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Structure-function analysis of the dopamine transporter in the presence of synthetic cathinones and amphetamines

Shari Melissa Radford

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Is approved by the final examining committee:

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12/2/2016
STRUCTURE-FUNCTION ANALYSIS OF THE DOPAMINE TRANSPORTER 
IN THE PRESENCE OF SYNTHETIC CATHINONES AND AMPHETAMINES 

A Dissertation 
Submitted to the Faculty 
of 
Purdue University 

by 
Shari Melissa Radford 

In Partial Fulfillment of the 
Requirements for the Degree 
of 
Master of Science 

December 2016 
Purdue University 
West Lafayette, Indiana
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I would like to acknowledge my primary mentor Dr. Eric Barker and thank Doctors Nick Noinaj and Angeline Lyon for unofficially adopting me into their labs as I learned to express and purify proteins.
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ABBREVIATIONS

\(\alpha\)-CaMKII  \(\alpha\) calcium/calmodulin-dependent protein kinase II
\(\alpha\)-PVP  \(\alpha\)-pyrrolidinopentiophenone
4-MMC  4-methyl methcathinone
5-HT  serotonin
ADHD  attention deficit hyperactivity disorder
AMPH  amphetamine
COMT  catechol-O-methyl transferase
cryo-EM  cryogenic electron microscopy
DA  dopamine
DAT  dopamine transporter
dDAT  \textit{Drosophila melanogaster} dopamine transporter
DEA  United States Drug Enforcement Agency
DEER  double electron-electron resonance
dSERT  \textit{Drosophila melanogaster} serotonin transporter
EDMA  ethylenedioxymethamphetamine
EDMC  ethylenedioxy methcathinone
FRET  fluorescence resonance energy transfer
GABA  \(\gamma\)-aminobutyric acid
hDAT  human dopamine transporter
HEK  human embryonic kidney
HEPES  (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
hNET  human norepinephrine transporter
hSERT  human serotonin transporter
LeuT  leucine transporter
<table>
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<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>MDMA</td>
<td>3,4-Methylenedioxyamphetamine</td>
</tr>
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<td>MDMC</td>
<td>methylenedioxy methcathinone</td>
</tr>
<tr>
<td>MDPV</td>
<td>3,4-Methylenedioxytyrovalerone</td>
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<tr>
<td>meth</td>
<td>methamphetamine</td>
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<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MTS</td>
<td>Methanethiosulfonate</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NET</td>
<td>norepinephrine transporter</td>
</tr>
<tr>
<td>NSS</td>
<td>neurotransmitter:sodium symporter</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PTSD</td>
<td>post-traumatic stress disorder</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERT</td>
<td>serotonin transporter</td>
</tr>
<tr>
<td>TAAR1</td>
<td>trace amine associated receptor 1</td>
</tr>
<tr>
<td>VMAT</td>
<td>vesicular monoamine transporter</td>
</tr>
<tr>
<td>VMAT2</td>
<td>vesicular monoamine transporter 2</td>
</tr>
<tr>
<td>V_{max}</td>
<td>maximal velocity</td>
</tr>
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<td>WT</td>
<td>wild-type</td>
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ABSTRACT

Radford, Shari M. M.S., Purdue University, December 2016. Structure-Function Analysis of the Dopamine Transporter in the Presence of Synthetic Cathinones and Amphetamines. Major Professor: Eric L. Barker.

The dopamine transporter (DAT) is a monoamine transporter that regulates dopamine (DA) neurotransmission by clearing DA from the synapse. DAT is acted on by a number of psychostimulant drugs, which block reuptake and potentiate DA signaling. Several inhibitors of DAT, both amphetamines and cathinones, also cause reversal of transport. Previous research has shown the importance of a conserved salt bridge in another monoamine transporter, the serotonin transporter, in amphetamine-induced efflux. In our studies here, we engineered a mutant Drosophila melanogasterDAT (dDAT D475N) designed to interrupt this salt bridge and examine the effects on amphetamine- and cathinone-induced efflux in vitro.

