

Torres GE, Carneiro A, Seamans K, Fiorentini C, Sweeney A, Yao WD, Caron MG (2003) Oligomerization and trafficking of the human dopamine transporter. Mutational analysis identifies critical domains important for the functional expression of the transporter. *J Biol Chem* 278:2731-2739.

Torres GE, Yao WD, Mohn AR et al. (2001) Functional interaction between monoamine plasma membrane transporters and the synaptic PDZ domain-containing protein PICK1. *Neuron* 30:121-134.

Ukairo OT, Bondi CD, Newman AH, Kulkarni SS, Kozikowski AP, Pan S and Surratt CK (2005) Recognition of benztropine by the dopamine transporter (DAT) differs from that of the classical dopamine uptake inhibitors cocaine,

methylphenidate and mazindol as a function of a DAT transmembrane 1 aspartic acid residue. *J Pharmacol Exp Ther* 314:575-583.

Vandenbergh DJ, Persico AM, Uhl GR (1992) A human dopamine transporter cDNA predicts reduced glycosylation, displays a novel repetitive element and provides racially-dimorphic TaqI RFLPs. *Brain Res Mol Brain Res* 15:161-166.

Venton BJ, Seipel AT, Phillips PE, Wetsel WC, Gitler D, Greengard P, Augustine GJ, Wightman RM (2006) Cocaine increases dopamine release by mobilization of a synapsin-dependent reserve pool. *J Neurosci* 26:3206-3209.

Volz TJ and Schenk JO (2005) A comprehensive atlas of the topography of functional groups of the dopamine transporter. *Synapse* 58:72-94.

Wang W, Sonders MS, Ukairo OT, Scott H, Kloetzel MK and Surratt CK (2003) Dissociation of high-affinity cocaine analog binding and dopamine uptake inhibition at the dopamine transporter. *Mol Pharmacol* 64:430-439.

Wise RA (1996) Neurobiology of addiction. *Curr Opin Neurobiol* 6:243-251.

Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E (2005) Crystal structure of a bacterial homologue of Na^+/Cl^--dependent neurotransmitter transporters. *Nature* 437:215-223.

Zahniser NR, Sorkin A (2004) Rapid regulation of the dopamine transporter: role in stimulant addiction? *Neuropharmacology* 47:80-91.