Fall 2005

Structural Determinants for Substrate and Inhibitor Recognition by the Dopamine Transporter

Okechukwu T. Ukairo

STRUCTURAL DETERMINANTS FOR SUBSTRATE AND INHIBITOR RECOGNITION BY THE DOPAMINE TRANSPORTER

A Dissertation

Presented to the Graduate School of Pharmaceutical Sciences

Of

Duquesne University

In Partial Fulfillment of the

Requirements for the Degree

Of

Doctor of Philosophy

Pharmacology and Toxicology

By

Okechukwu T. Ukairo

December 9, 2005

COPYRIGHT NOTICE

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Chris Surratt, my thesis advisor, for his years of support and guidance. Dr. Surratt has been a great mentor and I've become a better scientist under his guidance.

I am also deeply grateful to members of my dissertation committee, Drs. Paula Witt-Enderby, Wilson Meng, David Johnson and John Pollock for their invaluable suggestions and continued encouragement throughout the course of my research. I am particularly indebted to Dr Pollock for his help with confocal microscopy experiments. Dr. Pollock was unstinting in his help and I have constantly drawn from his expert knowledge and good advice. I extend special thanks to Dr. Simon Watkins of the University of Pittsburgh, PA for allowing me the use of his laboratory facilities. I gratefully acknowledge the help I received from Suneetha Ramanujapuram, Corry Bondi, Stephen Pan, and Mike Schmidt in carrying out some of the experiments. Drs. Doug Bricker, Fred Fotchman, Thomas Mattei, and Wilson Meng have been a source of good counsel and encouragement and I am deeply grateful to them. My sojourn at Duquesne University has been a wonderful and rewarding experience due to the love and support of great friends such as Nick and Lisa Radio, Dr. Viji Pokala, and Jeff Kovacs. They continuously challenged me in their respective ways to become a better and more focused graduate student.

My sincere and heartfelt thanks to my wife, Stella, and our kids, Lulu and Toby, for their selfless understanding and patience and without whom all these wouldn't have been possible.

TABLE OF CONTENTS **PAGES**

LIST OF TABLES **PAGES**

LIST OF FIGURES **PAGES**

LIST OF ABBREVIATIONS

AMPH= Amphetamine

BZT= Benztropine

DA= Dopamine

DAT= Dopamine transporter

DUIP= Dopamine uptake inhibition potency

MAZ= Mazindol

NET= Norepinephrine transporter

NSS= Neurotransmitter: sodium symporter

NUIP= Norepinephrine uptake inhibition potency

SERT= Serotonin transporter

I. INTRODUCTION

A. Overview

Dopamine, a major neurotransmitter in the mammalian central nervous system, is involved in the control of locomotor activity, and also in pathways regulating behavior and reward (Schultz, 2002). The dopamine transporter (DAT) plays a critical role in the synaptic clearance of dopamine by mediating the reuptake of dopamine from the extracellular space. Following the invasion of the nerve terminal by an action potential (in dopaminergic neurons), dopamine is released into the synaptic cleft by Ca^{2+}-mediated exocytosis. The released dopamine binds to receptors on the postsynaptic membrane and activates dopaminergic signal transduction pathways. The neurotransmission of dopamine is terminated primarily by reuptake of dopamine into the presynaptic cell via the dopamine transporter (Fig. 1). Thus, DAT is, in part, responsible for the maintenance of dopaminergic homeostasis in neuronal cells. The dopamine transporter belongs to a large class of transporters referred to as Na^+/Cl^--dependent transporter or neurotransmitter: sodium symporter (NSS) family. Members of this class include: the serotonin transporter (SERT), norepinephrine transporter (NET), epinephrine transporter (ET), and also transporters for betaine, creatine, GABA, glycine, proline, and taurine (Amara and Arriza, 1993; Amara and Kuhar, 1993). For these transporters, the reuptake process involves the translocation of substrate and cosubstrates Na^+ and Cl^- across the plasma membrane. This transport process also involves a conformational change in the

transporter protein (Rudnick and Clark, 1993). In the case of DAT, the outward-facing transporter binds one Cl^- and two Na^+ ions and one dopamine molecule. These binding events permit the transporter to undergo a conformational change to an inward-facing conformation. This internal-facing form dissociates Na^+, Cl^-, and dopamine to the cytoplasm. On binding internal K^+, the transporter undergoes another conformational change (to an outward-facing conformation) which allows the carrier to dissociate K^+ on the cell exterior. Consequently, the original form of the transporter is generated which can then initiate another round of transport by binding external Na^+, Cl^-, and dopamine.

DAT is expressed almost exclusively in dopaminergic neurons of the substantia nigra and the ventral tegmental area of the brain (Ciliax et al., 1995; Freed et al., 1995). The areas of the brain where dopaminergic neurons are found to project include the striatum, nucleus accumbens and the prefrontal cortex. DAT is also expressed throughout the cell on axons, dendrites and the soma (Nirenberg et al., 1997).

DAT is the primary target of psychostimulant drugs including methylphenidate, cocaine, and amphetamines; such drugs bind to DAT and inhibit the reuptake of dopamine (Kuhar et al., 1991). Thus, a convincing clinical rationale exists for understanding the structure and function of this transporter protein. In addition to inhibiting uptake of extracellular dopamine, amphetamines also stimulate efflux of intracellular dopamine (Jones et al., 1998). The resultant effect of psychostimulant drug action on DAT is increased accumulation of dopamine in the synaptic cleft causing potentiated neurotransmission of

dopamine in those areas of the brain associated with reward and reinforcement. Consequently, the motor and reward pathways of the midbrain are activated, triggering the increased locomotor activity and euphoria associated with psychostimulant drug use.

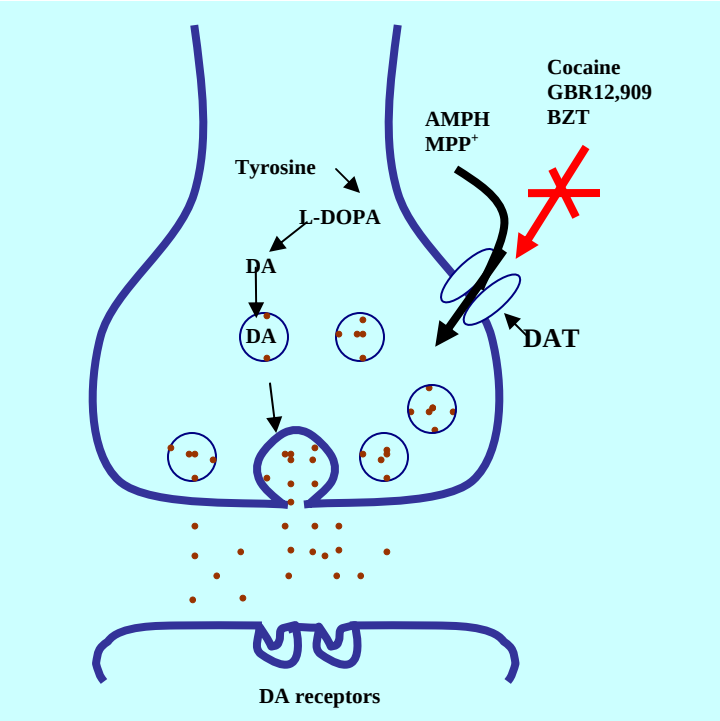


Figure 1. Schematic representation of dopamine synaptic terminal.
DAT is localized to presynaptic sites where it is vital for the termination of dopaminergic neurotransmission and maintenance of presynatic dopamine storage. DA, dopamine; AMPH, amphetamine; DAT, Dopamine transporter; BZT, benztropine.

Experiments using mice lacking the dopamine transporter have demonstrated the importance of the transporter in psychostimulant action (Giros et al., 1996). High doses of cocaine did not increase locomotor activity above the already elevated levels in these DAT knock-out mice. Nonetheless, it is pertinent to note that not all the behavioral effects of cocaine can be ascribed solely to DAT. Subsequent studies evaluating both cocaine self-administration (Rocha et

al., 1998) and conditioned place preference (Sora et al., 1998) in DAT knock-out mice showed that cocaine self-administration and conditioned place preference were intact. Mice with a double disruption of both dopamine and serotonin transporter genes exhibited no conditioned place preference in response to cocaine. The serotonin transporter may contribute to cocaine reward and reinforcement naturally (Sora et al., 2001) or in compensation for the absence of DAT.

B. Statement of the problem

Cocaine abuse is growing at an alarming rate in the U.S. It is estimated that more than 23 million people in the U.S. have tried cocaine, nearly 40,000 people use it daily, and 5,000 new users are added each day (SAMHSA, 1997). Drug abuse generally inflicts severe medical, social, and judicial costs upon the society. The central problem with cocaine addiction is that even after treatment and extended periods of abstinence the risk of relapse is high. The primary feature of cocaine addiction is the compulsive seeking of the drug that results in failure to resist taking the drug. The abuse liabilities of cocaine result from its euphorigenic and reinforcing properties. Neurochemical studies on effects of cocaine show that the mesolimbic and mesocortical dopamine systems of the brain are involved in psycholocomotor stimulant reward function. The behavioral profile that is associated with cocaine addiction results from the accumulation of dopamine in the synapse and its actions at one or more of the D1- D5 dopamine receptors. Thus, the reinforcing property of cocaine is caused by potentiated

neurotransmission of dopamine in mesolimbic pathways. Cocaine binds to the dopamine transporter and inhibits dopamine reuptake leading to a distortion in dopamine homeostasis. No definitive information exists on the conformation of neurotransmitter transporter proteins, as no crystallographic or high resolution data are available. At present the binding sites for dopamine or cocaine on DAT have not been well elucidated. Using site-directed mutagenesis and chimeric studies, investigators have identified certain amino acid residues in the primary structure of the dopamine transporter that are important for substrate and/or inhibitor binding to DAT. Of the several DAT mutants published so far, aspartate-79 (D79) of the rat DAT is still the only residue whose mutation caused an increase in both K_m for dopamine uptake and K_i for dopamine to compete for the cocaine analog [^{3}H]WIN35,428 binding site (Kitayama et al., 1992). This suggested a critical role for D79 in DAT function. In a separate study, Barker and coworkers (1999) showed that in both human norepinephrine and rat serotonin transporters, the replacement of the aligned aspartates with uncharged amino acids resulted in the disruption of substrate transport. For the reason that dopamine and cocaine possess a protonated nitrogen atom at physiological pH, it was postulated that the positively charged amino group of dopamine or protonated nitrogen of cocaine directly interacts with the negatively charged carboxylate of D79 and that ionic competition between cocaine and dopamine is the mechanism of cocaine inhibition of dopamine uptake (Carroll et al., 1992). Several lines of evidence, however, directly contradict this hypothesis. In their studies with neutral 8-oxa analogs of WIN35, 428 and cocaine, Madras et al.

(1996) and Kozikowski et al. (1999) have proven that both compounds are quite potent inhibitors of dopamine transport by DAT relative to the charged, nitrogen-based parent compounds. Nevertheless, the D79 residue appears to be crucial to DAT function. The D79 residue is one of two charged residues that reside in the hydrophobic lipid bilayer. Furthermore, amino acid sequence alignment of transmembrane domain 1 from various members of the NSS transporter gene family, which the DAT belongs to, shows that an aspartic acid at this position is conserved only in those members whose substrates have an aromatic moiety. It is likely that the role of the D79 side chain may be to contribute to an aromatic binding pocket for DAT ligands. Therefore, the primary focus of this study was to determine whether D79 was involved in the recognition of some feature of the DAT substrate or inhibitor structure. The specific hypothesis to be tested here is that the D79 residue is pivotal to DAT function and that the side chain of this residue may form intramolecular contacts that supported an aromatic binding pocket.

In the course of this study using mammalian cell lines stably-transfected with WT DAT cDNAs, it was observed that the dopamine uptake inhibition potency (DUIP) of a given inhibitor will fluctuate even though the apparent binding affinity of the drug remained static. The nature of this phenomenon was investigated by examining three possible influences on DAT function at the level of the cultured cell: the age of the cell line (measured by cell passage number), the density of the cell monolayer (*i.e.,* percent confluence), and the effect of varying DAT expression level by manipulation of transfection conditions. The

specific hypothesis to be tested in this case is that the DUIP of DAT inhibitors in CHO cells stably transfected with WT DAT fluctuate as a function of cell state.

C. Literature Survey

1. Structure of the Dopamine Transporter

The human DAT contains 620 amino acids in its primary sequence. As mentioned earlier, the three-dimensional structure of DAT, or any other member of the family of mammalian neurotransmitter transporters, is unknown due to the absence of X-ray crystallographic or high-resolution structural information. Nevertheless, the topological assignment of the dopamine transporter has been made possible by the use of analytical techniques such as substituted cysteine accessibility method (SCAM), site-directed mutagenesis, and hydrophobicity studies. Hydrophobicity analysis of the primary sequences of mammalian monoamine transporters indicates that monoamine transporters are polytopic membrane proteins containing 12 transmembrane domains connected by alternating extracellular and intracellular loops with the N- and C-termini located in the cytosol (Fig. 2). The cytoplasmic location of N-terminal and the extracellular location of the sequence between transmembrane (TM) domain 3 and 4 of DAT has been demonstrated (Nirenberg et al., 1996). In addition, the topological assignments of four cysteine residues have been verified using SCAM analysis (Ferrer and Javitch, 1998). C90 and C306 are located extracellularly, and C135 and C342 appear to be intracellular (Ferrer and Javitch, 1998). Furthermore, three residues (two histidines and on glutamate) predicted

to be on the extracellular face of the human dopamine transporter, H193 (located in extracellular loop 2), H375 (located in TM 7), and E396 (TM 8), have been shown to constitute three coordinates of an endogenous Zn^{2+}-binding site. The participation of these residues in binding the small Zn^{2+} ion implies a nearness of extracellular loop 2, TM 7, and TM 8 in the tertiary structure of the human DAT (Norregaard et al., 1998; Loland et al., 1999). DAT is a heavily glycosylated protein with an apparent molecular mass of ~80 kDa as determined by gel electrophoresis. The N-deglycosylation of mature DAT reduced the molecular mass to ~50 kDa (Patel, 1997). The bulk of cloned dopamine transporters have at least three consensus sites for N-linked glycosylation in the second extracellular loop located between TMs 3 and 4. Mutational studies have shown that this glycosylation is very important for the activity and stability of the transporters at the surface, but does not seem to be important for the regulation of the intrinsic transport activity (Torres et al., 2003). The stability and targeting of the human norepinephrine transporter to the cell surface largely depend on glycosylation of the transporter at the consensus sites (Melikian et al., 1996). This effect may also be applicable to the dopamine transporter protein.

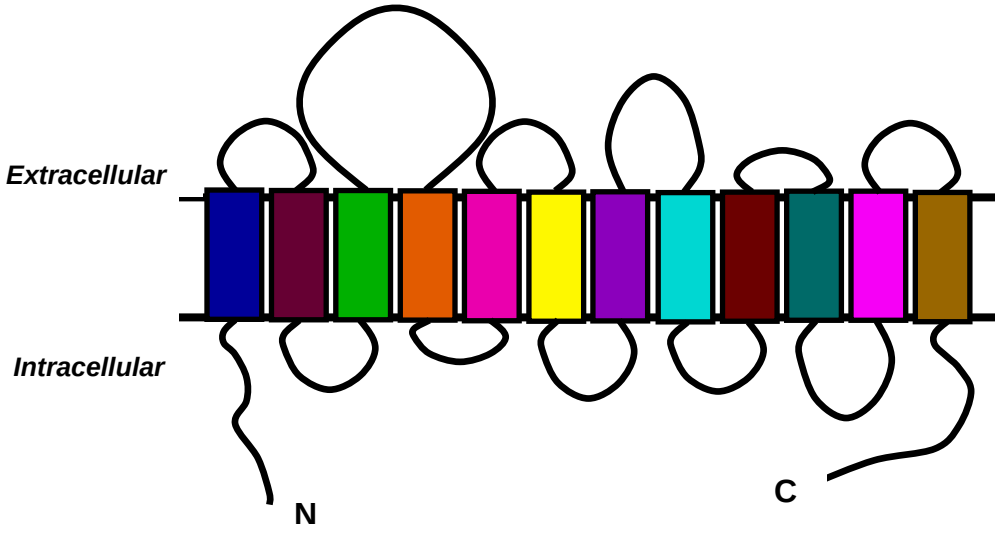


Figure 2. Schematic illustration of plasmalemmal monoamine transporters topology.

Two cysteines (C180 and C189) in the second extracellular loop are predicted to form a disulfide bridge important to DAT cell surface expression (Wang et al., 1995). Substitutions of these cysteines severely decreased the expression of the mutant transporters. Immunocytochemistry data showed that COS cells transfected with C180A and C189A mutant DAT displayed reduced membrane staining and prominent staining in perinuclear regions consistent with Golgi apparatus (Wang et al., 1995). These results suggest that cysteines in the DAT second extracellular loop may provide sulfide residues crucial to full transporter expression.

DAT is phosphorylated in stably expressing cells and striatal synaptosomes (Vaughan et al., 1997). Protein kinase C-mediated DAT phosphorylation (Vaughan et al., 1997) causes internalization of the transporter

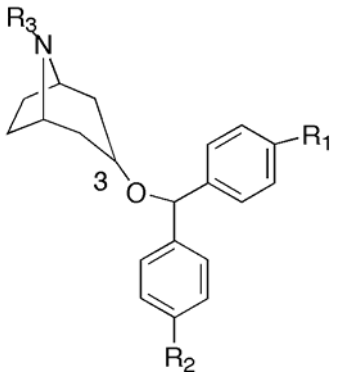
protein (Pristupa et al., 1998; Melikian and Buckley, 1999) and down-regulation of transporter activity (Kitayama et al., 1994; Vaughan et al., 1997; Zhang et al., 1997). There are several DAT consensus sites for protein kinase phosphorylation (Giros et al., 1992); nonetheless, DAT function does not appear to be regulated by phosphorylation at these sites. Deletion of all possible protein kinase C sites did not affect the ability of protein kinase C activators or inhibitors to regulate dopamine transport (Granas et al., 2003). It is possible, therefore, that direct dopamine transporter phosphorylation might occur at nonconsensus phosphorylation sites. It is also likely that DAT phosphorylation is considerably influenced by ligand occupancy.

2. Substrate and Inhibitor Selectivity of the DAT

DAT cloning and characterization has been achieved for several species (Giros et al., 1991, 1992; Miller et al., 2001; Jayanthi et al., 1998; Kilty et al., 1991; Usdin et al., 1991; Porzgen et al., 2001; Gallant et al., 2003; Shimada et al., 1991). The DAT has a well-defined and distinctive pharmacological profile. Dopamine uptake by DAT is inhibited by a variety of compounds including the psychostimulants cocaine, mazindol, and methylphenidate, and a few selective compounds that include the GBR compounds 12909 and 12935 and the muscarinic antagonist benztropine. In addition to clearing dopamine, DAT can transport amphetamines and the dopaminergic neurotoxin 1-methyl-4-phenylpyridinium ion (MPP+), which triggers a Parkinson's disease-like syndrome. A brief description of selected DAT inhibitors is given below.

Benztropines

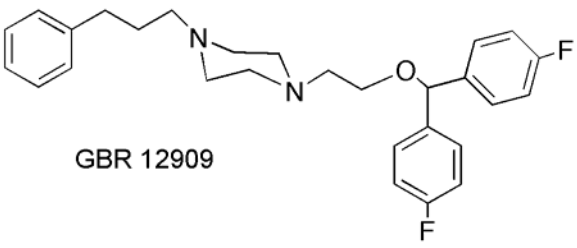
Benztropine (3α-diphenylmethoxytropane, cogentin1) is a tropane-based dopamine uptake inhibitor. It is an anticholinergic agent which is used for the treatment of symptoms associated with Parkinson's disease. Benztropine is different from cocaine by possessing a diphenylmethoxy group attached to the C-3 position of the tropane ring. Para-substitution of a halogen-group on one of the phenyl rings of benztropine augments its potency for inhibition of dopamine uptake. It has been shown that optimal binding affinity at the DAT was accomplished when small halogens such as fluorine and chlorine were placed in the para- and/or meta- positions of one or both phenyl rings. This modification caused a diminution in potency for inhibition of serotonin and norepinepherine uptake, resulting in a considerably more dopamine-selective compound than the parent benztropine. The following structure–activity relationships for the benztropines have been established: the diphenyl ether must be in the α-stereochemistry and both aryl rings are required and must be free to rotate. Assessment of benztropine analogs in animal models of cocaine abuse demonstrated that regardless of binding to DAT with high affinity and exhibiting potent inhibition of dopamine uptake, *in vitro*, these compounds did not demonstrate a cocaine-like behavioral profile (for a review of the structure-activity studies of benztropine analogs, see Newman and Kulkarni, 2002).



Benztropine

GBR Compounds

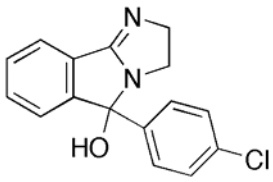
GBR compounds (aryl 1,4-dialkyl piperazines) were modeled after benztropine. The key structural dissimilarity between benztropine and GBR compounds is that the GBR compounds have a piperazine ring in place of the tropane nucleus present in benztropine. One of the most potent compounds in this group is GBR 12909. In contrast with cocaine, GBR 12909 is not a central nervous system stimulant. The mechanism of action of GBR 12909 is identical to that of cocaine. GBR 12909 binds to DAT and inhibit dopamine uptake. GBR 12909 differs from cocaine in that it produces a relatively moderate and long-lasting increase in dopamine, which does not produce the same degree of euphoria compared to the effect of cocaine.