Understanding the mechanism of action of structurally similar yet functionally diverse psychostimulants necessitates information about their binding sites at the transporter. A second mutant D. melanogaster DAT (dDAT D121G) was engineered to more closely mimic the substrate binding site of human DAT. By using already existing crystal structure data, compounds were computationally docked to the identified binding sites, allowing prediction of binding poses and binding free energies. dDAT D475N was found to have similar inhibition of uptake by synthetic cathinones relative to wild-type. No significant drug-induced efflux was observed in dDAT wild-type or dDAT D475N, while the D121G mutant restored efflux activity beyond that of human DAT. These results show that the presence of a salt bridge at the external gate of DAT, as well as a hydrophobic environment within the DA binding site, are important for drug-induced efflux.
1. PSYCHOSTIMULANT DRUGS AND THE DOPAMINE TRANSPORTER

1.1 The Synapse

Nerve cells communicate with one another through chemical signaling and the release of neurotransmitters across the space between cells, known as the synapse. Classical neurotransmitters include acetylcholine, the amino acids glutamate, γ-aminobutyric acid (GABA), and glycine, and the monoamines dopamine (DA), norepinephrine (NE), and serotonin (5-HT) [1]. Monoamines are synthesized from the amino acids tyrosine or tryptophan through a series of enzymatic reactions. Monoamines are then packaged into vesicles by vesicular monoamine transporters (VMATs) [2]. An action potential causes depolarization of the neuronal membrane at the axon terminal, triggering fusion of vesicles to the presynaptic membrane [3]. Monoamines are then released into the synapse, where they bind to postsynaptic receptors and pass on a signal. Neurotransmission is halted by removing neurotransmitter from the synapse. Neurotransmitters may be degraded by enzymes, taken up by nearby glial cells, or recycled into the presynaptic cell by transporter proteins. The monoamine transporters—dopamine transporter (DAT), norepinephrine transporter (NET), and serotonin transporter (SERT)—are responsible for reuptake of DA, NE, and 5-HT, respectively, from the synapse (Figure 1.1) [4]. VMATS have a higher substrate turnover rate than monoamine transporters, keeping the cytosol free of neurotransmitters and repacking monoamines into vesicles for later release. Monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) act within the cell to break down free catecholamine neurotransmitters.
Psychostimulant drugs influence mood by increasing synaptic neurotransmitter levels, potentiating signal and stimulating neurons in reward pathways of the brain. Cocaine and amphetamines produce their psychoactive and addictive effects primarily by inhibiting monoamine transporters, increasing synaptic levels of DA, NE, and 5-HT [5,6]. An initial buildup of dopamine in the mesolimbic system gives rise to euphoria and a desire to take the drug again. Blockade of DAT is necessary for cocaine and amphetamine reward, demonstrated by mutagenesis and DAT knockout studies in mice. Mice lacking a functional dopamine transporter show no conditioned place preference, locomotor activity, or DA release upon exposure to cocaine or amphetamine, indicating that the dopamine transporter is a target of psychostimulants [7,8]. The short time course of DA release in the nucleus accumbens plays a role in reward and behavior [9]. The nucleus accumbens receives information from the amygdala, prefrontal cortex, and hippocampus that is then converted to behavior through connections with the extrapyramidal motor system [10]. Long-term psychostimulant use leads to an accumulation of the transcription factor ΔFosB in the limbic system and long-term changes in neural structure, which has been correlated with addiction-like behaviors in mice [11,12]. While psychostimulant use has acute effects on synaptic neurotransmitter levels, chronic use can lead to permanent changes in the brain.

1.2 Psychostimulant Drugs

According to the the United Nations World Drug Report 2016, 247 million people used illicit drugs within the past year, and 29 million of those people were addicted [13]. The United States 2014 National Survey on Drug Use and Health found that 27 million, or 10.2%, of Americans had used an illicit drug within the past year [14]. Commonly abused drugs include cannabis, alcohol, heroin, cocaine, and methamphetamine. The drugs are generally categorized as either stimulants or depressants. Here, I will discuss the mechanism of action of some classical and
Figure 1.1.: Schematic interaction of DAT and amphetamine in the synapse.
Vesicles release DA from the presynaptic cell, which crosses the synapse to interact with DA receptors. DAT terminates signaling by clearing DA from the synapse. In the presence of amphetamines, DA transport is inhibited through competition for uptake with drug. Amphetamines also induce efflux, a reversal of the DAT transport cycle.
Figure 1.2.: Structure of monoamine neurotransmitters, amphetamine, cathinone, and novel psychoactive substances.
relatively new psychostimulants, including amphetamine, cathinone, and derivative structures which act on monoamine transporters (Figure 1.2). A more complete understanding of psychostimulant activity in the brain could lead to novel pharmacological interventions for the treatment of substance addiction.