GBR 12909

Mazindol

Mazindol is a dopamine uptake inhibitor with very low abuse liability. Currently, mazindol, 5-(4-chlorophenyl)-2,3-dihydro-5-hydroxy-5*H*-imidazo[2,1-*a*]isoindole, is marketed in the United States as an anorexic agent (exogenous obesity) and as an orphan drug for the treatment of Duchenne muscular dystrophy. It is an effective inhibitor of uptake and binding at DAT, NET and SERT sites. It blocks the uptake of dopamine and also inhibits [^{3}H]cocaine and [^{3}H]WIN 35,428 binding.

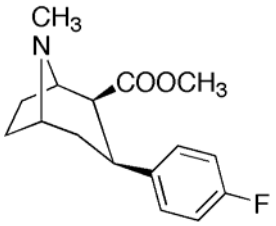


Mazindol

WIN-type of compounds

The key structural feature of phenyltropanes (WIN-type of compounds) is that they lack the 3α-benzoyl ester function group present in cocaine. The phenyl ring is directly attached to the tropane ring. The phenyltropanes were first made with the intention of obtaining an effective stimulant or antidepressant with diminished toxicity. These compounds bind to DAT and block dopamine uptake. WIN35,428 is the prototype compound in this class. Inhibition of [^{3}H]WIN35,428 binding to the DAT is the common radioligand used to determine ligand binding

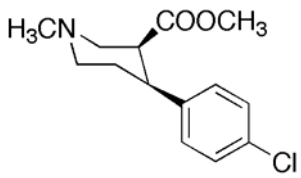
affinity due to sufficient specificity to DAT. [^{3}H]WIN35,428 labels both the high- and low-affinity binding sites of DAT, just like cocaine, and is resistant to metabolic and chemical degradation. It also has a high signal-to-noise ratio.



WIN 35,428

4-ARA-127

4-ARA-127 is a piperidine-based ligand that has been fast-tracked by NIDA's Medication Development Group for use as a substitute medication for cocaine dependence. This compound displays the characteristics of a partial agonist at the dopamine transporter. The most important pharmacological point of distinction between this compound and cocaine is its low activity at the serotonin transporter (with a slight action at other receptor systems as found through the NIMH screening program). In primate studies, it was established that this piperidine-based ligand was more weakly reinforcing than cocaine.



4-ARA-127

3. Structure-Function Analysis of the Dopamine Transporter

a. Transmembrane (TM) domains.

TM 1

A TM 1 aspartic acid residue common to the plasma membrane monoamine transporters but not shared by other neurotransmitter: sodium symporter (NSS) family members is the side chain most frequently proposed to directly contact both inhibitors and substrates of the dopamine transporter. Of all the several DAT mutants published so far, aspartate-79 (D79) of the rDAT is the only residue whose mutation (to glutamate) was reported to cause an increase in both K_m for dopamine uptake, and an increase in K_i for dopamine in competing for the cocaine analog [^{3}H]WIN35,428 binding site (Kitayama et al., 1992). In contrast, recent findings with the same D79E DAT mutant showed no effect on dopamine affinity when compared with WT values. Three-fold losses in cocaine, WIN 35,428, mazindol and methylphenidate affinities were observed, with little or no effect on the dopamine uptake inhibition potency (DUIP) for these drugs

(Wang et al., 2003). On the other hand, binding affinities and DUIPs for benztropine and its analogs were altered substantially by the D79E mutation (Ukairo et al., 2005). Barker et al. (1999) showed that for the human norepinephrine transporter (NET) and rat serotonin transporter (SERT), the replacement of the analogous aspartate residue with uncharged amino acids resulted in the disruption of substrate transport. These findings led investigators to believe that the positively charged amino group of dopamine directly interacts with the negatively charged carboxylate of D79, an approach based on the G protein-coupled receptor model of a salt bridge between the positively charged agonist amino group and negatively charged TM domain carboxylate side chain (Strader et al., 1988). Consequently, it has been postulated that ionic competition between cocaine and dopamine is the mechanism by which cocaine inhibited dopamine uptake (Carroll et al., 1992). The neutral “8-oxa” analogs of WIN35,428 and cocaine still inhibit DAT, albeit less potently than the charged, nitrogen-based parent compounds (Madras et al., 1996 and Kozikowski et al., 1999). Thus, the idea that D79 of the plasma membrane dopamine transporter is a counter ion for the positively charged substrate amino group is speculative. A glycine side chain is found at the analogous position in the other NSS family members including transporters for GABA, betaine, glycine and proline. The substrates for these transporters share with the monoamines the positively charged amino group but lack aromatic groups (Wang et al., 2003). The presence of an aspartic acid side chain at this position in only those transporters recognizing aromatic substrates may indicate that the aspartic acid residue

serves as a ‘strut’ supporting an aromatic binding site for the ligand (Ukairo et al., 2005). Recent data were not in agreement with, but did not rule out, formation of a salt bridge between aspartate and either dopamine, cocaine or amphetamine (Wang et al., 2003; Ukairo et al., 2005). The aspartate (D75) in the analogous position in NET was intolerant to mutation, and like the DAT, only glutamate substitution of D98 yielded a functional SERT (Barker et al., 1999). In an effort to find DAT TM residues capable of directly interacting with the positively charged moieties of substrates and inhibitors, conserved acidic and tryptophan hDAT residues were separately mutated and the mutant transporters characterized, including the TM 1 residues D68 and W84 (Chen et al., 2001). The mutation of D68 to asparagine (D68N) resulted in 3 - 4 fold losses in affinity for WIN 35,428 and in cocaine DUIP (Chen et al., 2001). The mutation did not appreciably affect recognition of the classic DAT inhibitor GBR-12909 or most of the hydroxypiperidine GBR-like analogs tested; however, one such analog, (+)-*R,R*-D-84, sustained a 17-fold affinity loss. (+)-*R,R*-D-84 differs from one of the analogs unaffected by the mutation only in the position of a hydroxyl group. Thus, it is possible that DAT interacts with GBR analogs by way of hydrogen bonding between its D68 residue and the hydroxyl groups of these compounds (Zhen et al., 2004).

Human (W84L) and rat (W84A) DAT substitutions of this tryptophan residue increased WIN 35,428 affinity and cocaine DUIP without affecting dopamine K_m values (Chen et al., 2001; Lin et al., 2000). It is envisaged that W84 may contribute to maintaining an intracellular-facing DAT conformation

(Chen et al., 2001). The Na^+-dependent conformational changes required for DAT function were impaired in hDAT W84L (Chen et al., 2004). This mutant also displayed Na^+ sensitivity differences between cocaine and the diphenylmethoxy-bearing compounds benztropine and GBR-12,909. Taken together with the aforementioned rDAT D79E results, the hDAT W84L findings suggest that TM 1 residues may provide discrimination between diphenylmethoxy-bearing compounds and classic inhibitors such as cocaine, WIN 35,428 and mazindol. Moreover, of several endogenous hDAT cysteine residues surveyed for accessibility to the methanethiosulfonate alkylating agent MTSET, the benztropine-induced DAT alkylation pattern deviated from those of cocaine, WIN 35,428, mazindol and dopamine only at C90, a residue immediately extracellular to TM 1 (Reith et al., 2001). It is unclear whether these TM 1-associated inhibitor selectivities are solely due to DAT conformational differences or are indicative of TM 1 contributions to inhibitor binding sites.

TM 2

Mutation of rDAT F98 to alanine (F98A), a residue presumed to be located at the TM 2 extracellular boundary, decreased WIN 35,428 binding 6-fold. The mutation had no effect on the dopamine uptake affinity, but dopamine turnover rate was significantly diminished (Lin et al., 1999). F98 is largely conserved in the NSS family and possibly less likely to directly contact specific uptake inhibitors. A mouse/*Drosophila* DAT chimera study led to identification of mDAT F105 as the residue primarily responsible for the 10-fold higher DUIP of cocaine

at the mDAT; this position is occupied by methionine in the dDAT. Of several mutations tested, only the presence of an aromatic side chain at mDAT position 105 retained wildtype-like DUIPs for cocaine. Substitutions of F105 with nonaromatic amino acids alanine, serine, threonine, leucine, isoleucine, asparagine, and glutamine resulted in mutants with diminished cocaine binding affinity. Whether the effect of nonaromatic substitution of mDAT F105 on cocaine DUIP was direct or indirect was not investigated (Wu and Gu, 2003). Intriguingly, WIN 35,428 affinity at F105A mutant rDAT decreased by only 2-fold relative to wildtype rDAT (Lin et al., 1999). In contrast with the observation made in the studies of DAT TM2, substituted cysteine accessibility method (SCAM) analysis of rSERT TM 2 did not identify residues that directly affected substrate binding or were accessible to alkylating agents (Sato et al., 2004). Most recently, random mutagenesis of mDAT TM 2 residues in the vicinity of F105 generated the triple mutant L104V/F105C/A109V, which suffered 69- and 47-fold DUIP losses for cocaine and methylphenidate. The DUIPs for the substrates amphetamine and methamphetamine at the triple mutant were not significantly different from those at the wildtype mDAT (Chen et al., 2005).

TM 3

A region of the dopamine transporter surrounding the 3rd transmembrane domain is considered to be vital for cocaine recognition. Using human/bovine DAT chimeras a 54 residue segment encompassing TM 3 was identified as especially crucial for dopamine uptake and WIN 35,428 binding (Lee et al.,

1998). Remarkably, replacement of the bDAT TM 3 residue I152 with its conservative valine counterpart in the hDAT was found to almost single-handedly confer the superior substrate transport and WIN 35,428 binding characteristics of the hDAT (Lee et al., 2000). Two positions away, FI54 in TM 3 also appears to be a cocaine-selective residue in DAT. Mutation of F154 to alanine (F154A) lowered cocaine affinity by about 10-fold while retaining normal dopamine uptake activity (Lin and Uhl, 2002). The F154A mutation also altered cocaine stereoselectivity. V152 and F154 should be located on opposite faces of DAT TM 3, implying that both cannot directly contact the ligand. SCAM analysis of TM 3 of rSERT indicated that I172, the residue analogous to hDAT V152, is on the helical face accessible to ligands and external agents (Chen et al., 1997). Moreover, I172 and Y176 of rSERT are in or near the binding sites for serotonin and cocaine (Chen et al., 1997; Chen and Rudnick, 2000). Assuming that TM 3s of the DAT and SERT have comparable orientations in the plasma membrane, V152 would be expected to face the ligand pore and F154 would face the lipid bilayer. F155 of DAT could still face the ligand pore, a residue conserved among DATs but replaced by tyrosine in SERTs and NETs. The rDAT F155A mutant sustained a profound loss in apparent affinity for dopamine, but only a mild decrease in WIN 35,428 affinity (Lin et al., 1999).

TM 5

Replacement of W267 hDAT with leucine forms a mutant DAT with a 3-fold decrease in cocaine DUIP when compared with WT hDAT (Chen et al.,

2001). Uptake kinetics suggest that W267 contributes to an outward-(extracellular-) facing DAT conformation (Chen et al., 2001). This residue is presumed to border the cytoplasm. Glycine replacement of rDAT P272 modestly reduced Vmax for dopamine uptake, but decreased WIN 35,428 binding affinity 10-fold without a reduction in rDAT plasma membrane expression. DUIPs for cocaine, BTCP (1-[1-(2-benzo[b]thiopheneyl)cyclohexyl]piperidine hydrochloride), mazindol and trihexyphenidyl decreased by over 100-fold (Kopajtic, 1997). A subsequent study characterized rDAT P272A mutant in uptake and binding studies. The alanine mutant decreased the affinities for both dopamine and WIN 35,428 binding relative to wildtype rDAT (Lin et al., 2000). Alanine replacement of the analogous hNET residue P270 yielded undetectable specific binding of nisoxetine and 11-, 3- and 3-fold decreases in the norepinephrine uptake inhibition potencies (NUIPs) of nisoxetine, desipramine and cocaine, respectively. Of 10 hNET proline residues mutated, only P270A decreased recognition of uptake inhibitors by 3-fold or more (Paczkowski and Bryan-Lluka, 2004). The number of TM proline residues is observed to be inexplicably large in transport proteins when compared with other integral membrane proteins, yet it is not clear how such TM proline residues affect transporter protein structure and function (Williams and Deber, 1991; Brandl and Deber, 1996). In general, it has been postulated that proline residues disrupt α-helices, whereas alanine residues promote α-helix formation (Barlow and Thornton, 1988). The extent of the α-helical "kink" induced by proline, however, is reliant on its environment (Li et al., 1996), and especially on neighboring residues (Ri et al., 1999; Visiers et al.,

2003). Thus, proline residues may serve a structural role in determining protein infrastructure by influencing helix-helix packing. Functional roles for TM proline residues may include providing hinges that facilitate signal transduction, mediating conformational changes via *cis-trans* isomerization of the bond linking the proline to the preceding residue of the polypeptide, and providing a geometry that allows neighboring amide carbonyl oxygen atoms of the polypeptide to serve as cation binding sites (Sansom et al., 2000; Eisenman and Dani, 1987; Sansom, 1992). The latter functional role is most likely for the monoamine transporters. Thus, P272 may provide a direct ligand binding site, a key Na^+ binding site that modulates transport or substrate or inhibitor recognition, or simply a kink necessary to the ligand or ion binding pocket.

TM 6

Mutation of hDAT W311 to leucine (W311L) decreased WIN 35,428 affinity 10-fold and cocaine DUIP over 3-fold while dopamine inhibition of WIN 35,428 binding decreased by over 100-fold (Chen et al., 2001). This mutation had no effect on the plasma membrane expression of hDAT. Conversely, alanine replacement of the rDAT counterpart (W310A) increased WIN 35,428 affinity 4-fold, and dopamine displacement of the cocaine analog was over 200 times more effective (Lin et al., 2000). The rDAT W310A mutation caused decreased plasma membrane expression, increased Km and diminished Vmax for dopamine uptake. The discrepancy in the data obtained for hDAT and rDAT could be because the rDAT binding was conducted at 4^{o}C, compared to 37^{o}C in

the hDAT study (Chen et al., 2001). Two residues away, the hDAT D313N mutant did not markedly affect WIN 35,428 or cocaine binding under normal assay conditions, and dopamine affinity was diminished (Chen et al., 2001). Although it is unlikely to be part of the substrate or inhibitor binding sites, D313 may nevertheless regulate access to external dopamine in a Na^+-dependent fashion. This residue and W84 have been shown to be involved in cation interactions, and controls, in part, the ability of Na^+ to drive the DAT between inward- and outward-facing conformations, in turn influencing dopamine access and Na^+-dependent cocaine affinity (Chen et al., 2002; Chen and Reith, 2003).

TM 7

Simultaneous mutation of the rDAT S356 and S359 residues to glycine or alanine resulted in reductions in WIN 35,428 binding affinity and decreases in dopamine uptake. These residues are hypothesized to form hydrogen bonding interactions with the catechol hydroxyl groups of dopamine (Kitayama et al., 1992). This model was borrowed from that of Strader and colleagues, who demonstrated an association between α-adrenergic receptor TM serine residues and agonist hydroxyl groups (Strader et al., 1989). The fact that a serine side chain is found at this same position throughout the NSS family, including transporters for non-catechol substrates, argues against this model.

Finally, alanine replacement of rDAT F361 decreased WIN 35,428 binding affinity by an order of magnitude without affecting dopamine uptake kinetics (Lin

et al., 1999). Positioned at the midpoint of TM 7, this side chain is largely conserved in the NSS family.

TM 8

For the rDAT, alanine substitution of W406 (W406A) decreased WIN 35,428 binding affinity by 3-fold; decreased V_{max} for dopamine uptake by ~10-fold and increased dopamine uptake and binding affinities. DAT plasma membrane expression was altered in this mutant (Lin et al., 2000). This residue is not far from the extracellular boundary and is largely conserved in the NSS family, although a phenylalanine side chain is found at the same position in the SERT.

b. Extracellular loops (ECL)

Many mutations of recognized extracellular and intracellular residues have been generated for the purpose of detecting conformational shifts in, or mapping tertiary structure of, NSS family members. A few of these mutations were demonstrated to affect uptake inhibitor recognition. The hDAT C90A mutant was prepared toward generating a DAT species lacking endogenous methanethiosulfonate-reactive cysteine residues (Ferrer and Javitch, 1998). This mutation did not alter affinity for WIN 35,428, but selectively diminished the DUIP of benztropine; DUIPs of cocaine, WIN 35,428 and mazindol were unaffected by the mutation. This very highly conserved ECL 1 residue is not expected to directly contact benztropine, but rather to differentially contribute toward stabilizing benztropine- versus cocaine-preferring DAT conformations (Reith et

al., 2000; Ferrer and Javitch, 1998). Gether and colleagues have mutated many extratransmembranous hDAT residues in elucidating the endogenous Zn^{2+} binding site of the DAT, and in creating new Zn^{2+} sites toward mapping TM domain proximities (Loland et al., 2004; Norgaard-Nielsen et al., 2002; Loland et al., 1999). In the course of this work, the extracellular hDAT mutants E218Q (ECL 2), E307Q (ECL 3) and D385N (ECL 4) were found to sustain 4- to 5-fold losses in WIN 35,428 affinity (Loland et al., 1999). Treatment of M371C and A399C mutant hDATs with MTSET caused a profound reduction in [^{3}H]dopamine uptake (Norregaard et al., 2003). Cocaine, but not dopamine, protected A399C DAT from MTSET-induced inactivation. On the other hand, dopamine, and not cocaine protected M371C from MTSET. This observation may imply that A399 is in the DAT binding site of cocaine or that cocaine induces a conformational change that diminishes the reactivity of A399C. In addition, the finding that protection of A399C occurs selectively with inhibitors and protection of M371C selectively with substrates supports the notion that inhibitors promote and bind conformational states of the transporter that differ from those adopted in the presence of substrates (Norregaard et al., 2003).

Regarding other ECL mutations, WIN 35,428 affinity decreased 5-fold as a result of the ECL 4 rDAT F390A mutation; dopamine uptake was virtually eliminated (Lin et al., 1999). Asparagine substitution of hDAT D476, a residue at the ECL 5 / TM 10 border, decreased WIN 35,428 affinity 4-fold, cocaine DUIP 3-fold, and apparent affinity (measured by K_m value) for dopamine 7-fold (Chen et al., 2001). For the above ECL loop mutants, it is likely that the inhibitor binding

affinity and uptake inhibition potency losses are due to mutation-induced conformational changes; however, modifications in the actual inhibitor binding sites have not been ruled out.

c. Intracellular loops (ICL)

The tyrosine residue at position 335 of the hDAT may be important for cocaine binding. Mutation of this residue to alanine (Y335A) caused defects in the function of high affinity Zn^{2+} binding to DAT (Loland et al, 2002). Zn^{2+} binding to WT DAT normally reduces dopamine uptake. In Y335A DAT, binding of Zn^{2+} to its high affinity sites resulted in potentiation of dopamine uptake. In addition, Y335A mutation caused a 150-fold reduction in apparent affinity of DAT for cocaine and related inhibitors when compared with WT DAT values. Loland and colleagues proposed that Y335 may be part of a network of intramolecular interactions that is important for stabilizing the transporter in a conformational state that maintains the structural integrity of the inhibitor binding site and to which extracellular substrate can bind and initiate transport. Further analyses of the intracellular loops of hDAT identified other residues that, when mutated to alanine, exhibited similar phenotype as Y335A (Loland et al, 2004). Akin to Y335A, the mutants K264A (IL2), D345A (IL3), and D436A (IL4) were characterized by lower affinities for cocaine and other inhibitors as well as a low uptake capacity that was potentiated by Zn^{2+}. In a separate study, Chen et al, 2004 showed that D345N DAT also exhibited a phenotype similar to Y335A. D345N mutation decreased cocaine analog binding by over 90% but uptake

inhibition by various DAT inhibitors was preserved in contrast to what was observed with Y335A mutation.

4. Regulation of DAT Function

a. Post-translational modification

DAT function is regulated largely by way of rapid shuttling of DAT to and from the plasma membrane. Phosphorylation of DAT by kinases leads to rapid redistribution and internalization of DAT away from the plasma membrane causing diminished cellular uptake of dopamine (Pristupa et al., 1998, Melikian and Buckley, 1999; Daniels and Amara, 1999). There are several consensus sites on the primary structure of the DAT for protein kinase phosphorylation of the transporter (Giros et al., 1992). Acute exposure of hDAT expressing Sf9 cells to PMA, a PKC activator, reduced Vmax for [^{3}H]dopamine uptake by ~ 40% when compared with control cells treated with αPDD (an inactive phorbol ester) (Pristupa et al., 1998). This inhibition of uptake was blocked by the protein kinase inhibitor staurosporine (Pristupa et al., 1998). Furthermore, confocal microscopy data revealed that the observed decline in [^{3}H]dopamine uptake was associated with redistribution of surface DAT to the internal environment. Recruitment of the internalized transporters back to the cell surface was responsible for the observed augmentation in [^{3}H]dopamine uptake in cells treated with the PKC inhibitor (Pristupa et al., 1998). Nonetheless, proof that the direct phosphorylation of the transporter modifies its intrinsic activity or triggers the downregulation and internalization of the dopamine transporter is

inconclusive. The deletion of all the consensus sites for protein kinase C did not put a stop to protein kinase C-induced internalization (Chang et al., 2001). In a different study, deletion of the first 22 amino acids (N-terminus) from DAT eliminated ^{32}P incorporation into DAT in response to PKC activation (Granas et al., 2003). This shortened mutant DAT still transported substrate and was internalized normally subsequent to protein kinase C activation (Granas et al., 2003). These data mean that N-terminal phosphorylation of DAT is not necessary for transporter internalization. Recent evidence indicates that the transport capacity and intracellular trafficking of DAT is controlled by mitogen-activated protein kinase (MAPK) (Moron et al., 2003). Incubation of striatal synaptosomes or epitope-tagged hDAT HEK 293 cells with MAPK kinase inhibitors diminished dopamine uptake in a concentration dependent manner; decreased Vmax for dopamine uptake but had no effect on dopamine uptake affinity (Moron et al., 2003). Biotinylation and confocal microscopic studies showed that the observed effect of MAPK kinase inhibitors on dopamine transport kinetics was due to clathrin-mediated redistribution of hDAT from the plasma membrane to the internal environment of the cell (Moron et al., 2003).

It has been demonstrated recently that PKC can control MAPK activation and that the mechanism of activation shows isotype specificity (Schonwasser et al., 1998). Some species of PKC, such as PKC-α, show an ability to activate the MAPK cascade via c-Raf, while other isoforms activate this cascade by a mechanism independent of c-Raf1 activation. Thus, distinct subclasses of PKC

may account for two independent signalling pathways to MEK and, hence, MAPK activation.

b. Protein-protein interactions

Monoamine transporters have sequence motifs that are known to guide protein-protein interactions, including a leucine repeat in TM 2, a PDZ binding site at the extreme carboxyl termini, and presumed tyrosine-based and di-leucine internalization motifs (Torres et al., 2003). By means of the yeast two-hybrid system, a direct interaction between α-synuclein and DAT has been determined (Lee et al., 2001). α-synuclein is a pre-synaptic protein that has been associated with Parkinson's disease (Gwinn- Hardy, 2002). α-synuclein augments the functional activity of DAT when it is co-expressed with DAT in cells (Lee et al., 2001). On the contrary, coexpression of α-synuclein and DAT in L*tk*– fibroblasts manifestly decrease the reuptake of dopamine by DAT by ~30–50% (Wersinger and Sidhu, 2003; Wersinger et al., 2003; Sidhu et al., 2004). The diminution in DAT activity was due to a reduction in Vmax for dopamine uptake by the transporter without any change in DAT expression levels. An outcome of the α-synuclein-mediated reduction of DAT activity was that upon exposure of cotransfected cells to dopamine, there was decreased dopamine-induced oxidative stress and cytotoxicity. In the presence of α-synuclein, the DAT was trafficked away from the plasma membrane into the cytoplasm. This is evident from biotinylation experimental data showing reduced DAT presence at the plasma membrane by (Wersinger et al., 2003). α-Synuclein interacts directly with

DAT, creating a stable protein: protein heteromeric complex in cotransfected cells, mesencephalic neurons, and substantia nigra. The interactions were observed between the nonamyloid β component (NAC) domain (residues 58-107) of α-synuclein and the last 22 amino acids of the carboxyl terminal tail of DAT (Wersinger et al., 2003). Analogous to wild-type, the missense A30P mutant form of α-synuclein decreased DAT function, trafficking DAT away from the plasma membrane and contributing to the formation of stable protein: protein complexes, again through the NAC domain (residues 58-107) of A30P and the last 22 amino acids of the CT tail of DAT (Wersinger et al., 2003a). The other missense A53T mutant was unable to alter DAT function, and succeeding studies demonstrated that this protein interacted only weakly with the transporter (Wersinger et al., 2003a).

The PDZ domain-containing protein PICK 1 has been shown to interact with the carboxyl termini of DAT and NET (Torres et al., 2001). In culture, PICK 1 colocalizes with DAT and NET in dopaminergic and noradrenergic neurons respectively. PICK 1 additionally stimulates DAT and NET clustering in transfected cells thereby increasing the activity of the transporters. Thus, PICK 1 may play a part in targeting of these transporters to the nerve terminals (Torres et al., 2001). In cultured neurons, a DAT mutant lacking the PICK 1 binding site failed to localize to neuronal processes and remained confined primarily in cell bodies. There are indications that PICK 1 interacts with PKC. It is possible then that PICK 1 may be involved in the PKC-mediated trafficking of monoamine

transporters. Recent experience also suggest an interaction between DAT and the LIM homeodomain-containing protein, Hic-5 (Carneiro et al., 2002).

c. Transporter oligomerization

The oligomerization of DAT has been well studied and there are indications that DAT function is also regulated via transporter oligomerization. In mammalian cells co-expressing differentially tagged DAT molecules, HA-tagged DAT co-purified with 6His-tagged DAT signifying a physical contact between transporter proteins (Torres et al., 2003). Verification of the functional oligomerization of DAT was achieved using dominant-negative mutants of DAT. Two loss-of-function mutant transporters (Y335A and D79G) that were expressed at the cell surface blocked wild-type DAT uptake activity without affecting the membrane targeting of the wildtype transporter. Furthermore, non-functional amino and carboxyl termini-truncated mutants of DAT blocked wild-type DAT function by impeding the normal processing of the wild-type transporter to the cell membrane (Torres et al., 2003). Mutations in the leucine repeat of the second transmembrane domain of the transporter removed the dominant-negative effect of all these mutants. Additionally, a small fragment comprising the first two transmembrane domains of DAT blocked wild-type transporter function but not when the leucine repeat motif was mutated. Taken together, these results indicate that the assembly of DAT monomers plays a vital role in the expression and function of the transporter (Torres et al., 2003). Hastrup et al., 2001 have also identified a well characterized dimerization motif, GxxxG, in the intracellular

half of TM 6 of DAT. DAT dimers are formed as a result of a symmetrical cross-linking between cysteine residues located on the extracellular face of TM 6. The precise contribution of oligomer formation to transporter function remains uncertain. Nonetheless, oligomerization has clearly been established to play a role in facilitating the trafficking of the transporters to the surface. Data from Javitch's (Hastrup et al., 2001) and Caron's research groups (Torres et al., 2003) have shown that mutants in which the domains involved in oligomer formation have been mutated are not efficiently delivered to the cell surface.

II. MATERIALS AND METHODS

A. Materials and Equipment

1. Facilities

Laboratories – Mellon Hall of Science, Room 456

Office – Mellon Hall of Science, Rooms 420 and 456

2. Cell lines

Chinese Hamster Ovary (CHO) cells stably expressing wildtype and D79E mutant DAT.

COS 7 cells

3. Chemicals and Drugs

Bovine serum albumin
Fisher Scientific, Pittsburgh, PA

Complete Mini protease inhibitor cocktail tablets
Roche Diagnostics Corp., Indianapolis, IN

Compressed carbon dioxide
Air Products, Pittsburgh, PA

Dimethylsulfoxide
Sigma Chemical Co., St. Louis, MO

Ampicillin Na salt
Fisher Scientific, Pittsburgh, PA

Ethanol, HPLC grade
Fisher Scientific, Pittsburgh, PA

Isopropanol, DNase-free
Fisher Scientific, Pittsburgh, PA

LB agar
Fisher Scientific, Pittsburgh, PA

LB broth
Fisher Scientific, Pittsburgh, PA

Methanol, HPLC grade
Fisher Scientific, Pittsburgh, PA

Ribonuclease A
Fisher Scientific, Pittsburgh, PA

Precast polyacrylamide gels, 4 – 20%
BioRad Laboratories, Hercules, CA

dNTP mix
Stratagene, La Jolla, CA

Plasmid mini-prep kit
Stratagene, La Jolla, CA

Polymerase, Pfu Turbo
Stratagene, La Jolla, CA

Quickchange mutagenesis kit
Stratagene, La Jolla, CA

XL1 Blue cells
Stratagene, La Jolla, CA

Dpn1 enzyme
Stratagene, La Jolla, CA

Plasmid maxi-prep kit
Qiagen Inc., Valencia, CA

Plasmid mini-prep kit
Qiagen Inc., Valencia, CA

Qiaquick gel extraction kit
Qiagen Inc., Valencia, CA

Qiaquick PCR extraction kit
Qiagen Inc., Valencia, CA

D-glucose
Sigma Chemical Co., St. Louis, MO

Tetracycline-HCL
Sigma Chemical Co., St. Louis, MO

F-12 Nutrient Mixture
GIBCO-BRL, Grand Island, NY

Glycine
Sigma Chemical Co., St. Louis, MO

HEPES
Fisher Scientific, Pittsburgh, PA

HRP-conjugated goat anti-rat IgG
Chemicon International, Temecula, CA

Agarose
Invitrogen, Carlsbad, CA

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

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

DH5α cells
Invitrogen, Carlsbad, CA

PolyFect Transfection Reagent
Qiagen Inc., Valencia, CA

SOC media
Invitrogen, Carlsbad, CA

Not 1 restriction enzyme
Invitrogen, Carlsbad, CA

Bgl2 Restriction Enzyme
Invitrogen, Carlsbad, CA

Psh1 restriction enzyme
Invitrogen, Carlsbad, CA

BsiW1 restriction enzyme
Invitrogen, Carlsbad, CA

Rhodamine phalloidin
Molecular Probes, Eugene, OR

T4 DNA ligase
Invitrogen, Carlsbad, CA

Penicillin-Streptomycin
GIBCO-BRL, Grand Island, NY

Fetal bovine serum
GIBCO-BRL, Grand Island, CA

Sodium Dodecyl Sulfate
Sigma Chemical Co., St. Louis, MO

Sodium Hydroxide
Sigma Chemical Co., St. Louis, MO

Trichloroacetic Acid
Sigma Chemical Co., St. Louis, MO

Trizma Base
Sigma Chemical Co., St. Louis, MO

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

ScintSafe scintillation fluid
Fisher Scientific, Pittsburgh, PA

PBS, Ca/Mg-free
Fisher Scientific, Pittsburgh, PA

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

Protein MW marker
Fisher Scientific, Pittsburgh, PA

Sodium chloride
Sigma Chemical Co., St. Louis, MO

[^{3}H]WIN35, 428
Perkin Elmer, Foster City, CA

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

GVA Mounting Solution
Zymed, San Francisco, CA

Goat Serum
Biomeda, Foster City, CA

4. Materials

BioRad Protein Assay Kit
BioRad, Hercules, CA

Cryogenic vials
Nalgene, Rochester, NY

Polaroid Film
Fisher Scientific, Pittsburgh, PA

Pasteur pipettes
Fisher Scientific, Pittsburgh, PA

Scintillation vials
Fisher Scientific, Pittsburgh, PA

Test tubes, 12x75mm
Fisher Scientific, Pittsburgh, PA

PVDF membrane
BioRad Laboratories, Hercules, CA

Sterile Tissue culture plates (6-, 12-, 24-well)
Fisher Scientific, Pittsburgh, PA

Sterile disposable serological pipettes (5, 10, 25 ml)
Fisher Scientific, Pittsburgh, PA

Eppendorf tubes (1.5µl)
Fisher Scientific, Pittsburgh, PA

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

Glass microscope slides
Fisher Scientific, PA

Kodak T-Max 400 film
Eastman Kodak Co., Rochester, NY

Fisherbrand Redi-TipsTM disposable pipette tips (1, 10, 200, 1000μl)
Fisher Scientific, Pittsburgh, PA

Pipette Aid
Drummond Scientific Company, Broomall, PA

5. Equipment

Accumet pH meter, Model 291
Fisher Scientific, Pittsburgh, PA

BioRad Power Pac 3000
BioRad, Hercules, CA

Hot plate with stirrer
Fisher Scientific, Pittsburgh, PA

Leica Confocal Laser Microscope TCS-SP2
Leica Microsystems, San Diego, CA

Liquid Scintillation Counter Tri-Carb 2100TR
Packard Instrument Co., Downers Grove, IL

UV/VIS Spectrophotometer DU530
Beckman Instruments, Fullerton, CA

Analytical Balance
Mettler-Toledo Inc., Highstown, NJ

Centrifuge 5810R (Large)
Eppendorf North America Inc., Westbury, New York.

Centrifuge 5415C (Small)
Eppendorf North America Inc., Westbury, New York.

Universal Vacuum System UVS400
Savant Company Inc., Irvine, CA

PCR Mastercycler
Eppendorf North America Inc., Westbury, New York.

Weighing Balance
Denver Instrument, Denver, CO.

Freezer (-20^{o}C)
Forma Scientific, Waltham, MS

Liquid Nitrogen Tank

Laboratory Refrigerator
REVCO Thermo Electron Corporation, Asheville, NC.

Horizontal Gel Electrophoresis System
GIBCO-BRL, Grand Island, NY.

Gel Apparatus
BioRad, Hercules, CA.

6. Computer Software

GraphPad Prism 3.0
GraphPad, San Diego, CA

Microsoft Excel, Microsoft Office 2003
Microsoft Corporation, Orem, UT

Microsoft Word, Microsoft Office 2003
Microsoft Corporation, Orem, UT

Adobe Photoshop Version 5.0
Adobe Systems, Seattle, WA

Adobe Acrobat (Reader and Writer)
Adobe Systems Inc., Seattle, WA

MDL-ISIS Draw 2.5
Elsevier MDL, San Leandro, CA

B. Methodology and Procedures

1. Cell culture

CHO-K1 cells stably transfected with WT DAT or D79E DAT were used in the present study. These cells were grown at 37^{O}C and 5% CO_2 in F12K medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100mg/ml streptomycin and 100µg/ml G-418. COS-7 cells were employed for all experiments requiring transient transfection of plasmid DNA. The COS-7 cells were maintained in DMEM media supplemented with 10% FBS, 100 units/ml penicillin, 100mg/ml streptomycin, and 20 mM L- glutamine. All cells were grown in 75 cm^2 flasks and subcultured twice weekly. Cells were subcultured by aspirating media from the flask and washing the confluent monolayer with 10 mL Hanks buffered salt solution (HBSS). The cells were then detached by addition of 3 mL trypsin-EDTA solution to the monolayer. The trypsin-EDTA solution was swirled to coat the monolayer and 2 mL of the trypsin-EDTA was aspirated from the flask. The cells were left to stand at room temperature (under a sterile hood) in 1 mL trypsin-EDTA for 3 min or until the cells detach from the flask. The trypsinization reaction was quenched by addition of 9 mL of appropriate “complete media” (media supplemented with FBS and antibiotics). The trypsinate was gently mixed to create an even suspension of cells. Two milliliters, out of a total volume of 10 mL, of the trypsinate was transferred into a new flask and 18 mL of “complete media” was added to it; the cell solution was then mixed gently. The flask was capped and kept flat in the

incubator (37^{o}C and 5% CO_2) and cells were allowed to grow to confluence. Cells were typically subcultured on Mondays and Thursdays.

2. Site- directed mutagenesis

All site-directed mutagenesis was conducted using the QuikChange mutagenesis kit (Stratagene). rDAT in Bluescript vector was used as template for the PCR-based mutagenesis. Sense and antisense oligonucleotides were designed to contain the desired mutations. The oligonucleotide primers were synthesized by Sigma-Genosys Inc., U.S.A. The sequences of primers used for the generation of D79L DAT were: 5'-GTCATCGGCTTTGCTGTGCTCCTGGCCAATGTCTG-3' for the forward primer and 5'-CCAGACATTGGCCAGGAGCACAGCAAAGCCGATGAC-3' for the reverse primer. These complementary primers were annealed to the template cDNA and extended with the proofreading polymerase *pfu* Turbo (Stratagene) with the following PCR parameters: 30 sec at 95^{o}C, 30 sec at 95^{o}C, 1 min at 55^{o}C, and 6 min at 72^{o}C for 25 cycles. Subsequently, the PCR product was digested with Dpn1 to eliminate the methylated, nonmutated parental strands. The mutant cDNA was then transformed into XL1-Blue supercompetent *E. coli* cells using the heat-shock method. The bacterial cell solution was then plated on agar plates containing 50 µg/ mL ampicillin and incubated overnight at 37^{o}C. The following day, colonies were selected and plasmid preparation was carried out using the Stratagene miniprep system to produce pure supercoiled plasmid DNA. Mutagenesis was confirmed by DNA sequence analysis of the miniprep

product (University of Pittsburgh sequencing facility). The fragment (~ 500 bp) containing the mutation in rDAT-pBluescript was isolated by digestion with *Not1* and *Bgl2* restriction enzymes, gel purified, and ligated into the *Not1/Bgl2*-digested wild type rDAT-pC1 vector. The ligation product was transformed into MC1061 cells using the heat shock method and cells were plated on agar plates containing ampicillin and tetratcycline and incubated overnight at 37^{o}C. Colonies were again selected and the plasmid DNA was purified using the Stratagene mini-prep kit according to manufacturer's guidelines. The fragment containing the mutation in rDAT-pC1 was isolated by double digestion with *PshA1* and *BsiW1* enzymes. This 1.8 kb fragment was then ligated into the *PshA1/BsiW1*- digested wildtype rDAT-pIRES vector. The ligation product was transformed into DH5α supercompetent *E. coli* cells by way of the heat shock method and cells were plated on agar plates containing ampicillin. The agar plates were then incubated overnight at 37^{o}C to allow growth of bacterial colonies. A few bacterial colonies were selected, lysed, and the supercoiled DNA was purified using the Stratagene mini-prep kit. The presence of supercoiled plasmid DNA was verified by agarose gel electrophoresis. Following confirmation of successful mutagenesis by DNA sequence analysis, a sample from the mini-prep product was again transformed into DH5α cells, plated on agar plates and incubated overnight at 37^{o}C. Plasmid preparations were carried out, this time, using the Qiagen maxi-prep kit to produce a larger volume of pure supercoiled plasmid DNA sufficient for transient transfection of cells. The DNA concentration and purity (measured as A_{260}:A_{280} ratio) were estimated by

spectrophotometric analysis. Plasmid DNA having an A_{260}:A_{280} ratio greater than or equal to 1.7 was typically used for transient transfection of COS 7 cells. All other D79 mutant DATs used in the present study were previously generated by Wenfei Wang in the laboratory of Dr. Surratt at Albert Einstein College of Medicine (AECOM), Bronx, New York.

3. Cell Transfections

A modification of the calcium phosphate method or the use of PolyFect reagent (Qiagen, Los Angeles, CA) was employed for transient transfections of COS 7 cells. For the calcium phosphate method, confluent COS 7 cells were subcultured in 6-well plates such that the monolayer would be 40 - 50% confluent when the transfection commenced the next day. Twenty micrograms of plasmid were used for the transient transfections. Two tubes were prepared, with the contents of one tube slowly added to the second tube. The first tube contained plasmid DNA, Millipore water, 10x Tris- EDTA (TE) buffer pH 8.0, and 0.5M $CaCl_2$ mixed together to achieve a final amount of DNA of 20 µg. The second tube contained 2x HEPES- buffered saline (HBS) solution. The contents of the first tube were added to the second tube dropwise with continuous vortexing. For an individual well of the 6-well plate containing 40% confluent cells, 200 µl of the above mixture was added. The cells take up the exogenous plasmid via endocytosis of the calcium phosphate/DNA complex. On the next day, the transfection mixture is removed and the replaced with 2 ml fresh DMEM. The cells were used for pharmacological studies 48 hours later.

For the PolyFect reagent-mediated transient transfection, a slight modification of the manufacturer's protocol was employed. The day before transfection, cells were seeded in 6-well plates and incubated overnight at 37^{O}C and 5% CO_2 such that the cells will be 50-80% confluent the next day. On the day of transfection, cell monolayers in 6-well plates were washed with 1 x 1mL phosphate buffered saline (PBS), pH 7.3, and 1.5 mL of "complete" DMEM media was added to each well. These cells were then incubated at 37^{O}C and 5% CO_2 until the commencement of transfection. The transfection mixture was prepared as follows: plasmid DNA (1.5 μg) was diluted with 100 μL of DMEM containing no serum, proteins, or antibiotics and mixed by vortexing for 10 seconds. Ten microliters of PolyFect transfection reagent was then added to the DNA solution, mixed by vortexing for 10 sec, and incubated at room temperature for 8 min to allow DNA-PolyFect complex formation. Subsequently, 600 μL of complete DMEM (containing serum and antibiotics) was added to the reaction tube containing the transfection complexes and mixed by pipetting up and down twice. The total volume of the transfection mixture was immediately transferred to a single well of the 6-well plate containing 80% confluent cells at room temperature. The transfected cells were incubated at 37^{o}C and 5% CO_2 and used for pharmacological studies 48 hours after transfection. When performing transient transfections of cells in more than one well (which was more often the case), the volumes described above were multiplied by the number of wells used.

The stably-transfected DAT-CHO cell lines used in this study were prepared in the laboratory of Dr. Surratt at AECOM, New York. Lipofectamine-

mediated transfection was used to prepare the stable cell lines; stable transfectants were selected in the presence of 500 µg/ml G-418 in F-12K HAM medium. Cell lines were maintained as described above.

4. Immunocytochemistry and confocal microscopy

COS-7 cells were seeded on coverslips placed in 6-well plates and grown to 40 - 60% confluence. Cells were transiently transfected on the following day with WT or D79 mutant DAT plasmids, or the vector control plasmid, using PolyFect reagent (Qiagen, Los Angeles, CA). After 48 hours, cells were fixed in 4% paraformaldehyde solution in PBS at room temperature for 15 min, rinsed once with PBS, and incubated with blocking-permeabilizing solution (5% goat serum, 1% BSA, and 0.1% Triton X-100 in PBS buffer solution) for 45 min at room temperature. Cells were next incubated with rat monoclonal anti-DAT antibody (MAB369; Chemicon, Temecula, CA) at 1:1000 dilution for 1 hr. The anti-DAT antibody solution was aspirated and cells were washed five times with PBS containing 0.1% Triton X-100 (TPBS), and incubated with a mixture of secondary antibody (goat anti-rat Alexa Fluor 488; Molecular Probes, Eugene, OR) at 1:500 dilution and rhodamine phalloidin (Molecular Probes) at 1:250 dilution for 1 hr. After three washes in TPBS followed by two washes in PBS, coverslips were mounted on slides using GVA mounting solution (Zymed, San Francisco, CA) and left to dry overnight in the dark at 4^{O}C. DAT protein was visualized using the Leica TCS-SP2 confocal laser microscope with a glycerin immersion 63x objective. Alexa 488 was excited at 488 nm with an

argon/krypton 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.

5. [^{3}H]-Dopamine saturation uptake assays

All [^{3}H]-dopamine uptake assays were conducted with cell monolayers seeded in 6-well plates. In dopamine saturation uptake assays, cell monolayers in six-well plates were washed 2 X 1 mL with KRH buffer (25mM HEPES, pH 7.3, 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 5.6 mM glucose) supplemented with 50 mM ascorbic acid (KRH/AA) and then incubated with increasing concentrations (0.5 – 16 μM) of a fixed ratio of [^{3}H]dopamine: nonradioactive dopamine (~0.1 Ci/mmol) for 5 min. Dopamine uptake was quenched by aspirating the dopamine solution and washing the monolayers with 2 X 1 mL KRH/AA buffer. Subsequently, cells in each well were lysed with 1 ml of 1% SDS, incubated at room temperature for 1 hr with gentle shaking, and transferred to scintillation vials for determination of incorporated tritium. The results of liquid scintillation counting and protein determinations were used to calculate uptake of [^{3}H]dopamine expressed as pmol/mg protein/ min. In each experiment, the mean result from duplicate wells for each treatment was used and the experiments were repeated at least four times. Specific uptake was calculated as the difference between uptake of [^{3}H]dopamine in the absence and

presence of 10 μM mazindol. K_M and V_{MAX} values were calculated from non-linear regression analysis of the data for each individual experiment using the GraphPad Prism 3.0 software.

6. [^{3}H]-Dopamine uptake inhibition assays

The monolayer was washed twice with KRH/AA buffer (2 x 1 mL). Dopamine uptake inhibition experiments incubated the cells with inhibitors for 10 minutes, followed by the addition of 10nM [^{3}H]-dopamine for a further five minutes. The range of concentrations of inhibitors or substrates employed was as follows: cocaine, 1 nM - 100 μM; WIN35,428, 1 nM - 10 μM; 8-oxa-norcocaine, 1 nM - 1 mM; 4-ARA-127, 10 nM - 100 μM; mazindol, 3 nM - 10 μM; methylphenidate, 3 nM - 10 μM; benztropine, 0.1 nM - 30 μM; 3β-4'-chlorobenztropine, 1 nM - 30 μM; 4'-chlorobenztropine, 0.1 nM - 30 μM; 4',4"-difluorobenztropine, 0.1 nM - 10 μM; GBR-12,909, 0.1 nM - 10 μM; rimcazole, 1 nM - 30 μM; N-formyl-4,4'-difluorobenztropine, 10 nM - 1 mM; and 4-trifluoromethylbenztropine, 1 nM - 30 μM. Uptake was quenched by aspirating the inhibitor/ radioligand solution and washing the monolayer with 2 x 1 ml of KRH containing ascorbic acid (KRH/AA). Nonspecific binding was assessed with 10μM mazindol, or with 30 μM cocaine if the drug tested was mazindol. The cells in each well were then lysed with 1 mL of 1% SDS and incubated at room temperature for 1 hour with gentle shaking. The lysate was transferred into scintillation vials containing 5 mL ScintSafe fluid and radioactivity was counted

using a liquid scintillation counter. K_i values for [^{3}H]dopamine uptake inhibition were determined with GraphPad Prism 3.0.

7. [^{3}H]WIN35,428 saturation binding assays

All binding assays were conducted with stable DAT-CHO cells or transient DAT-COS 7 cells subcultured in six-well plates. [^{3}H]WIN35,428 was the radioligand used in all binding assays. This compound is structurally similar to cocaine, labels both the high- and low- affinity binding sites just as cocaine, but is more stable *in vitro*. For [^{3}H]WIN35,428 saturation binding assays, serial dilutions of WIN35,428 (1 nM - 10 μM) were made in KRH/AA buffer. [^{3}H]WIN35,428 was added to each dilution of drug to achieve a final concentration of approximately 1 nM for the radioligand. Cell monolayers in 6-well plates were then washed with 2 x 1mL of KRH/AA buffer and incubated with the mixture of [^{3}H]WIN35,428 and WIN35,428 for 15 minutes. The drug solution was removed and the cell monolayers were washed with 2 x 1 mL KRH. Cells were lysed with 1% SDS solution and the ^{3}H content of the cell lysates was determined in a scintillation counter. B_{max} and K_d values for [^{3}H]WIN35,428 binding were determined with GraphPad Prism 3.0 software.

8. [^{3}H]WIN35,428 binding inhibition assays

All competition binding assays were conducted exactly as described for the dopamine uptake inhibition assays except that [^{3}H]-dopamine was replaced

with 1nM [^{3}H]WIN35,428 and cells were incubated with a mixture of radioligand and nonradioligand competitor for 15min. Briefly, serial dilutions of the drugs to be examined were made in KRH/AA buffer. [^{3}H]WIN35,428 was added to each dilution of drug to achieve a final concentration of 1 nM for the radioligand. The range of concentrations of drugs employed were as indicated above for dopamine uptake inhibition: cocaine, 1 nM - 100 μM; WIN35,428, 1 nM - 10 μM; 8-oxa-norcocaine, 1 nM - 1 mM; 4-ARA-127, 10 nM - 100 μM; mazindol, 3 nM - 10 μM; methylphenidate, 3 nM - 10 μM; benztropine, 0.1 nM - 30 μM; 3β-4'-chlorobenztropine, 1 nM - 30 μM; 4'-chlorobenztropine, 0.1 nM - 30 μM; 4',4"-difluorobenztropine, 0.1 nM - 10 μM; GBR-12,909, 0.1 nM - 10 μM; rimcazole, 1 nM -30 μM; N-formyl-4,4'-difluorobenztropine, 10 nM - 1 mM; and 4-trifluoromethylbenztropine, 1 nM - 30 μM). To begin with, cell monolayers in 6-well plates were washed with 2 x 1mL of KRH/AA buffer and incubated with the mixture of radioligand and nonradioactive competitor for 15 minutes. The experiment was quenched by aspirating the test solution and washing the cell monolayers twice (2 x 1 mL) with KRH/AA buffer. Nonspecific binding was assessed by addition of 10μM mazindol except when mazindol was the drug tested, in which case 30μM cocaine was substituted. The cell monolayers were then lysed with 1 mL of 1% SDS solution and incubated at room temperature for 1 hour with gentle shaking. Cell solution from each well was then transferred to scintillation vials containing 5 mL of ScintSafe fluid and radioactivity was counted using a scintillation counter. K_i, K_d, and B_{MAX} values were determined with GraphPad Prism 3.0 software.

9. Protein assays

Stable DAT-CHO cells or transient DAT-COS 7 cells subcultured in 6-well plates were used for protein assays. For every experiment carried out that required estimates of protein content, two wells were reserved for this purpose. The confluent cell monolayers in 6-well plates were washed with 2 x 1 mL PBS. Cells in each well were then lysed with 0.5 mL of 0.2N NaOH solution, scraped with a rubber policeman, and incubated for 30 min at 4^{o}C with gentle shaking. The cell lysates were transferred into Eppendorf tubes for storage at -20^{o}C or for immediate estimation of protein content. Protein assays were conducted according to the method of Bradford. For each assay, carried out in duplicate, 780 µL of water was added to 20 µL of cell lysate in a test tube and mixed gently. 200 µL of BioRad "micro-Bradford" reagent was then added to the cell solution, vortexed for 10 sec, and incubated at room temperature for 5 min. The absorbance of the protein/ dye solution was measured at 595 nm using a spectrophotometer. The concentration of protein was calculated by interpolating the absorbance reading of the sample protein using protein standard curve.

This curve was generated using BSA (1mg/mL stock solution) as protein standard. A serial dilution of the BSA stock solution in water was performed to make six different concentrations of BSA such that the final concentrations were 1, 2, 3, 4, 5, and 6 µg/ mL. The initial volume for each dilution was 800 mL (e.g. for 2µg/ mL: 2µL of 1mg/mL BSA + 798 µl of water). 200 µL of BioRad reagent was then added to each test tube containing the protein dilutions (bringing the total volume of reaction to 1 mL), vortexed for 10 sec, and incubated at room

temperature for 5 min. The absorbance of each protein dilution was read at 595 nm using a spectrophotometer. A protein standard curve (plot of absorbance versus amount of protein) was generated using the Excel software.

III. RESULTS AND DISCUSSION

A. Results

1. Expression, localization and function of WT DAT and D79 mutant DATs

WT DAT and four D79 mutant DATs (D79A, D79E, D79L, D79N) were screened for their abilities to bind [^{3}H]WIN35,428, take up [^{3}H]dopamine, or target to the cell membrane. The mutant DATs were made by site-directed mutagenesis and confirmed by DNA sequencing. These mutants were used to characterize features of the TM1 aspartic acid residue important for dopamine uptake and inhibitor recognition. A mutation from aspartate to alanine (D79A) results in a large decrease in size and a loss of charge and polarity of the residue. The mutation of aspartate 79 in rDAT to glutamate (D79E) is a conservative exchange as these two amino acids differ from one another only by one methylene group. The D79L mutation provides a leucine side chain that causes a loss in the negative charge and hydrogen bonding potential of aspartate but retains a size similar to that of aspartate. An asparagine for aspartate substitution at position 79 of DAT (D79N) eliminates the negative charge but retains the hydrogen bonding potential of the residue. All constructs were assessed in parallel using a one point binding assay. Relative to WT DAT, only the D79E DAT mutant displayed detectable specific binding of [^{3}H]WIN35,428 ($P<0.05$, compared with vector transfected cells) (Fig. 3).

The D79 mutant DATs were also tested for functional uptake by incubating COS 7 cells transiently transfected with the cDNA of WT DAT or the D79 mutant

DATs with 10 nM [^{3}H]dopamine for 5 min at 22^{o}C. The D79E DAT mutant was yet again the only mutant DAT that displayed functional uptake of [^{3}H]dopamine ($P<0.001$, compared with vector-transfected cells) (Fig. 4).

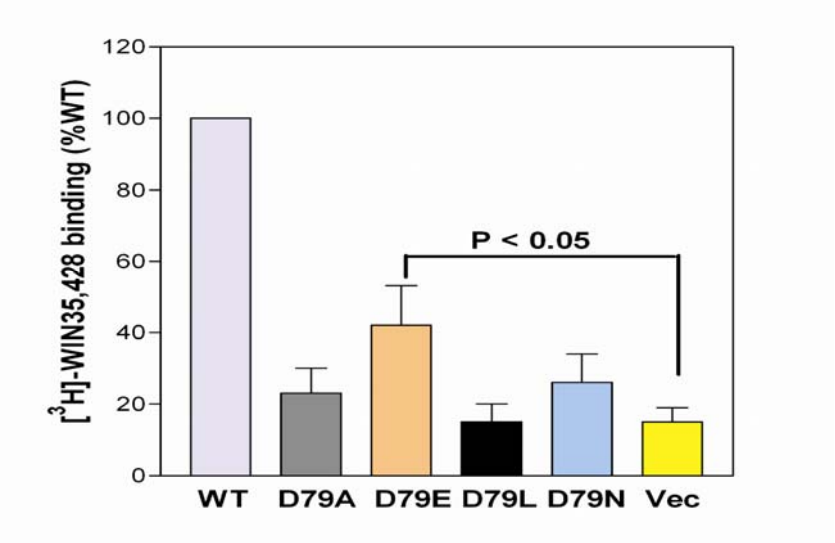


Figure 3. Determination of [^{3}H]-WIN35,428 specific binding by COS-7 cells expressing WT or D79 mutant DAT proteins.

The extent of specific [^{3}H]-WIN35,428 binding as a percentage of WT DAT was assessed for cells transiently transfected with plasmids encoding D79A, D79E, D79L or D79N DAT, or the plasmid vector lacking DAT sequence ("Vec"). The data represent an average of 5 separate experiments. * $P < 0.05$ (Student T test) relative to "vector alone" control. Reprinted with permission from ASPET from Ukairo et al., 2005.

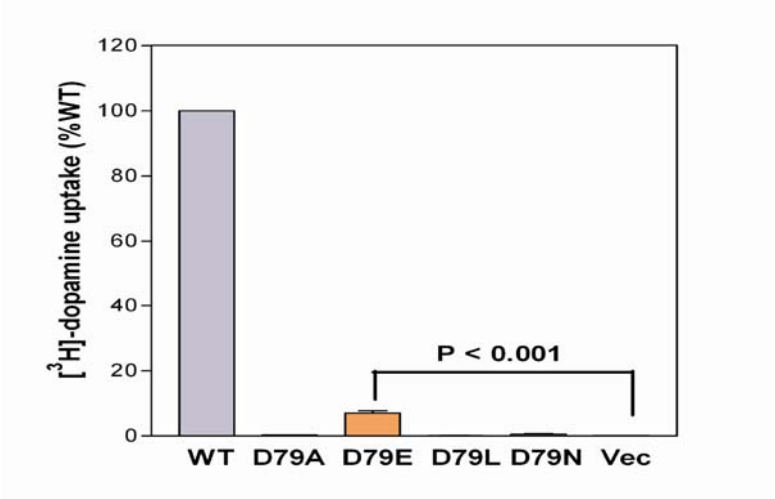


Figure 4. Determination of [^{3}H]-dopamine uptake ability by COS-7 cells expressing WT or D79 mutant DAT proteins.

The extent of total [^{3}H]-dopamine uptake as a percentage of WT DAT was assessed for cells transiently transfected with plasmids encoding D79A, D79E, D79L or D79N DAT, or the plasmid vector lacking DAT sequence ("Vec"). The data represent an average of 5 separate experiments. *P< 0.001 (Student T test) relative to "vector alone" control.

Confocal microscopy experiments, however, showed that all the DAT protein constructs tested were expressed at the cell surface (Fig. 5). Cell surface expression of WT DAT and D79 mutant DAT proteins had earlier been confirmed by biotinylation and western blotting data from our laboratory; D79N DAT, nevertheless, displayed a diminished cell surface expression when compared to WT DAT (Wang et al., 2003). The fact that all D79 mutant DATs were expressed on the cell plasma membrane suggests that the functional and radioligand binding deficits observed were not due to an inability to target to the cell surface. Only the WT DAT and D79E DAT mutant were further characterized due to the failure to detect either radioligand specific binding (Fig. 3) or [^{3}H]-dopamine uptake (Fig. 4) in the other mutant constructs.

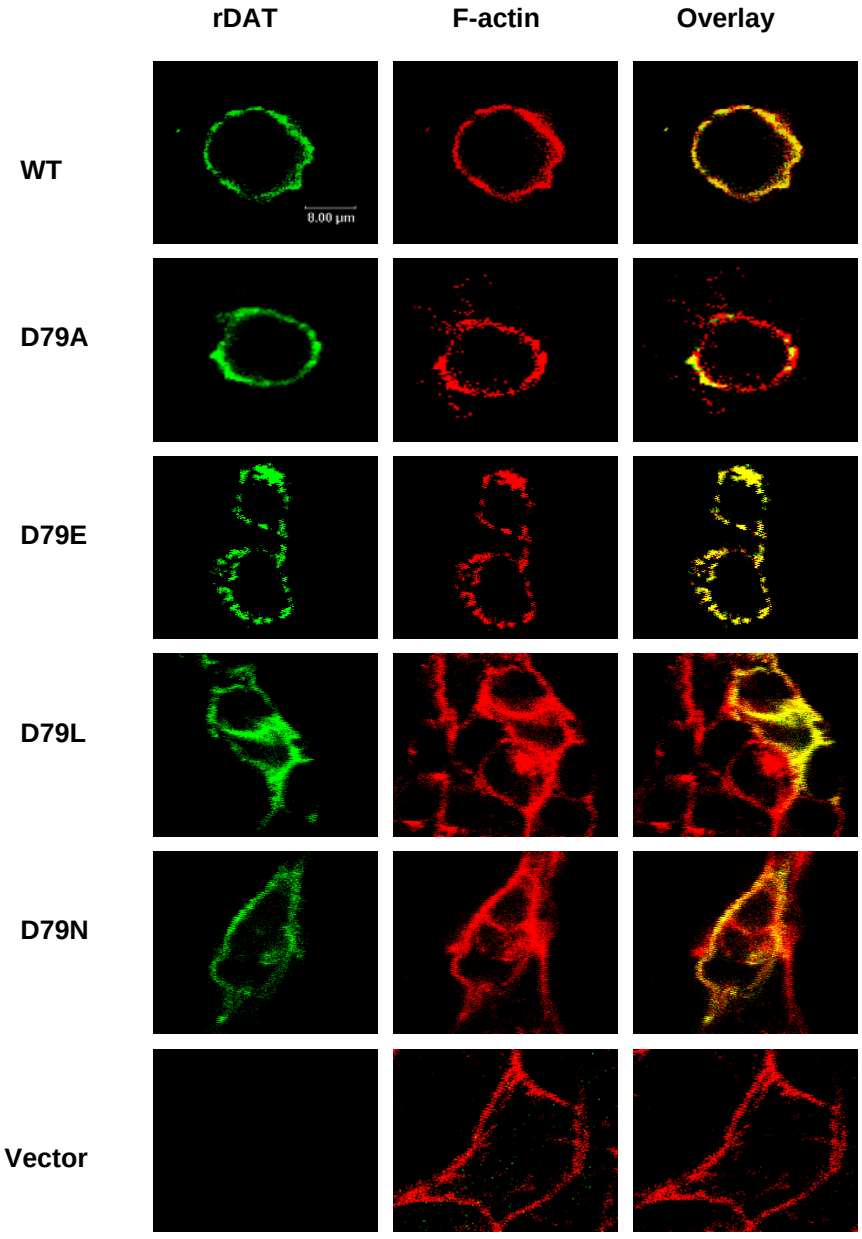


Figure 5. Localization of WT or D79 mutant DAT in DAT-COS 7 cells under confocal laser-scanning microscope.

COS 7 cells transiently transfected with WT DAT or D79 mutant DAT were stained with monoclonal anti-DAT antibody and visualized with Alexa Fluor 488 (green signal). The cells were also stained with rhodamine phalloidin (red signal) to label cortical F-actin, a marker at the cell membrane. Overlay view (yellow signal) shows the co-localization of WT DAT or D79 mutant DAT with F-actin. Shown are representative confocal images of four different experiments. Scale bar = 8.00µm for all images. Reprinted with permission from ASPET from Ukairo et al., 2005.

2. Assessing the role of DAT TM 1 D79 residue in dopamine uptake

The dopamine uptake inhibition potencies (DUIPs) and binding affinities of various dopamine analogs at WT DAT and D79E DAT were tested. This study investigated the effect of modifying the catechol moiety of dopamine to overall substrate transport and also verified whether the D79 side chain of DAT was involved in recognition of some feature of the substrate catechol ring moiety. The drugs tested were: (+)-amphetamine, (-)-amphetamine, p-tyramine, m-tyramine, norepinephrine, and dopamine (Fig. 6).

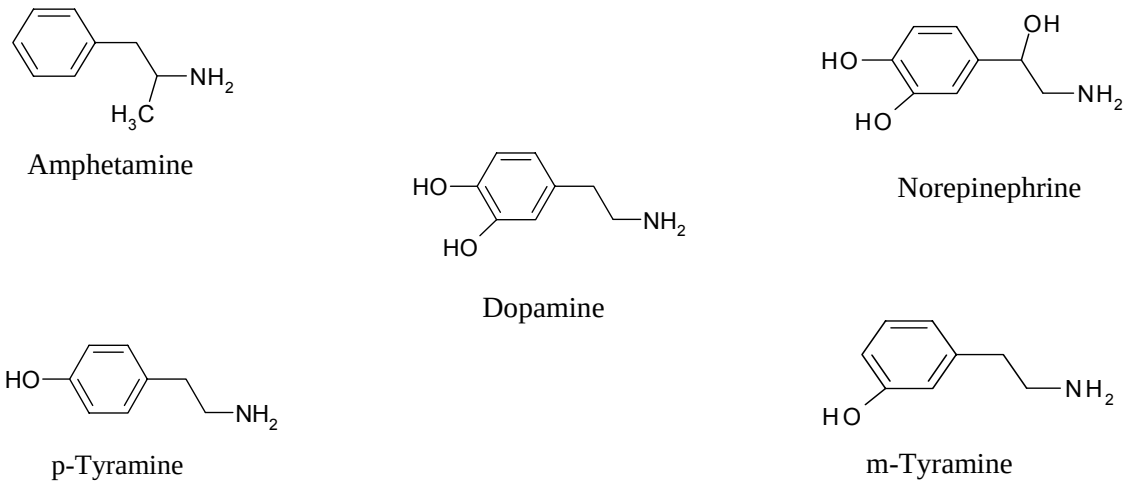


Figure 6. Chemical structures of DAT substrates employed in [^{3}H]-WIN35,428 and [^{3}H]-dopamine displacement assays.

The K_m for dopamine uptake and the K_i for dopamine inhibition of [^{3}H]WIN35,428 binding were unaffected by the D79E mutation (Table 1). These data contradict the long-held hypothesis of an ionic interaction between dopamine and aspartate-79 as being the principal means of interaction between dopamine and the DAT. Norepinephrine (addition of a β-OH group to dopamine) was equipotent at both WT DAT and D79E DAT at inhibiting dopamine uptake. On the other hand, the D79E mutation caused a two-fold loss in the binding affinity of norepinephrine (Table 1). The D79E mutation had little or no effect on the DUIPs of m-tyramine or p-tyramine (each lacking one of the catechol OH groups found in dopamine); the mutation, nevertheless, decreased the binding affinities of both compounds by more than 5-fold (Fig 7, and Table 1). The D79E mutation induced a 5-fold loss in the DUIP and a 9-fold loss in the binding affinity of *S*(+)-amphetamine (lacking both catechol OH groups and adding an α–methyl group) (Table 1). The DUIP of the less psychoactive *R*(-)-amphetamine isomer was not significantly affected by the D79E mutation, but its binding affinity was diminished by about 4-fold (Table 1). The DUIPs for m-tyramine, p-tyramine and (-)-amphetamine at both DAT constructs suggest that loss of one or both catechol OH groups was tolerated by D79E DAT, while the binding affinities of the three substrates were markedly diminished by the mutation.

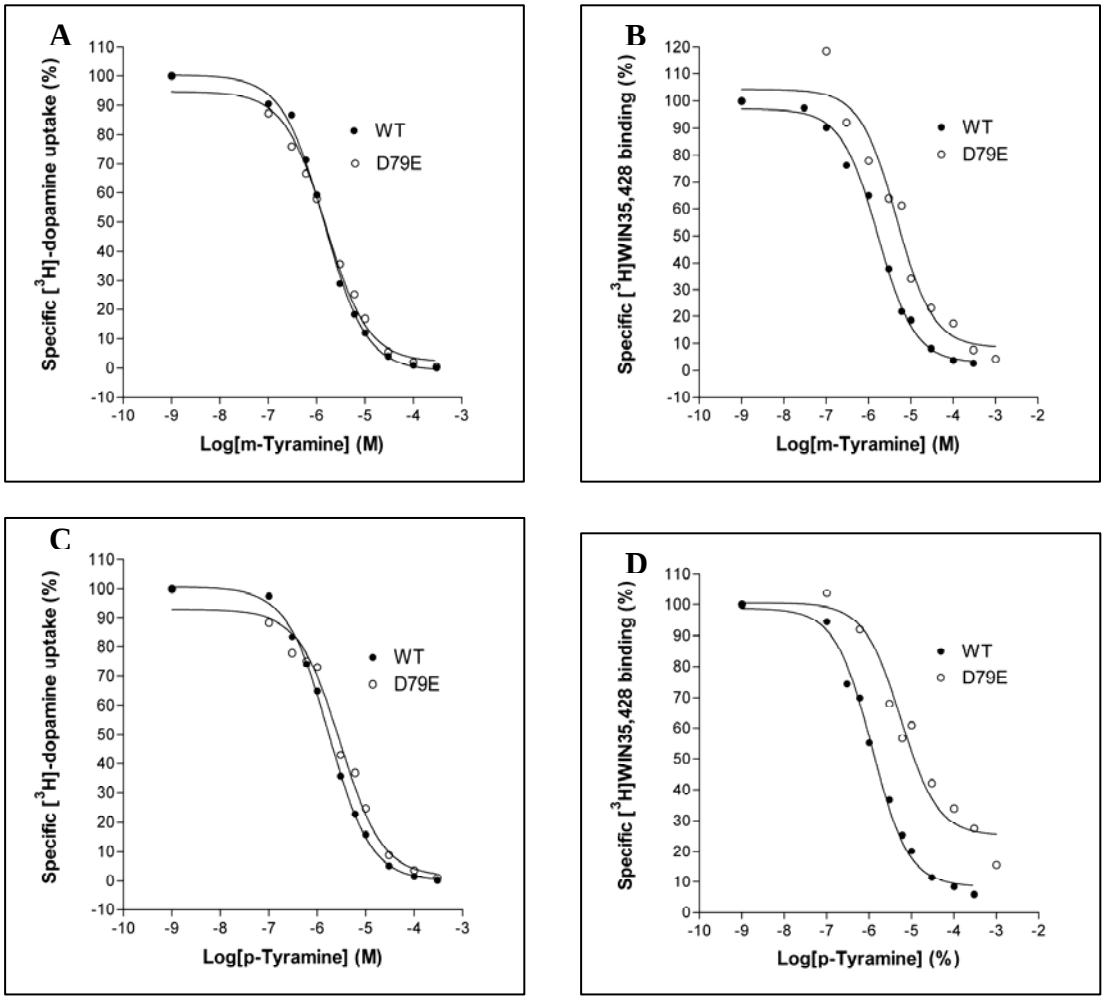


Figure 7. m-Tyramine or p-Tyramine inhibition of [^{3}H]-dopamine uptake or [^{3}H]WIN35,428 binding.

The [^{3}H]-dopamine uptake inhibiton (A and C) and inhibition of [^{3}H]WIN35,428 binding (B and D) experiments were done under identical conditions at CHO cells stably transfected with WT DAT (filled symbols) or D79E DAT (open symbols). The data are representative of at least three independent experiments.

Table 1. Effect of DAT substrates on CHO cells stably expressing WT and D79E DAT.

K_M and K_i values were derived from experiments incubating the stably transfected cells with nonradioactive substrates in the presence of [^{3}H]-dopamine or [^{3}H]-WIN35,428 at 22^{O}C in KRH/AA buffer. Values are presented as mean ± S.E.M. for three to six independent experiments. Reprinted with permission from ASPET from Wang, Sonders, Ukairo et al., 2003.

	K_i or K_M	
	WT DAT	**D79E DAT**
	(μM)	
[^{3}H]- dopamine uptake		
Dopamine	1.7 ± 0.3	2.3 ± 0.3
Norepinephrine	5.4 ± 0.5[a]	5.2 ± 0.3[a]
m-Tyramine	1.4 ± 0.3	1.2 ± 0.3[a]
p-Tyramine	1.7 ± 0.1	3.3 ± 0.4[b]
(+)-Amphetamine	0.5 ± 0.1	2.4 ± 0.3[b]
(-)-Amphetamine	2.8 ± 0.5	4.2 ± 0.5[a]
[^{3}H]-WIN35,428 inhibition		
Dopamine	2.1 ± 0.2	2.5 ± 0.4
Norepinephrine	2.3 ± 0.5[c]	5.5 ± 0.7[a,b]
m-Tyramine	1.0 ± 0.2[a]	6.6 ± 1.2[a,b,c]
p-Tyramine	0.8 ± 0.1[a,c]	4.3 ± 0.5[b]
(+)-Amphetamine	0.4 ± 0.1[a]	3.7 ± 0.8[b]
(-)-Amphetamine	2.0 ± 0.2	8.1 ± 0.5[a,b,c]

[a] P<0.05 versus dopamine for the given assay and DAT construct (Student's T test)
[b] P<0.05 versus WT DAT for that assay (Student's T test)
[c] P<0.05 for [^{3}H] for WIN inhibition versus dopamine uptake at the same DAT construct (Student's T test)

3. Effect of DAT inhibitors on CHO cells stably expressing WT or D79E DAT

WT DAT and D79E DAT stably-transfected CHO-K1 cell lines were employed to characterize the DUIPs and binding affinities of various DAT inhibitors (Fig 8). In order to better assess the key functional groups important for DAT recognition, DAT inhibitors containing various pharmacophore modifications of cocaine were selected for analysis. The K_i values for DUIP and binding affinity were obtained from inhibition of [^{3}H]-dopamine uptake and [^{3}H]-WIN35,428 binding inhibition experiments respectively; both assays were carried out under the same conditions. Previously, it was shown that the D79E DAT mutation had little or no effect on the DUIPs of the classical DAT inhibitors cocaine, WIN35,428, methylphenidate and mazindol (Wang et al., 2003). Except for benztropine (structurally different from cocaine by having a diphenylmethoxy group at the C-3 position), all other drugs studied here displayed this same DUIP pattern. The DUIP of 8-oxa-norcocaine, an analog of cocaine lacking the tropane nitrogen and thus a positive charge, was unaffected by the D79E mutation (Table 2). Furthermore, the DUIPs of 4-ARA-127, rimcazole and GBR-12909 (all compounds containing the piperidine or piperazine substitution of the tropane ring) were unaffected by the D79E DAT mutation (Fig. 9, and Table 2). In contrast, the DUIP of benztropine was 8 fold higher at D79E DAT than at WT DAT, a deviation from the trend observed for the other drugs (Fig 9 and Table 2).

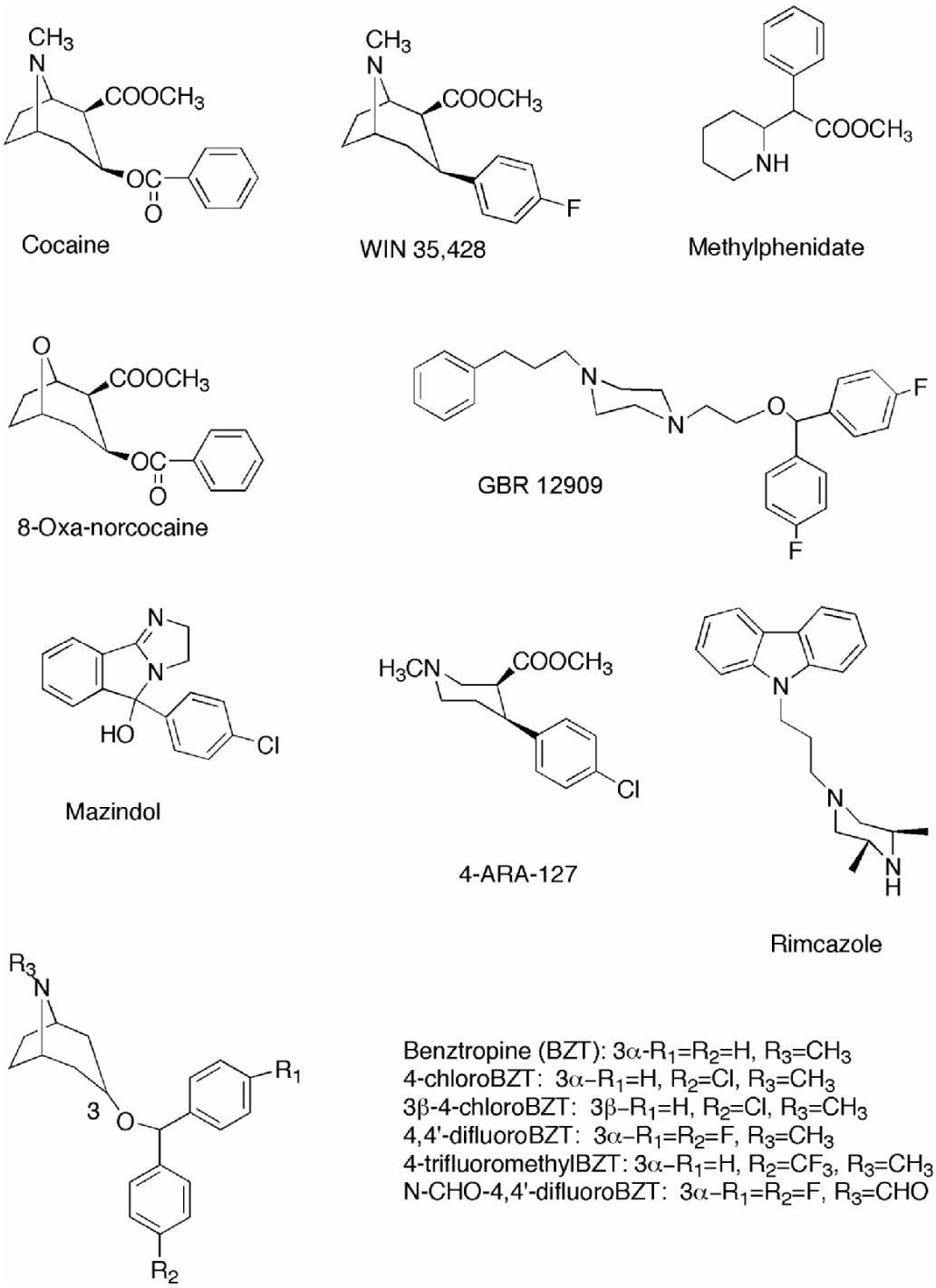


Figure 8. Chemical structures of DAT inhibitors employed in [^{3}H]-WIN35,428 and [^{3}H]-dopamine displacement assays.

Reprinted with permission from ASPET from Ukairo et al., 2005.

The mutation had a different effect on the binding affinities of the drugs studied. The binding affinities of cocaine, WIN35,428, 8-oxa norcocaine, mazindol, and methylphenidate were about 3-8 fold less at D79E DAT when compared with WT DAT values. The mutation had no effect on the binding affinities of GBR-12,909 and rimcazole but elevated the binding affinity of ARA-127 by 2-fold (Table 2). As was earlier reported for cocaine, WIN35,428, methylphenidate, and mazindol, the binding affinities for rimcazole and 8-oxanorcocaine were 3-6 fold higher than their DUIPs at WT DAT. The binding affinities for 4-ARA-127 and GBR-12,909 at WT DAT were 2 fold lower than their DUIPs (Table 2). On the other hand, benztropine was equipotent at inhibiting [^{3}H]WIN35,428 binding and [^{3}H]dopamine uptake at WT DAT (Tables 2 and 3). The binding affinities and DUIPs of cocaine, mazindol, methylphenidate, WIN35,428, and 4-ARA-127 at D79E DAT were identical (Table 2). For 8-oxa-norcocaine and rimcazole, however, the binding affinity at D79E DAT was 2 - 4 fold higher than DUIP. Benztropine and GBR-12,909 were 3 fold less potent as inhibitors of [^{3}H]WIN35,428 binding than as inhibitors of [^{3}H]dopamine uptake at D79E DAT (Tables 2 and 3).

Seeing that the D79E mutation affected only the DUIP of benztropine, the pharmacological profiles of structural analogs of this drug were further examined at WT and D79E DAT. Except for 4',4"-difluoroBZT and GBR-12,909, the DUIPs of all other benztropine analogs were diminished by the D79E mutation but to different extents (Table 3). Intriguingly, the considerably different DUIPs of the

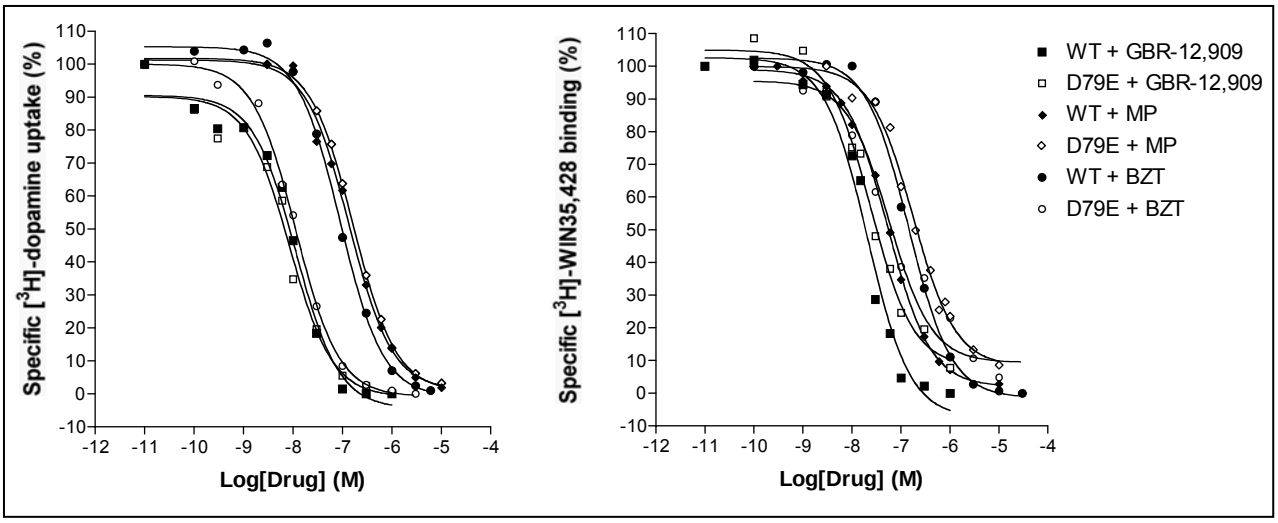


Figure 9. Effect of DAT inhibitors on CHO cells stably expressing WT or D79E DAT

GBR-12,909 (squares), methylphenidate (diamonds) and benztropine (circles) inhibition of [^{3}H]-dopamine uptake (left graph) or [^{3}H]-WIN 35,428 binding (right graph) under identical conditions at CHO cells stably transfected with WT DAT (filled symbols) or D79E DAT (open symbols). The data are representative of at least 3 independent experiments. Reprinted with permission from ASPET from Ukairo et al., 2005.

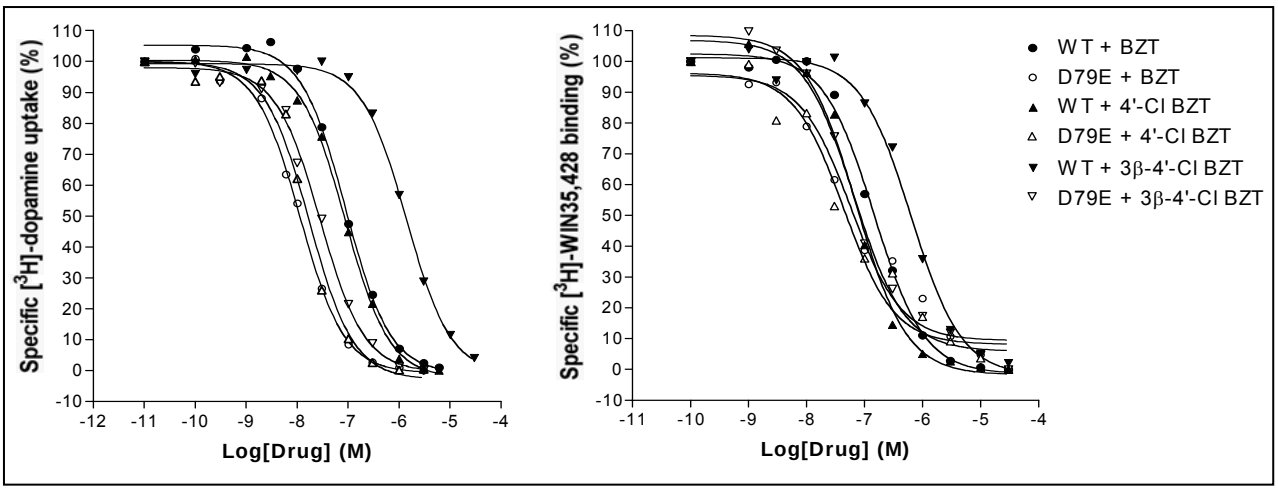


Figure 10. Effect of benztropine and BZT analogs on CHO cells stably expressing WT or D79E DAT.

Benztropine (circles), 4'-chlorobenztropine (upright triangles) and 3β-4'-chlorobenztropine (inverted triangles) inhibition of [^{3}H]-dopamine uptake (left graph) or [^{3}H]-WIN 35,428 binding (right graph) under identical conditions at CHO cells stably transfected with WT (filled symbols) or D79E DAT (open symbols). The data are representative of at least 3 independent experiments. Reprinted with permission from ASPET from Ukairo et al., 2005.

benztropine analogs (ranging from 15 – 964 nM) at the WT DAT were unified to approximately 20 nM at D79E DAT (Fig. 10 and Table 3). Additional benztropine analogs were investigated to assess the effects of adding larger groups to the tropane and phenyl rings. Replacement of the N-methyl group of 4',4"-difluoroBZT with the charge-neutral N-formyl group (N-CHO-4,4'-diFBZT) caused a profound reduction in DUIPs at both WT and D79E DAT (Table 3). Although the D79E mutation increased the DUIP of this compound, the K_i for inhibiting dopamine uptake diverged from the K_i of 20 nM at D79E DAT observed for other benztropine analogs. The binding affinity of N-CHO-4,4'-diFBZT was increased by 3 fold by the mutation. 4'-trifluoromethylBZT, a benztropine analog having a larger aryl group on one of the phenyl rings, also diverged from the 20 nM DUIP D79E DAT pattern. The D79E mutation increased the DUIP of 4'-trifluoromethylBZT by about 4 fold but had no effect on its binding affinity (Table 3). Interestingly, the 3β-4-chlorobenztropine compound (with its diphenyl ether group in the β position) also yielded a DUIP of approximately 20 nM at D79E DAT. The mutation caused a 37-fold increase in the DUIP of 3β-4-chlorobenztropine in order to achieve the 20 nM DUIP.

For most of the benztropine analogs, the D79E mutation had no effect on their binding affinities. Increased binding affinities due to the mutation were observed only for 3β-4-chloroBZT (3 fold) and N-formyl-difluoroBZT (5-fold) (Table 3). The DUIP data of benztropine and its analogs suggested a connection between modifications of the tropane ring C-3 position of cocaine and modifications of the DAT D79 side chain.

Table 2. Dopamine uptake inhibition potencies and binding affinities of structurally diverse DAT inhibitors at CHO cells stably expressing WT or D79E DAT.

K_d (WIN35,428 only) and K_i values were derived from experiments incubating the stably-transfected cells with nonradioactive ligands in the presence of [^{3}H]dopamine or [^{3}H]WIN35,428 at 22^{o}C in KRH/AA buffer. Values are presented as Mean ± SEM for 3 - 6 independent experiments. Of the 9 structurally diverse DAT blockers studied, only the DUIP of benztropine was substantially altered by the D79E mutation. Reprinted with permission from ASPET from Ukairo et al., 2005.

	K_i (nM)	
	WT	**D79E**
[^{3}H]- Dopamine uptake		
WIN35,428[a]	74 ± 17	71 ± 12
ARA-127	1630 ± 200	1540 ± 120
Cocaine[a]	555 ± 29	1126 ± 32[b]
Oxa-norcocaine	9777 ± 1489	8196 ± 340
Mazindol[a]	31 ± 3	47 ± 4
Methylphenidate[a]	152 ± 20	161 ± 9
Benztropine	160 ± 37	19 ± 3[b]
GBR-12,909	15 ± 3	40 ± 9
Rimcazole	643 ± 43	691 ± 76
[^{3}H]-WIN35,428 inhibition		
WIN35,428[a]	20 ± 1[c]	68 ± 4[b]
ARA-127	3300 ± 40[c]	1570 ± 210[b]
Cocaine[a]	128 ± 5[c]	1066 ± 84[b]
Oxa-norcocaine	1564 ± 212[c]	5533 ± 653[b,c]
Mazindol[a]	13 ± 1[c]	41 ± 3[b]
Methylphenidate[a]	57 ± 3[c]	167 ± 2[b]
Benztropine	127 ± 22	51 ± 5[b,c]
GBR-12,909	31 ± 5	40 ± 9
Rimcazole	180 ± 26[c]	226 ± 38[c]

[a] Wang et al. (2003) *Mol. Pharmacol*. 64: 430 - 439
[b] P<0.05 versus WT DAT for that assay
[c] P<0.05 for WIN inhibition vs. dopamine uptake at the same DAT construct

Table 3. Dopamine uptake inhibition potencies and binding affinities of benztropine and its analogs at CHO cells stably expressing WT or D79E DAT.

K_i values were derived from experiments incubating the stably-transfected cells with nonradioactive inhibitors in the presence of [^{3}H]dopamine or [^{3}H]WIN35,428 at 22^{o}C in KRH/AA buffer. Values are presented as mean ± SEM for 3 - 6 independent experiments. Reprinted with permission from ASPET from Ukairo et al., 2005.

	K_i (nM)	
	WT DAT	**D79E DAT**
[^{3}H] - Dopamine uptake		
Benztropine	160 ± 37	19 ± 3[a]
4-chloroBZT	83 ± 6	19 ± 3[a]
3β-4-chloroBZT	964 ± 161	26 ± 4[a]
4,4'-difluoroBZT	22 ± 4	18 ± 3
4-trifluoromethylBZT	1282 ± 79	330 ± 36[a]
N-CHO-4,4'-diFBZT	21760 ± 2857	1575 ± 452[a]
GBR-12,909	15 ± 3	14 ± 3
[^{3}H]–WIN35,428 inhibition		
Benztropine	127 ± 22	51 ± 5[a,b]
4-chloroBZT	45 ± 6[b]	44 ± 10[b]
3β-4-chloroBZT	680 ± 123	120 ± 35[a,b]
4,4'-difluoroBZT	8 ± 1[b]	9 ± 2[b]
4-trifluoromethylBZT	541 ± 71	359 ± 25
N-CHO-4,4'-diFBZT	12730 ± 1996	3840 ± 746[a]
GBR-12,909	31 ± 5	40 ± 9

[a]P<0.05 versus WT DAT for that assay
[b]P<0.05 for WIN inhibition vs. dopamine uptake at the same DAT construct

4. Effects of cell age, density and DAT cell surface expression on the DUIPs of classical DAT inhibitors

Previous observations indicated that the DUIP of cocaine at WT DAT varied with cell age (C.K. Surratt, unpublished data). The study was enlarged to determine whether this phenomenon was unique to cocaine or could be extended to other classical DAT inhibitors such as mazindol, methylphenidate, and benztropine. The influence of cell density (%confluence) and DAT cell surface expression levels on inhibitor DUIP fluctuation was also investigated. The WT DAT-CHO K1 stable cell line was used for the study. Cells in passages 9 - 20, 25 - 36, and 40 - 54 were described as "low", "medium", and "high" passage cells, respectively. The DUIPs for these classical DAT blockers differed between "low passage" (P9 - P20) and "high passage" (P40 - P54) WT DAT CHO cells in a statistically significant fashion (Fig 11 and Table 4). The K_i value for inhibition of [^{3}H]dopamine uptake (for all the drugs tested) was about 2 fold less at low passages when compared with high passage values. In contrast, assays conducted in parallel indicated that apparent binding affinities (measured by displacement of [^{3}H]-WIN35,428) of these 4 inhibitors were not subject to cell passage number. WT DAT B_{max} values for [^{3}H]-WIN35,428 binding varied significantly between the "low" and "high" passage cells; however, the K_d values for all treatment groups remained unchanged (Table 4).

The DUIP of cocaine at WT DAT-CHO cells grown to different densities in 6-well plates was also investigated. Neither the DUIP nor the apparent binding affinity of cocaine was influenced by the density (% confluence) of the cell

monolayer (Table 5). The cells were grown to 20%, 100% and 150% confluence. The monolayer is defined as “100% confluent” when all cells appeared to be in contact with neighboring cells, so as not to leave open spaces on the culture dish.

Manipulation of WT DAT expression level via transient transfection of COS-7 cells with an amount of DAT plasmid cDNA 4-fold lower than optimal resulted in a statistically significant fluctuation in the dopamine uptake inhibition potency of cocaine (Table 6).

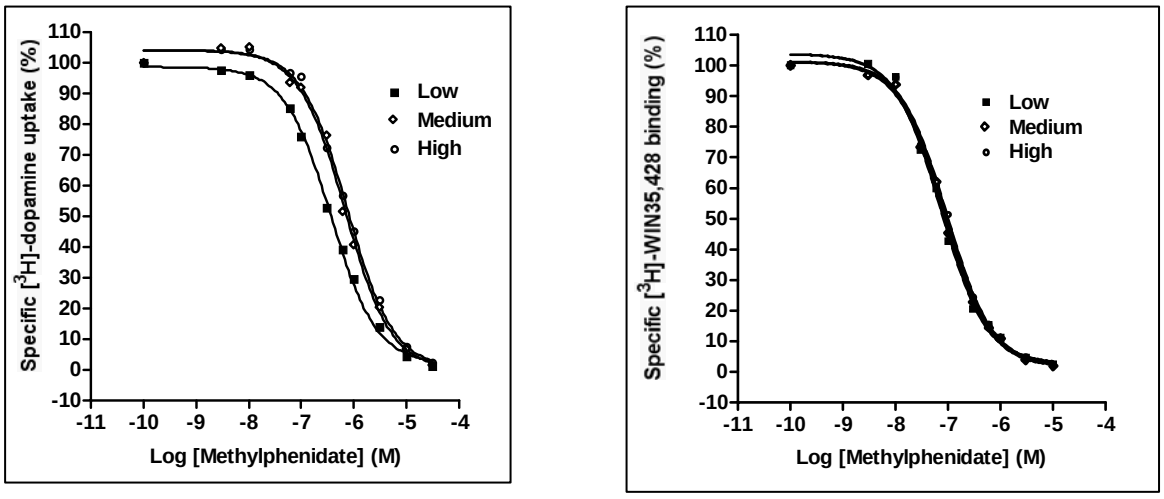


Figure 11. Effect of methylphenidate on WT DAT-CHO cells of different passage numbers. Methylphenidate inhibition of [^{3}H]-dopamine uptake (left panel) or [^{3}H]-WIN35,428 binding (right panel) under identical conditions at WT DAT CHO cells of different passage number. The data are representative of at least 3 independent experiments. Passages of 9 -20, 25 - 36 and 40 - 54 were classified as “low”, “medium” and “high”, respectively.

Table 4. Effect of DAT blockers on WT DAT-CHO cells of different passage numbers.

Inhibition of [^{3}H]-dopamine uptake or [^{3}H]-WIN35,428 binding by classical DAT blockers under identical conditions at WT DAT CHO cells of different passage number. Ki values were derived from experiments incubating the stably-transfected cells with nonradioactive DAT inhibitors in the presence of [^{3}H]-dopamine or [^{3}H]-WIN 35,428 at 22^{O}C in KRH buffer. Passages of 9 -20, 25 - 36 and 40 - 54 were classified as "low", "medium" and "high", respectively. (The ranges for an individual drug were narrower, as testing with a given inhibitor was completed before the next inhibitor was addressed). Mean ± SEM for at least 3 independent experiments.

	Cell Passage		
	Low	**Medium**	**High**
		K_i (nM)	
[^{3}H]-DA uptake inhibition			
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]
[^{3}H]-WIN35,428 inhibition			
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
[^{3}H]-WIN35,428 binding			
K_d (nM)	17 ± 1	17 ± 2	18 ± 1
B_{max} (pmol/mg)	9 ± 2	11 ± 2	19 ± 3[b]

[a]$P < 0.05$ vs. low passages for the same drug in the uptake assay (one-way ANOVA, Newman-Keuls post hoc test)

[b]$P < 0.05$ vs. low passages in the [^{3}H]WIN35,428 saturation binding assay (one-way ANOVA, Newman- Keuls post hoc test).

Table 5. Effect of cell density on the DUIP and binding affinity of cocaine at WT DAT-CHO cells.

Cocaine inhibition of [^{3}H]-dopamine uptake or [^{3}H]-WIN35,428 binding under identical conditions at WT DAT CHO cells as a function of cell density (% confluence). Experiments were carried out with intact cell monolayers at 20%, 100%, and 150% confluence. K_i values were derived from experiments incubating the stably-transfected cells with cocaine in the presence of [^{3}H]-dopamine or [^{3}H]-WIN35,428 at room temperature in KRH buffer. Data are mean ± SEM for 3 - 4 experiments.

	Confluence (Cell Density)		
	20%	**100%**	**150%**
	K_i (nM)		
Cocaine			
[^{3}H]-DA uptake inhibition	505 ± 108	534 ± 109	540 ± 62
[^{3}H]-WIN35,428 inhibition	260 ± 25	240 ± 12	201 ± 16

Kinetic analysis of [^{3}H]dopamine uptake revealed a significant increase ($P<0.05$, one-way ANOVA; n = 4) in V_{max} of dopamine uptake in WT DAT-CHO cells with increasing cell passage number (low<medium<high) (Fig 12 and Table 7). This increase in V_{max} was associated with an increase in WT-DAT cell surface expression (B_{max}) at higher passage numbers. The turnover number of WT DAT (expressed as V_{max}/B_{max}), however, did not vary significantly between the “low”, “medium” and “high” passage cells (Table 7). A statistical comparison of the V_{max}/B_{max} values for the different groups was not possible as these are ratios of mean values. “Low”, “medium” and “high” passage cells showed no significant changes in K_m values for [^{3}H]dopamine uptake.

Table 6. Effects of DAT cell surface expression on the DUIP of cocaine

Cocaine inhibition of [^{3}H]-dopamine uptake by COS-7 cells transiently transfected with various amounts of a plasmid cDNA encoding WT DAT. "PolyFect"-mediated transient transfections were carried out using 375, 750 or 1500 ng WT DAT - pIRES plasmid per 35 mm well containing a confluent monolayer of COS-7 cells. Cell monolayers were assayed for [^{3}H]-dopamine uptake and its inhibition by cocaine at room temperature in KRH buffer 48 hours after transfection. Ki values are the Mean ± SEM for 3 experiments.

	WT DAT – pIRES Plasmid		
	ng DNA / 35 mm well		
	375	750	1500
		K_i (nM)	
[^{3}H]-DA uptake inhibition			
Cocaine	228 ± 7	353 ± 20	558 ± 75[a]
[^{3}H]-WIN35,428 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]$P < 0.05$ vs. dopamine uptake inhibition Ki values for assays employing 375 ng plasmid transfections (one-way ANOVA, Newman-Keuls post hoc test)

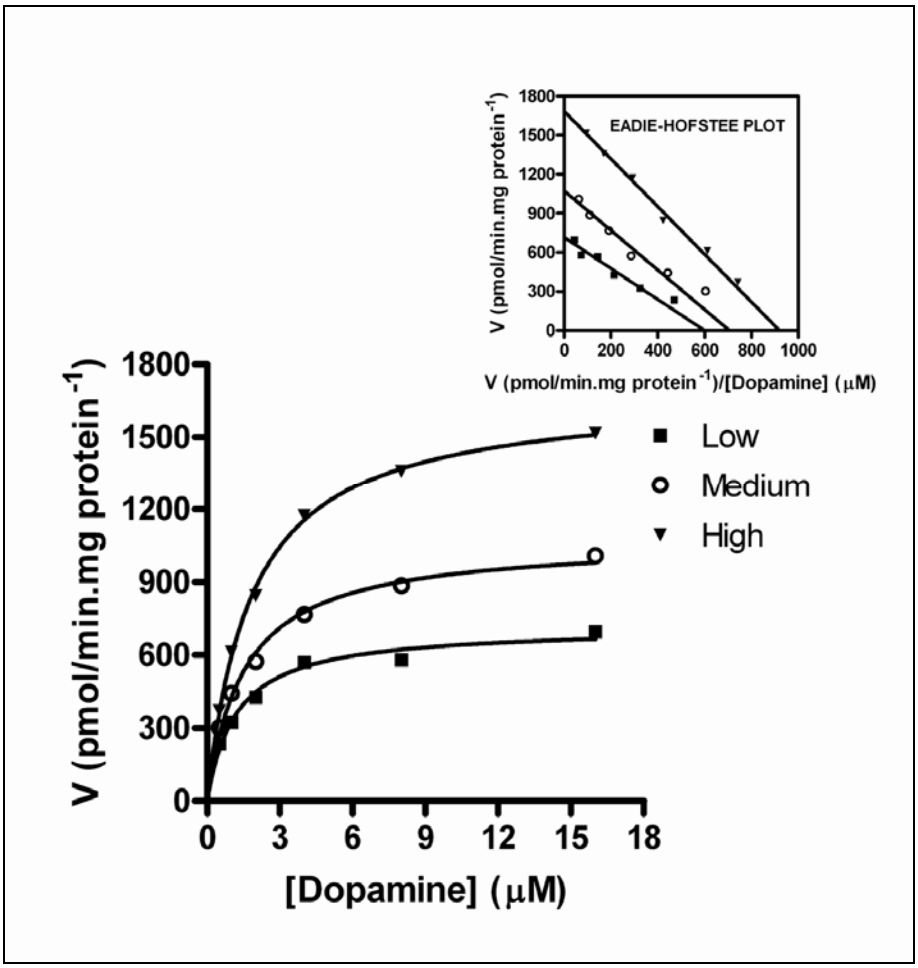


Figure 12. Dopamine uptake saturation kinetics of WT DAT CHO cells of different passage numbers.

CHO cells stably transfected with WT DAT were incubated with increasing concentrations of a fixed ratio of [^{3}H]dopamine: nonradioactive dopamine (0.5 – 16 μM dopamine at 0.1 Ci/mmol) as indicated in the method section. The data are representative of 4 independent experiments. Passages of 9 -20, 25 - 36 and 40 - 54 were classified as "low", "medium" and "high", respectively. K_m and V_{max} values were determined by fitting the rate versus concentration data. The inset shows an Eadie- Hofstee plot of the data with lines drawn from the derived kinetic constants. See Table 7 for a summary of the calculated data.

Table 7. Kinetic and binding characteristics of rDAT wild-type in CHO cells of different passage numbers.

K_m and V_{max} values were derived from uptake experiments incubating the WT DAT cells with dopamine at 0.1 Ci/mmol for 5 min at 22°C. Nonlinear regression analysis was employed to accurately determine K_m and V_{max} values. B_{max} values were derived from [^{3}H]WIN35,428 saturation binding experiments. K_m, V_{max}, and B_{max} values are the mean ± SEM for at least three independent experiments.

	Cell Passage		
	Low	**Medium**	**High**
V_{max}(pmol/min.mg protein^{-1})	681 ± 18	1136 ± 85[a,b]	1645 ± 202[a]
K_m (μM)	1.1 ± 0.2	1.4 ± 0.2	2.0 ± 0.4
B_{max} (pmol/mg)	9 ± 2	11 ± 2	19 ± 3[c]
V_{max}/B_{max}	76/min	103/min	87/min

[a]P < 0.05 vs. low passages in the uptake assay (one-way ANOVA, Newman-Keuls post hoc test)
[b]P < 0.05 vs. high passages in the uptake assay (one-way ANOVA, Newman-Keuls post hoc test)
[c]P < 0.05 vs. low passages in the [^{3}H]WIN35,428 saturation binding assay (one-way ANOVA, Newman- Keuls post hoc test).

B. Discussion

A TM 1 aspartic acid residue (Position 79 in the DAT) is conserved among the NSS proteins that conduct the aromatic monoamine neurotransmitters dopamine, norepinephrine, epinephrine and serotonin across the plasma membrane. This negative charge on aspartic acid has been proposed to serve as the counter-ion for the positively charged nitrogen atom present in all classical DAT inhibitors including cocaine (Carroll et al., 1992). In the present study alanine, leucine, asparagine or glutamate substitutions at this position were generated in order to assess the importance of side chain length, hydrogen bonding potential and negative charge for recognition of DAT inhibitors. Only the D79E DAT mutant displayed detectable specific binding of [^{3}H]-WIN35,428 (Fig. 1) or functional uptake of [^{3}H]dopamine (Fig. 2). Consequently, only WT DAT and D79E DAT stably transfected cell lines were employed in the present study with a range of DAT substrates as well as DAT blockers either structurally dissimilar to cocaine or containing variations of the 3 primary components of the cocaine pharmacophore, the positively charged tropane nitrogen atom, the tropane ring, and the C-3 aromatic substituent.

Using various dopamine analogs, the possibility that D79 is involved in recognition of some feature of the substrate catechol ring moiety was tested. The dopamine uptake inhibition potencies for the tyramines and (-)-amphetamine at WT versus D79E DAT indicated, however, that loss of one or both catechol hydroxyl groups were essentially tolerated by D79E DAT. Binding affinities for these three substrates, on the other hand, were reduced several fold by the

mutation. The result suggests that different DAT conformations or populations are responsible for DUIP and high affinity binding. Thus, one DAT conformation or population is apparently more sensitive to alterations in the substrate catechol moiety than is a second DAT conformation/population. An "aromatic pocket" role for the DAT D79 residue is consistent with the fact that the aspartate is conserved among the NSS proteins whose substrates bear an aromatic moiety. A glycine residue is found in the analogous position for members of the same transporter family that recognize nonaromatic substrates (eg. Glycine, GABA, betaine) that nevertheless retain the protonated amino group (Wang et al., 2003). As stated above, the aromatic moiety of dopamine would be the most logical substrate functional group to show dependence on the D79 DAT residue.

To investigate the importance of the positive charge of cocaine to its action at the DAT, we employed a compound for which the N-8 of the tropane ring was replaced with a charge-neutral oxygen atom, a modification that has previously been shown to spare dopamine uptake inhibition potency (DUIP) and specific binding at WT DAT (Kozikowski et al., 1999). The K_i for inhibition of dopamine uptake by 8-oxa-norcocaine at D79E DAT was similar to WT values; binding affinity was, however, diminished by almost 4 fold (Table 1). The glutamate-for-aspartate switch is often thought of as a conservative mutation. Recent findings indicate that although these two amino acids differ only by one methylene group, they have different spatial preferences (Johnson and Peterson, 2001). Furthermore, aspartic acid has been shown to favor interactions between the side chain carbonyl group and a backbone carbonyl group resulting in a ring-

like structure which is not observed for glutamic acid (Deane et al., 1999). Consequently, if D79 is actually situated in the binding pocket of the DAT, a D79E mutation should introduce some steric hindrance to the ligand binding cavity considering the different spatial preferences of the two amino acids. The observation that the DUIP of this charge-neutral cocaine analog is unaltered by an allegedly more crowded binding pocket is not in agreement with (but not exclusive of) the postulate that a salt bridge between the D79 residue and the N-8 atom is the principal, governing DAT - cocaine interaction. The 8-oxa-norcocaine compound paralleled cocaine in that K_i values suggest its affinity to be several fold greater than its DUIP at WT DAT, and in that its affinity and DUIP values were less than 2 fold apart at D79E DAT.

To verify the significance of the cocaine tropane ring with respect to the D79 side chain, DAT inhibitors that possess a piperidine or piperazine in place of the tropane ring were employed in binding and uptake inhibition assays (Table 2). A number of piperidine-based cocaine analogs have been shown to be effective in blocking binding of DAT ligands or dopamine uptake at the DAT (Kozikowski et al., 1998; Tamiz et al., 2000), demonstrating that the tropane ring of cocaine is not absolutely required for cocaine action at the transporter. The compound employed in the present work, 4-ARA-127, is a WIN35,428 analog that lacks the 6,7-bridgehead of the tropane ring and contains a *p*-chlorophenyl instead of a *p*-fluorophenyl substituent on what would be C-3 of the tropane ring (Fig. 3). The D79E mutation had little or no effect on the pharmacology of this piperidine-based cocaine analog (Table 2). The D79E mutation also did not alter

the pharmacological profile of the piperazine-based compounds, GBR-12,909 and rimcazole (Table 2). These data indicate that the role of the D79 residue may not include recognition of the tropane ring (or piperidine/piperazine ring replacement) of these DAT inhibitors.

It was predicted that the third position of the cocaine pharmacophore, an aromatic group at the tropane C-3 position, would be the most sensitive to alterations of the DAT D79 side chain. For an inhibitor that directly blocks dopamine binding at a DAT site in which the D79 residue is crucial, an aromatic ring would be the more logical primary pharmacophore for this role. An aromatic moiety is obligatory at the tropane C-3 position of cocaine for DAT inhibition, and even for inhibitors missing the tropane structure an aromatic ring is necessary (Carroll et al., 1992, Newman and Kulkarni, 2002). Based on this fact, the contribution of the tropane C-3 substituent to DAT inhibition was investigated in detail by testing benztropine, possessing one of the more pronounced structural variations at this position, with the D79E mutant. Of 9 structurally diverse DAT blockers initially studied, only the DUIP of benztropine was altered to a large extent by the D79E mutation (Table 2).

It is possible that the D79E mutation influences recognition of the common diphenylmethoxy pharmacophore given that benztropine and several of its analogs yielded the same DUIP at D79E DAT despite often very different DUIPs at WT DAT (Table 3). Unlike WT DAT, the D79E DAT protein was by and large tolerant of diphenylmethoxy ring substituents, especially surprising considering the marked reorientation of the diphenylmethoxy moiety in the 3β-4'-

chlorobenztropine derivative. Optimal binding at the DAT is achieved when the diphenyl ether of benztropine is in the axial or α-stereochemistry; both aryl rings are required and cannot be held in a rigid cyclic structure (reviewed in Newman and Kulkarni, 2002). The pharmacological profile of the different benztropine analogs at D79E DAT suggests that the D79E substitution may have enhanced DAT interaction with the diphenylmethoxy moiety possibly by providing a "roomier" binding pocket for this pharmacophore that accommodates different halogen substituents and ring orientations. Both of the phenyl rings of benztropine may simultaneously contribute to inhibition of D79E DAT, but likely not when in the same plane. The phenyl rings of rimcazole are fused and thus constrained in space by a bond that creates an intervening pyrrole ring (Fig. 8). Comparing results (Table 2) for rimcazole to those for GBR-12,909, its closest structural analog in this study, suggest that the phenyl rings must be able to rotate freely for potent dopamine uptake inhibition at the DAT. Furthermore, comparative molecular field analysis (CoMFA) of benztropines contends that the relative orientation of the phenyl rings is important for DAT affinity (Kline et al., 1997, Kulkarni et al., 2004).

There is accumulating evidence that benztropine employs a DAT binding site distinguishable from other DAT blockers. Newman and colleagues have identified dissimilarities between benztropine and cocaine actions at the DAT via distinctive structure-activity relationship profiles (Newman and Kulkarni, 2002). Benztropine altered the accessibility of alkylation agents to wildtype DAT cysteine residues in a pattern distinct from the pattern generated by cocaine,

WIN35,428 and mazindol (Reith et al., 2001). The latter study revealed a primary alkylation pattern difference at C90, a DAT extracellular loop cysteine residue that immediately follows TM 1. The notion that benztropine is recognized by a DAT protein region encompassing the TM 1 D79 residue is supported by other DAT structure-function findings. Chen and colleagues showed that the binding affinities for benztropine and GBR12,909, but not those of cocaine or WIN35,428, were Na^+ dependent at W84L DAT, a mutation of a TM 1 residue only 5 positions away from D79 (Chen et al., 2004).

In addition to different roles of the tropane ring nitrogen and the C-3 position aromatic functional groups between cocaine-like and benztropine-like compounds, the necessity and nature of the C-2 position substituent also differs between these structural classes of DAT inhibitors (Newman and Kulkarni, 2002). Cocaine and its analogues require a substituent in the C-2 position (*e.g.*, a 2-methyl ester for cocaine), a substituent not required for the benztropines to exhibit high affinity for DAT. Indeed, the only 2-methyl ester substituted benztropine with affinity for the DAT presents this substituent in a stereochemistry opposite to that of cocaine (Meltzer et al., 1994). These findings have been confirmed and expanded (Zou et al., 2002; Zou et al., 2003), and preliminary behavioral evaluation of the (+)-2-ester-substituted benztropines suggests that the C-2 position substituent plays a role in the behavioral profile of these compounds (Newman and Kulkarni, unpublished data).

From these SAR studies and the data herein, the influence of particular functional groups within the C-3 pharmacophore appears to be weighted toward

the aromatic ring system. In the benztropine class, the 4',4"-difluorophenyl ether provides optimal binding affinity at WT DAT. Molecular modeling of benztropine and its most divergent analogs with respect to WT versus D79E DAT pharmacology indicates that C-3 aromatic substituents that reduce DUIP at DAT fall outside of the optimal binding pocket (Ukairo et al., 2005). The same is true for the neutral tropane N-formyl group of AHN 2-032. Considering the significant DUIP increases for these compounds with the D79E mutation, it may be that the benztropine binding pocket is modified by the mutation to increase tolerance for these C-3 or tropane nitrogen modifications. With regard to the latter, it is possible that the cocaine and benztropine recognition sites of DAT differ in that D79 contributes to recognition of the C-3 pharmacophore of cocaine, but the tropane nitrogen of benztropine. Nevertheless, it should be taken into account that the D79E mutation-induced DUIP increase was only slightly more pronounced for N-formylbenztropine compared to benztropine itself (Table 2).

The mechanism by which the D79E mutation influences the DAT binding affinities of the various inhibitors tested remains uncertain. The D79E mutation had little or no effect on the DUIPs of cocaine, WIN35,428, mazindol and methylphenidate. Conversely, their binding affinities were reduced 3-4 fold by this mutation (Wang et al., 2003). The K_i value for displacement of [^{3}H]WIN35,428 binding at D79E DAT by these classical DAT inhibitors matched their K_i values for inhibition of [^{3}H]dopamine uptake. Binding K_i values for these drugs diverged from their DUIP K_i values at WT DAT. It appears that the mutation eliminates the DAT site, conformation or population responsible for

higher affinity binding of these drugs, while sparing the site/conformation/population primarily responsible for inhibition of dopamine uptake (Fig. 13) (Wang et al., 2003).

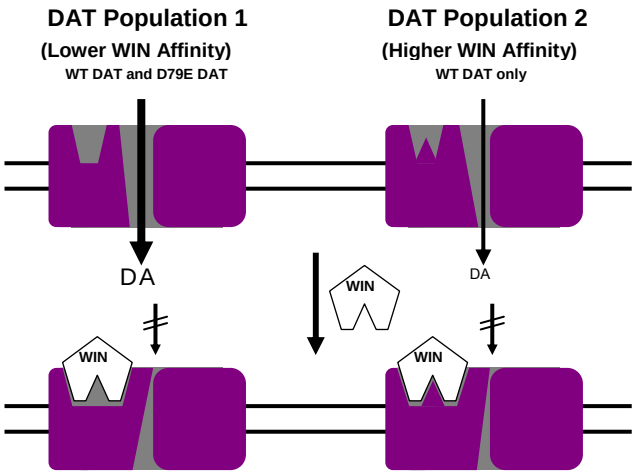


Figure 13. Model for dopamine uptake and WIN35, 428 binding at DAT

Two DAT populations (rectangular structures) are represented at the cell surface, within the plasma membrane (parallel horizontal lines). The large majority of [^{3}H]dopamine uptake is proposed to be mediated by DAT population 1; population 2 is thus under-represented with respect to total uptake of the dopamine radiotracer. Population 1 displays a lower affinity (~70 nM) for [^{3}H]WIN 35,428 (WIN, inscribed in quasi-pentagonal structure) than does population 2 (~20 nM); Population 1 is thus underrepresented with respect to total binding of the WIN 35,428 radiotracer. Higher levels (e.g., >500 nM) of WIN 35,428 (or other DAT inhibitors employed in the study) thoroughly eliminate dopamine uptake by both DAT populations, but the uptake inhibition curves reflect actions principally at population 1 (the primary [^{3}H]dopamine conduit). The D79E mutation eliminates DAT population 2, eliminating the higher affinity binding of DAT inhibitors but affording little or no effect on their dopamine uptake inhibition potencies. Reprinted from Wang, Sonders, Ukairo et al., 2003.

The 8-oxa-norcocaine data are also in conformity with this hypothesis (Table 2). In the case of benztropine and structurally analogous compounds, binding affinities and DUIPs at WT DAT were lower than, or at best, equal to,

those at D79E DAT (Table 3). Furthermore, binding affinities and DUIPs for these compounds at D79E DAT were dissimilar. For these drugs, the DAT site/conformation/population responsible for higher affinity inhibitor binding is likely to be different from that for cocaine, and may be affected differently by the D79E substitution.

The idea of separate DAT populations, as opposed to simply multiple binding sites or conformations, is supported by the observations that the DAT and other monoamine transporter proteins form functional homooligomeric complexes (Kilic and Rudnick, 2000; Hastrup et al., 2001; Schmid et al., 2001; Hastrup et al., 2003; Kocabas et al., 2003) as well as complexes with membrane and cytoskeletal proteins including Hic-5, PICK-1, α-synuclein and syntaxin 1A (Torres et al., 2001; Carneiro et al., 2002; Wersinger and Sidhu, 2003; Lee et al., 2004). In fact, the SNARE protein syntaxin 1A, inhibits substrate uptake via direct interaction with GABA, glycine, serotonin and norepinephrine transporters, all members of the NSS transporter family which includes the DAT (Beckman et al., 1998; Geerlings et al., 2000; Haase et al., 2001; Sung et al., 2003). It has been shown that the GABA transporter N-terminal aspartate residues directly interacts with syntaxin 1A to modulate transporter function (Hansra et al., 2004). It is conceivable, therefore, that the D79E mutation eliminates (or augments) DAT populations consisting of complexes with syntaxins or other endogenous factors (Ramsey and DeFelice, 2002). Some TM 1 mutations have been shown to alter Na^+ or Cl^- binding, in turn altering inhibitor affinities (Mager et al., 1996; Barker et al., 1999; Chen et al., 2004); possibly, the D79E mutation could

manipulate availability of DAT sites, conformations or populations in this way. It is intriguing to consider that structural modification of a DAT inhibitor may alter its preference for a certain DAT population, in turn selectively affecting its behavioral pharmacology.

The lack of correlation between DAT inhibitor DUIPs and apparent binding affinities at the WT DAT is well-documented (Pristupa et al. 1994; Eshleman et al. 1999) and has yet to be satisfactorily explained. In the course of this study using mammalian cell lines stably-transfected with WT DAT cDNAs, it was observed that the DUIP of a given inhibitor will fluctuate even though the apparent binding affinity of the drug does not fluctuate. In order to investigate the nature of this phenomenon, three possible influences on DAT function at the level of the cultured cell were investigated: the age of the cell line (measured by cell passage number), the density of the cell monolayer (*i.e.,* percent confluence), and the effect of varying DAT expression level by manipulation of transfection conditions. To ensure that comparisons between DUIP and apparent binding affinity were legitimate for a given DAT inhibitor, [^{3}H]-dopamine uptake assays, binding assays involving the cocaine analog [^{3}H]-WIN 35,428, and versions of each assay that included nonradioactive competitors were conducted under identical conditions. As reported above, a conservative glutamate-for-aspartate substitution in TM 1 of the rat DAT protein (D79E) decreased apparent binding affinities of several classical DAT inhibitors by 3 - 8 fold, yet yielded little or no effect on the DUIPs of these blockers (Wang et al., 2003). This finding implied that at least two discrete inhibitor-binding DAT sites,

conformations or populations exist, and that in this CHO cell system at least, the DAT site/conformation/population responsible for inhibitor high affinity binding is less responsible for dopamine uptake. The present findings indicate that the "age" of the WT DAT CHO cell line, as defined by cell passage number, appears to be relevant to the DUIP for the classical DAT blockers cocaine, mazindol, methylphenidate and benztropine. The DUIP of cocaine was also apparently subject to DAT expression levels. These results can best be explained by a model invoking two or more DAT populations, as opposed to two or more DAT binding sites or conformations within the same DAT population. The "cell passage number" experiment indicated that DUIPs for all inhibitors tested fluctuated suggesting that DAT function is regulated by an unidentified intracellular agent, the actions of which are sensitive to cell state (*e.g.,* cell age). Reducing DAT expression while holding cell passage number constant also caused cocaine DUIP to fluctuate. Taken together, these results lead us to postulate that inhibitor DUIP may be a property of the ratio of "naked" or unmodified DAT molecules to "complexed" or posttranslationally-modified DAT molecules. DAT complexes may include homooligomers or heterooligomers (with syntaxins or other endogenous proteins/factors capable of modulating DAT function). For example, cellular aging may convert most of a "complexed" or posttranslationally-modified population, which is less efficient in dopamine transport but more efficient in binding the inhibitor, to the naked or unmodified DAT population that is more efficient in dopamine transport but less efficient in binding the inhibitor. This hypothesis is the subject of further investigation in our

laboratory. Given the fact that DAT is a phosphoprotein as well as a glycoprotein, the use of inhibitors of DAT phosphorylation and glycosylation will be employed to determine the involvement of these pathways in the observed changes in inhibitor DUIP due to cell passages. In addition, the influence of DAT oligomerization state on the observed phenomenon will also be investigated.

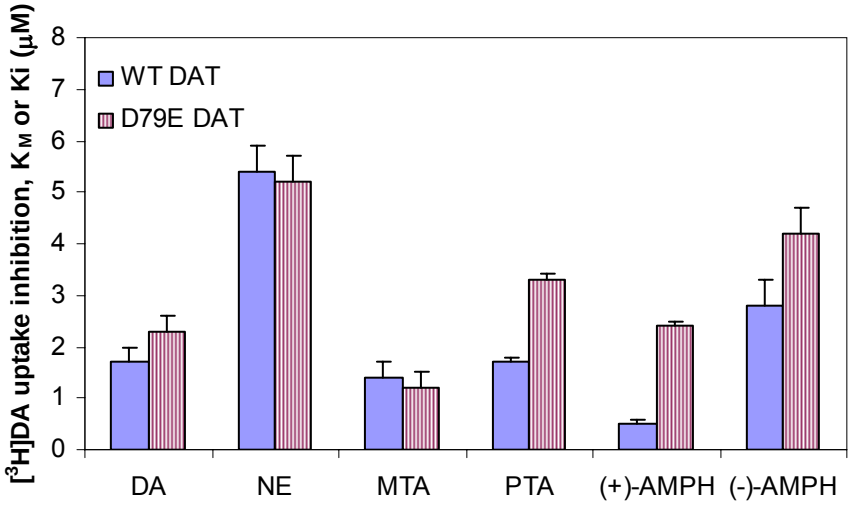
If certain DAT populations are more relevant to the mechanism(s) of action of cocaine, amphetamines and other psychostimulants, characterization of such populations may further development of novel therapeutics for these drugs of abuse.

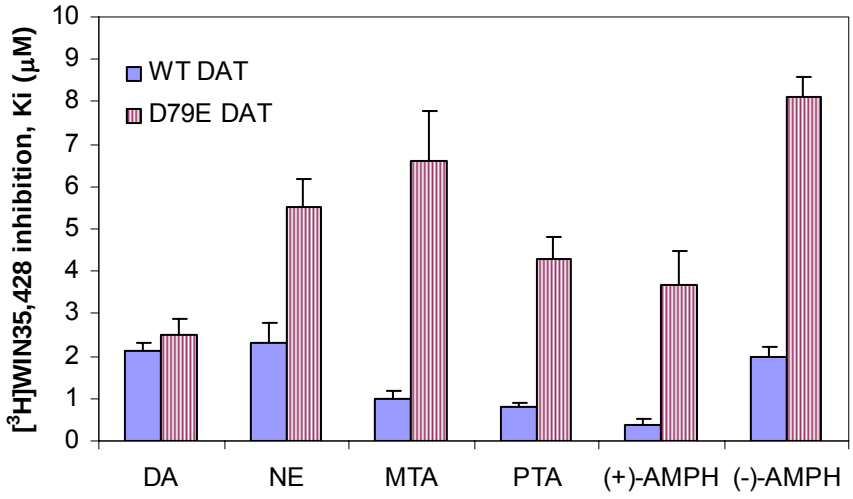
IV. CONCLUSIONS

Our results may be of significance to ongoing efforts in the development of anti-cocaine medications. In addressing whether the D79 side chain contributes to the DAT binding sites of other portions of the cocaine pharmacophore, only inhibitors with variations of the tropane ring C-3 substituent, i.e., benztropine and its analogs, displayed a considerably altered DUIP as a function of the D79E mutation. A single conservative amino acid replacement thus distinguished structural requirements for benztropine function relative to those for all other classical DAT inhibitors. Thus, it is possible to delineate the mechanism of action of this DAT inhibitor, which has a low abuse liability, using DAT mutagenesis and other structure-function studies. The development of drugs, including cocaine and benztropine analogs, that inhibit DAT and yet display a distinct behavioral profile from cocaine in animal models (Newman et al., 1994; Newman and Kulkarni, 2002; Woolverton et al., 2002; Kozikowski et al., 2003) suggest that an anti-cocaine medication is attainable.

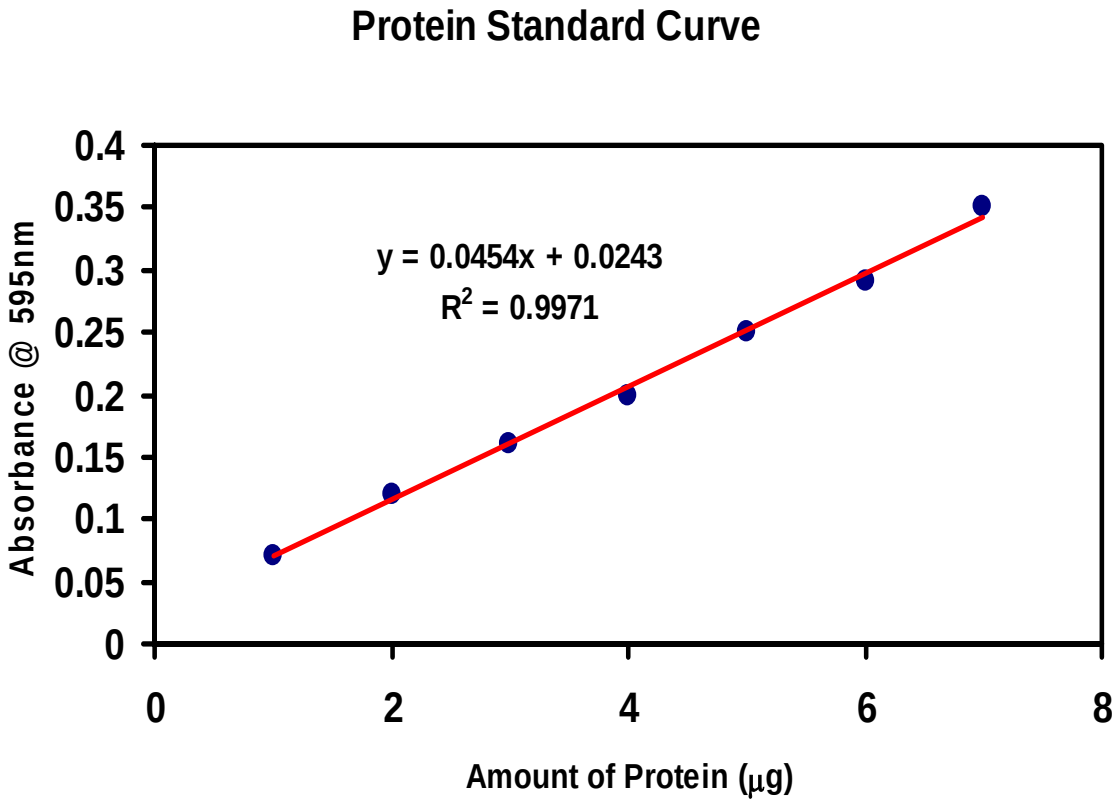
The present results also indicate that while apparent binding affinities of classic DAT blockers were unchanging at CHO cells stably transfected with the wildtype rat DAT, the DUIPs for these inhibitors fluctuated as a function of cell passage number. Manipulation of DAT levels in transiently-transfected COS-7 cells appeared to influence cocaine DUIP in these cells. These observations suggest that an unidentified cellular agent modulates DAT inhibitor DUIP but not binding affinity at the DAT protein.

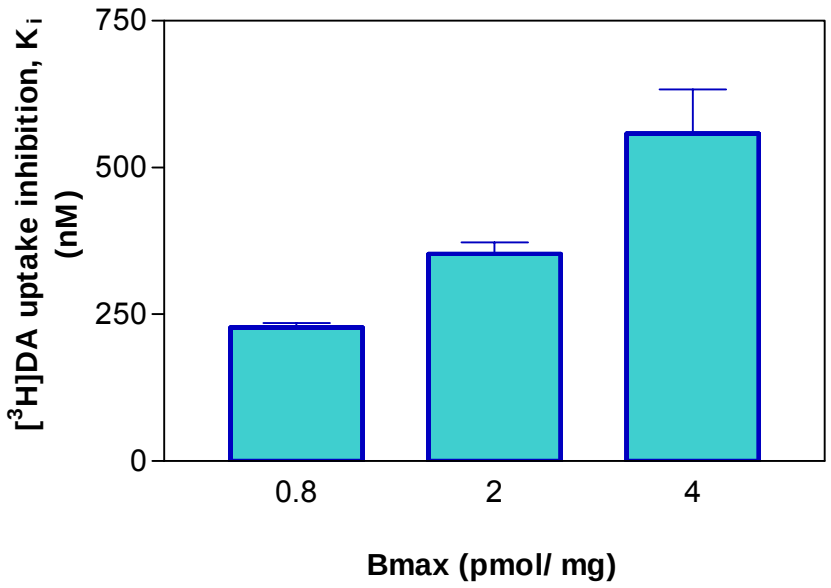
V. APPENDICES





Effect of DAT substrates on CHO cells stably expressing WT and D79E DAT. K_M and K_i values were derived from experiments incubating the stably transfected cells with nonradioactive substrates in the presence of [^{3}H]-dopamine (upper panel) or [^{3}H]-WIN35,428 (lower panel) at 22^{O}C in KRH/AA buffer. Values are presented as mean ± S.E.M. for three to six independent experiments. DA, dopamine; NE, norepinephrine; MTA, m-tyramine; PTA, p-tyramine; AMPH, amphetamine.


Protein Standard Curve
y = 0.0454x + 0.0243
R2 = 0.9971
Absorbance @ 595nm
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
Amount of Protein (µg)



Effects of DAT cell surface expression on the DUIP of cocaine. Cocaine inhibition of [^{3}H]-dopamine uptake by COS-7 cells transiently transfected with various amounts of a plasmid cDNA encoding WT DAT.

ASPET
AMERICAN SOCIETY FOR PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

9650 Rockville Pike
Bethesda, MD 20814-3995

Phone: (301) 634-7060
Fax: (301) 634-7061

info@aspet.org
www.aspet.org

August 30, 2005

Okechukwu Ukairo
Pharmacology and Toxicology
Duquesne University
600 Forbes Ave.
Pittsburgh, PA 15282

Fax: 412-396-4660

Dear Dr. Ukairo:

This is to grant you permission to include the following figures and tables in your doctoral thesis for Duquesne University:

> Figures 1-5 and Tables 1 and 2 from Okechukwu T. Ukairo, Corry D. Bondi, Amy Hauck Newman, Santosh S. Kulkarni, Alan P. Kozikowski, Stephen Pan, and Christopher K. Surratt, "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," *Journal of Pharmacology and Experimental Therapeutics*, vol. 314, no. 2, pp. 575-583, August 2005
>
> Table 2 from Wenfei Wang, Mark S. Sonders, Okechukwu T. Ukairo, Helen Scott, Megan K. Kloetzel, and Christopher K. Surratt, "Dissociation of High-Affinity Cocaine Analog Binding and Dopamine Uptake Inhibition at the Dopamine Transporter," *Molecular Pharmacology*, vol. 64, no. 2, pp. 430-439, August 2003

Permission is granted to use these figures and tables in all languages and in any format or medium including print and electronic. The authors and the sources must be cited.

Sincerely yours,

Rich Dodenhoff
Journals Director

VI. REFERENCES

Amara SG and Arriza JL (1993). Neurotransmitter transporters: three distinct gene families. *Curr. Opin. Neurobiol.* 3: 337-344.

Amara SG and Kuhar M (1993). Neurotransmitter transporters: recent progress. *Ann. Rev. Neurosci.* 16: 73-93.

Barker EL, Moore KR, Rakhshan F, Blakely RD. (1999). Transmembrane domain I contributes to the permeation pathway for serotonin and ions in the serotonin transporter. *J. Neurosci*. 19:4705-4717.

Beckman ML, Bernstein EM and Quick MW (1998) Protein kinase C regulates the interaction between a GABA transporter and syntaxin 1A. *J. Neurosci.* 18:6103-6112.

Brandl CJ, Deber CM. (1996). Hypothesis about the function of membrane-buried proline residues in transport proteins. *Proc Natl Acad Sci USA*. 83:917-921.

Barlow DJ, Thornton JM. Helix geometry in proteins. (1988). *J Mol Biol*. 201:601-619.

Carneiro, A.M., Ingram, S.L., Beaulieu, J.M., Sweeney, A., Amara, S.G., Thomas, S.M., Caron, M.G., Torres, G.E., (2002). The multiple LIM domain-containing adaptor protein Hic-5 synaptically colocalizes and interacts with the dopamine transporter. *J. Neurosci*. 22, 7045– 7054.

Carroll FI, Lewin AH, Boja JW, Kuhar MJ. (1992). Cocaine receptor: biochemical characterization and structure-activity relationships of cocaine analogues at the dopamine transporter. *J Med Chem*. 35:969-981.

Chang, M.Y., Lee, S.H., Kim, J.H., Lee, K.H., Kim, Y.S., Son, H., Lee, Y.S. (2001). Protein kinase C-mediated functional regulation of dopamine transporter is not achieved by direct phosphorylation of the dopamine transporter protein. *J. Neurochem.* 77: 754– 761.

Chen N, Rickey J, Berfield JL, Reith ME. (2004). Aspartate 345 of the dopamine transporter is critical for conformational changes in substrate translocation and cocaine binding. *J Biol Chem*. 279: 5508-5519.

Chen N, Vaughan RA, Reith ME. (2001). The role of conserved tryptophan and acidic residues in the human dopamine transporter as characterized by site-directed mutagenesis. *J Neurochem*. 77: 1116-1127.

Chen N, Zhen J, Reith ME. (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, Han DD, Gu HH. (2005). A triple mutation in the second transmembrane domain of mouse dopamine transporter markedly decreases sensitivity to cocaine and methylphenidate. *J Neurochem*. 94:352-359.

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

Chen J-G, Sachpatzidis A, Rudnick G. (1997). The third transmembrane domain of the serotonin transporter contains residues associated with substrate and cocaine binding. *J Biol Chem*. 272:28321-28327.

Chen J-G, Rudnick G. (2000). Permeation and gating residues in serotonin transporter. *Proc Natl Acad Sci USA*. 97:1044-1049.

Daniels, G. M., and Amara, S. G. (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

Deane CM, Allen FH, Taylor R and Blundell TL (1999). Carbonyl-carbonyl interactions stabilize the partially allowed Ramachandran conformations of asparagines and aspartic acid. *Protein Eng.* 12: 1025 – 1028.

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.

Eisenman G, Dani JA. (1987). An introduction to molecular architecture and permeability of ion channels. *Annu Rev Biophys Chem*. 16:205-226.

Eshleman AJ, Carmolli M, Cumbay M, Martens CR, Neve KA, 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.

Ferrer JV, Javitch JA. (1998). Cocaine alters the accessibility of endogenous cysteines in putative extracellular and intracellular loops of the human dopamine transporter. *Proc Natl Acad Sci USA*. 95:9238-9243.

Gallant, P., Malutan, T., McLean, H., Verellen, L., Caveney, S., Donly, C. (2003). Functionally distinct dopamine and octopamine transporters in the CNS of the cabbage looper moth. *Eur. J. Biochem*. 270, 664–674.

Geerlings A, Lopez-Corcuera B and Aragon C (2000). Characterization of the interactions between the glycine transporters GLYT1 and GLYT2 and the SNARE protein syntaxin 1A. *FEBS Lett* 470:51-54.

Giros, B., El Mestikawy, S., Bertrand, L., Caron, M.G. (1991). Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS Lett.* 295, 149–154.

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

Giros, B., Wang, Y.-M., Suter, S., McLeskey, S.B., Pifl, C., and Caron, M.G. (1994) Delineation of discrete domains for substrate, cocaine, and tricyclic antidepressant interactions using chimeric dopamine-norepinephrine transporters. *J. Biol. Chem*. 269:15985–15988.

Giros, B., Jaber, M., Jones, S.R., Wightman, R.M., Caron, M.G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* 379, 606–612.

Haase J, Killian AM, Magnani F and Williams C (2001) Regulation of the serotonin transporter by interacting proteins. *Biochem Soc Trans* 29:722-728.

Hansra N, Arya S and 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, 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.

Jayanthi, L.D., Apparsundaram, S., Malone, M.D., Ward, E., Miller, D.M., Eppler, M., Blakely, R.D. (1998). The Caenorhabditis elegans gene T23G5.5 encodes an antidepressant- and cocaine-sensitive dopamine transporter. *Mol. Pharmacol*. 54, 601– 609.

Johnson PH and Petersen SB (2001). A critical review of conservative mutations. *Protein Eng*. 14: 397 – 402.

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

Kilty, J.E., Lorang, D., Amara, S.G. (1991). Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science* 254: 578–579.

Kitayama S, Shimada S, Xu H, Markham L, Donovan DM, Uhl GR. (1992). Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. *Proc Natl Acad Sci USA*. 89:7782-7785.

Kitayama, S., Dohi, T., Uhl, G.R. (1994). Phorbol esters alter functions of the expressed dopamine transporter. *Eur. J. Pharmacol*. 268, 115–119.

Kline RH, Izenwasser S, Katz JL, Joseph DB, Bowen WD and Newman AH (1997). 3'-Chloro-3 alpha-(diphenylmethoxy)tropane but not 4'-chloro-3 alpha-(diphenylmethoxy)tropane produces a cocaine-like behavioral profile. *J Med. Chem.* 40:851-857.

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

Kozikowski AP, Araldi GL, Boja J, Meil WM, Johnson KM, Flippen-Anderson JL, George C and Saiah E (1998). Chemistry and pharmacology of the piperidine-based analogues of cocaine. Identification of potent DAT inhibitors lacking the tropane skeleton. *J Med Chem* 41:1962-1969.

Kozikowski AP, Simoni D, Roberti M, Rondanin R, Wang S, Du P, Johnson KM. (1999). Synthesis of 8-oxa analogues of norcocaine endowed with interesting cocaine-like activity. *Bioorg Med Chem Lett*. 9:1831-1836.

Kozikowski AP, Johnson KM, Deschaux O, Bandyopadhyay BC, Araldi GL, Carmona G, Munzar P, Smith MP, Balster RL, Beardsley PM and Tella SR (2003). Mixed cocaine agonist/antagonist properties of (+)-methyl 4beta-(4-chlorophenyl)-1-methylpiperidine-3alpha-carboxylate, a piperidine-based analog of cocaine. *J Pharmacol Exp Ther* 305:143-150.

Kulkarni SS, Grundt P, Kopajtic T, Katz JL and Newman AH (2004). Structure-activity relationships at monoamine transporters for a series of N-substituted 3alpha-(bis[4-fluorophenyl]methoxy)tropanes: comparative molecular field analysis, synthesis, and pharmacological evaluation. *J. Med. Chem.* 47:3388-3398.

Lee SH, Kang SS, Son H, Lee YS. (1998). The region of dopamine transporter encompassing the 3rd transmembrane domain is crucial for function. *Biochem Biophys. Res. Commun*. 246:347-352.

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

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.

Lee FJ, Pristupa ZB, Ciliax BJ, Levey AI, Niznik HB. (1996). The dopamine transporter carboxyl-terminal tail. Truncation/substitution mutants selectively confer high affinity dopamine uptake while attenuating recognition of the ligand binding domain. *J Biol Chem*. 271:20885-20894.

Li S-C, Goto NK, Williams KA, Deber CM. (1996). α-helical, but not β-sheet, propensity of proline is determined by peptide environment. *Proc Natl Acad Sci USA*. 93:6676-6681.

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

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

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

Loland, C.J., Norregaard, L., Gether, U., (1999). Defining proximity relationship in the tertiary structure of the dopamine transporter. *J. Biol. Chem*. 274: 36928–36934.

Loland CJ, Norregaard L, Litman T, 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 transport cycle. *Proc Natl Acad Sci USA*. 99:1683-1688.

Loland CJ, Granas C, Javitch JA, Gether U. (2004). Identification of intracellular residues in the dopamine transporter critical for regulation of transporter conformation and cocaine binding. *J Biol Chem*. 279:3228-3238.

Madras BK, Pristupa ZB, Niznik HB, Liang AY, Blundell P, Gonzalez MD, Meltzer PC. (1996). Nitrogen-based drugs are not essential for blockade of monoamine transporters. *Synapse*. 24:340-348.

Mager S, Kleinberger-Doron N, Keshet GI, Davidson N, Kanner BI and Lester HA (1996). Ion binding and permeation at the GABA transporter GAT1. *J Neurosci* 16:5405-5414.

Melikian, H.E., Ramamoorthy, S., Tate, C.G., Blakely, R.D., (1996). Inability to N-glycosylate the human norepinephrine transporter reduces protein stability, surface trafficking, and transport activity but not ligand recognition. *Mol. Pharmacol*. 50, 266–276.

Melikian, H. E., and Buckley, K. M. (1999). Membrane trafficking regulates the activity of the human dopamine transporter. *J. Neurosci.* 19**,** 7699–7710

Meltzer PC, Liang AY and Madras BK (1994). The discovery of an unusually selective and novel cocaine analog: difluoropine. Synthesis and inhibition of binding at cocaine recognition sites. *J Med Chem* 37:2001-2010.

Miller, G.M., Yatin, S.M., De La Garza, R., Goulet, M., Madras, B.K. (2001). Cloning of dopamine, norepinephrine and serotonin transporters from monkey brain: relevance to cocaine sensitivity. *Brain Res. Mol. Brain Res.* 87, 124–143.

Moron, J. A., Zakharova, I., Ferrer, J. V., Merrill, G. A., Hope, B., Lafer, E. M., Lin, Z. C., Wang, J. B., Javitch, J. A., Galli, A., et al. (2003). Mitogen-activated protein kinase regulates dopamine transporter surface expression and dopamine transport capacity. *J. Neurosci.* 23**,** 8480–8488

Newman AH, Allen AC, Izenwasser S and Katz JL (1994). Novel 3a-(diphenylmethoxy)tropane analogs: Potent dopamine uptake inhibitors without cocaine-like behavioral profiles. *J Med Chem* 37:2258-2261.

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

Nirenberg, M.J., Vaughen, R.A., Uhl, G.R., Kuhar, M.J., Pickel, V.M. (1996). The dopamine transporter is localized to dendritic and axonal plasma membranes of nigrostriatal dopaminergic neurons. *J. Neurosci.* 16, 436–447.

Norregaard, L., Frederiksen, D., Nielsen, E.O., Gether, U. (1998). Delineation of an endogenous zinc-binding site in the human dopamine transporter. *Eur. Mol. Biol. J.* 17: 4266–4273.

Norgaard-Nielsen K, Norregaard L, Hastrup H, Javitch JA, Gether U. (2002). Zn(2+) site engineering at the oligomeric interface of the dopamine transporter. *FEBS Lett*. 524:87-91.

Paczkowski FA, Bryan-Lluka LJ. (2001). Tyrosine residue 271 of the norepinephrine transporter is an important determinant of its pharmacology. *Brain Res Mol Brain Res*. 97:32-42.

Paczkowski FA, Bryan-Lluka LJ. (2004). Role of proline residues in the expression and function of the human noradrenaline transporter. *J Neurochem*. 88:203-211.

Patel, A.P., (1997). Neurotransmitter transporter proteins. In: Reith, M.E.A. Ed.., Neurotransmitter Transporters: Structure, Function, and Regulation. Humana Press, Totowa, NJ, pp. 241–262.

Porzgen, P., Park, S.K., Hirsh, J., Sonders, M.S., Amara, S.G., (2001). The antidepressant-sensitive dopamine transporter in Drosophila melanogaster: a primordial carrier for catecholamines. *Mol. Pharmacol.* 59, 83– 95.

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

Pristupa, Z. B., McConkey, F., Liu, F., Man, H. Y., Lee, F. J., Wang, Y. T., and Niznik, H. B. (1998). Protein kinase-mediated bidirectional trafficking and functional regulation of the human dopamine transporter. *Synapse* 30**,** 79–87

Ramsey IS and DeFelice LJ (2002). Serotonin transporter function and pharmacology are sensitive to expression level: evidence for an endogenous regulatory factor. *J Biol Chem* 277:14475-14482.

Reith ME, Berfield JL, Wang LC, Ferrer JV, Javitch JA. (2001). The uptake inhibitors cocaine and benztropine differentially alter the conformation of the human dopamine transporter. *J Biol Chem*. 276:29012-29018.

Ri Y, Ballesteros JA, Abrams CK, Oh S, Verselis VK, Weinstein H, Bargiello TA. (1999). The role of a conserved proline residue in mediating conformational changes associated with voltage gating of Cx32 gap junctions. *Biophys J*. 76:2887-2898.

Rocha, B.A., Fumagalli, F., Gainetdinov, R.R., Jones, S.R., Ator, R., Giros, B., Miller, G.W., Caron, M.G., (1998). Cocaine self-administration in dopamine-transporter knockout mice. *Nat. Neurosci.* 1, 132–137.

Rudnick G. and Clark J. (1993). From synapse to vesicle: the uptake and storage of biogenic amine neurotransmitters. *Biochim. Biophys. Acta* 1144: 249-263.

Sansom MS, Weinstein H. (2000). Hinges, swivels and switches: the role of prolines in signaling via transmembrane alpha-helices. *Trends Pharmacol Sci*. 21:445-451.

Sansom MSP. (1992). Proline residues in transmembrane helices of channel and transport proteins: a molecular modeling study. *Protein Engineering*. 5:53-60.

Sato Y, Zhang YW, Androutsellis-Theotokis A, Rudnick G. (2004). Analysis of transmembrane domain 2 of rat serotonin transporter by cysteine scanning mutagenesis. *J Biol Chem*. 279:22926-22933.

Schmid JA, Scholze P, Kudlacek O, Freissmuth M, Singer EA and 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.

Schonwasser DC, Marais RM, Marshall CJ, and Parker PJ (1998). Activation of the Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Pathway by Conventional, Novel, and Atypical Protein Kinase C Isotypes. *Mol. Cell. Biol.* 18(2): 790-798.

Shimada, S., Kitayama, S., Lin, C.L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M., Uhl, G. (1991). Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science* 254, 576– 578.

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

Sidhu A., Wersinger C., and Vernier P. (2004). Does α–synuclein modulate dopaminergic synaptic content and tone at the synapse? *FASEB J.* 18:637-647.

Sora, I., Wichems, C., Takahashi, N., Li, X.F., Zeng, Z., Revay, R., Lesch, K.P., Murphy, D.L., Uhl, G.R. (1998). Cocaine reward models: conditioned place preference can be established in dopa. *Proc. Natl. Acad. Sci. U. S. A.* 95, 7699–7704.

Sora, I., Hall, F.S., Andrews, A.M., Itokawa, M., Li, X.F., Wei, H.B., Wichems, C., Lesch, K.P., Murphy, D.L., Uhl, G.R. (2001). Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. *Proc. Natl. Acad. Sci. U. S. A.* 98, 5300– 5305.

Strader CD, Candelore MR, Hill WS, Sigal IS, Dixon RA. (1989). Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. *J Biol Chem*. 264:13572-13578.

Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS, Dixon RA. (1988). Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J Biol Chem*. 263:10267-10271.

Sucic S, Bryan-Lluka LJ. (2002). The role of the conserved GXXXRXG motif in the expression and function of the human norepinephrine transporter. *Brain Res Mol Brain Res*. 108:40-50.

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.

Tamiz AP, Zhang J, Flippen-Anderson JL, Zhang M, Johnson KM, Deschaux O, Tella S and Kozikowski AP (2000). Further SAR studies of piperidine-based analogues of cocaine. 2. Potent dopamine and serotonin reuptake inhibitors. *J Med Chem* 43:1215-1222.

Torres GE, Yao WD, Mohn AR, Quan H, Kim KM, Levey AI, Staudinger J, Caron MG. (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, 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.

Usdin, T.B., Mezey, E., Chen, C., Brownstein, M.J., Hoffman, B.J. (1991). Cloning of the cocaine-sensitive bovine dopamine transporter. *Proc. Natl. Acad. Sci. U. S. A*. 88: 11168– 11171.

Vaughan, R.A., Huff, R.A., Uhl, G.R., Kuhar, M.J. (1997). Protein kinase C-mediated phosphorylation and functional regulation of dopamine transporters in striatal synaptosomes. *J. Biol. Chem*. 272, 15541–15546.

Visiers I, Weinstein H, Rudnick G, Stephan MM. (2003). A second site rescue mutation partially restores functional expression to the serotonin transporter mutant V382P. *Biochemistry* 42:6784-6793.

Wang, J.B., Moriwaki, A., Uhl, G.R., (1995). Dopamine transporter cysteine mutants: second extracellular loop cysteines are required for transporter expression. *J. Neurochem.* 64, 1416–1419.

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

Wersinger, C., and Sidhu, A. (2003). Attenuation of dopamine transporter activity by alpha-synuclein. *Neurosci. Lett.* 340**,** 189–192

Wersinger, C., Prou, D., Vernier, P., and Sidhu, A. (2003). Modulation of dopamine transporter function by alpha-synuclein is altered by impairment of cell adhesion and by induction of oxidative stress. *FASEB J.* 17**,** 2151–2153

Wersinger, C., Prou, D., Vernier, P., Niznik, H. B., and Sidhu, A. (2003a). Mutations in the lipid-binding domain of α-synuclein confer overlapping, yet distinct, functional properties in the regulation of dopamine transporter activity. *Mol. Cell. Neurosci.* 24**,** 91–105

Williams KA, Deber CM. (1991). Proline residues in transmembrane helices: structural or dynamic role? *Biochemistry*. 30:8919-8923.

Woolverton WL, Ranaldi R, Wang Z, Ordway GA, Paul IA, Petukhov P and Kozikowski A (2002). Reinforcing strength of a novel dopamine transporter ligand: pharmacodynamic and pharmacokinetic mechanisms. *J Pharmacol Exp Ther* 303:211-217.

Wu X, Gu HH. (2003). Cocaine affinity decreased by mutations of aromatic residue phenylalanine 105 in the transmembrane domain 2 of dopamine transporter. *Mol Pharmacol*. 63:653-658.

Zhang, L., Coffey, L.L., Reith, M.E.A. (1997). Regulation of the functional activity of the human dopamine transporter by protein kinase C. *Biochem. Pharmacol.* 53, 677–688.

Zou MF, Agoston GE, Belov Y, Kopajtic T, Katz JL and Newman AH (2002). Enantioselective synthesis of S-(+)-2-beta-carboalkoxy-3-alpha-[bis(4-fluorophenyl)methoxy]tropanes as novel probes for the dopamine transporter. *Bioorg Med Chem Lett* 12:1249-1252.

Zou MF, Kopajtic T, Katz JL and Newman AH (2003). Structure-activity relationship comparison of (S)-2-beta-substituted 3-alpha-(bis[4-fluorophenyl]methoxy)tropanes and (R)-2-beta-substituted 3-beta-(3,4-dichlorophenyl)tropanes at the dopamine transporter. *J Med Chem* 46:2908-2916.

VII. ABSTRACT

A long-standing postulate holds that cocaine inhibits DAT-mediated dopamine transport via competition with dopamine for formation of an ionic bond with the DAT transmembrane 1 aspartic acid residue 79. A recent study from our laboratory indicated that mutation of this aspartate to glutamate (D79E) had little or no effect on dopamine affinity or dopamine uptake inhibition potencies for WIN35,428 and cocaine, and decreased WIN35,428 affinity by only 3 fold (Wang et al., 2003). The study cast doubt on the requirement of a dopamine-D79 ion pair, but did not address whether the residue plays a role in recognizing the cocaine pharmacophore. In the present study, DAT inhibitors containing variations of three primary components of this pharmacophore- the positively charged tropane nitrogen atom, the seven-carbon tropane ring itself, and the aromatic substituent at the tropane C-3 position- were assessed for binding affinity and dopamine uptake inhibition at the same D79 DAT mutants. Only inhibitors with modifications of the phenyl ring substituent of cocaine, i.e. benztropine and its analogs, displayed considerably altered dopamine uptake inhibition potency as a function of the D79E mutation. These observations may suggest that the side chain of the D79 residue is important for the recognition of the aromatic components of DAT ligands.

Furthermore, we investigated the influence of cell passage number, the density of the cell monolayer and the effect of varying DAT expression levels by manipulation of transfection conditions on DAT function. It was observed that the DUIPs of cocaine, mazindol, methylphenidate, and benztropine fluctuated as a

function of DAT-CHO cell passage number. The binding affinities of these DAT inhibitors, however, remained static. Also, the DUIP of cocaine fluctuated as a result of variations in DAT cell surface expression. It is therefore conceivable that an unidentified cellular mediator modulates DAT inhibitor DUIP but not binding affinity at the DAT protein.