1.2.1 Cocaine

Cocaine, a β-alkaloid found in the *Erythroxylon coca* plant, has been used by humans since at least 600 AD in Peru [15,16]. Cocaine was first isolated from coca leaves by Albert Neimann in 1855, and was the first recorded anesthetic [17]. Local administration of cocaine blocks voltage-gated sodium channels in nerve cells, preventing depolarization and blocking conduction of electrical impulses, leading to the anesthetic effect [1,18]. Cocaine also acts on monoamine transporters to block reuptake of DA, NE, and 5-HT when administered systemically, leading to a euphoric feeling. Noting its ability to elevate mood and increase alertness, companies began selling cocaine in a multitude of products. In the 1880s, cocaine could be found in cigarettes, wine, and solutions sold with hypodermic needles. Coca Cola’s original recipe containing cocaine was marketed as a temperance drink without the vices of alcohol. In 1879, cocaine was recommended for the treatment of morphine addiction [15,16]. Cocaine itself is a highly addictive and reinforcing drug, used in a multitude of animal models, and is one of the best studied drugs of addiction. Chronic repeated use leads to tolerance through a variety of mechanisms including increased density of alpha and beta adrenergic receptors in the brain and depletion of DA stores, resulting in drug cravings, lethargy, and anhedonia after cessation of use [19]. The addictive properties of cocaine were eventually realized and the 1914 Harrison Narcotics Act limited sale outside of prescription use. The 1970 Controlled Substances Act further restricted cocaine in the United States, placing it on Schedule II as a drug with accepted medical use but high abuse potential [20]. From 1970-1987, the high cost of cocaine hindered widespread use,
but lowered 1982-1984 black market pricing led to an increase in abuse. A 1988 study found that cocaine available on the street was only an average of 40% pure, adulterated with substances like sugar and caffeine [21]. Fluctuations in price and purity led to varying use statistics of the drug; 2014 cocaine use was estimated to be 1.5 million people, or 0.6% of the United States population, with 1.4% of those aged 18-24 having used cocaine within the past month [14].

1.2.2  Amphetamines

Amphetamine use can lead to feelings of euphoria similar to cocaine, and also has high potential for dependence. The structure of amphetamine is quite unique from cocaine, characterized by an $\alpha$-methyl-phenethyl-amine motif (Figure 1.2). The first synthetic amphetamine was made by Lazar Edeleanu at the University of Berlin in 1887 [22]. Amphetamine became commercially available without a prescription in 1936 as Benzedrine 10 mg tablets, sold by Smith Kline and French. The drug enhances attention and alertness, helping to ameliorate symptoms in patients with narcolepsy and attention deficit hyperactivity disorder (ADHD). Amphetamines have also been used historically in warfare, notably in boosting wakefulness in fighter pilots during World War II [23]. Widespread recreational use of the drug began after release of a publication from the University of Minnesota, where the Psychology Department did a study on alertness [24]. In 2014, 0.6% of Americans had used illegal stimulants within the past month, 0.2% of which were methamphetamine users [14]. Amphetamine use also has a number of detrimental side effects, including psychosis and restlessness. In fact, rodents chronically administered amphetamine are used as a model of schizophrenia [25]. Due to this and other dangerous effects, amphetamine and methamphetamine were placed on the United States Drug Enforcement Agency (DEA) Schedule II list of controlled substances in 1971 [20].
Amphetamine’s effect comes from a combination of factors in the central nervous system. The primary sites of action are at the monoamine transporters DAT, NET, and SERT [26]. Amphetamines have dual action at these transporters, causing both blockade and reversal of transport. Amphetamine drugs are most selective for DAT and NET, with lower potency at SERT [27]. Amphetamines also inhibit vesicular monoamine transporter 2 (VMAT2) [28]. Finally, amphetamines inhibit MAO, an enzyme that breaks down monoamines like DA, NE, and 5-HT. The combined effects of amphetamines lead to an increase in neurotransmitter levels at the synapse. Figure 1.1 demonstrates the effects of amphetamine at DAT. The Barker lab is interested in studying the effects of amphetamines and cathinones at the dopamine transporter, specifically the molecular mechanism of transport reversal, or efflux.

**Ecstasy (MDMA)**

Ecstasy, or 3,4-methylenedioxymethamphetamine (MDMA) is the classic party drug [? , 29]. Incidence of use is only a fraction of cocaine, where 0.2% of Americans over the age of 12 had used MDMA in the last month, with higher use (0.8%) in those age 18-25 [14]. MDMA, first synthesized at Merck Pharmaceuticals in 1912, is a ring substituted amphetamine that is also closely related to the hallucinogen mescaline [30]. Ecstasy is typically ingested orally in capsules or tablets.

Much is known about the mechanism of MDMA action, yet much remains to be studied. MDMA acts primarily at monoamine transporters, with similar potency at SERT, NET, and DAT [31]. Highly efficacious 5-HT release by MDMA at SERT potentiates serotonergic signaling, leading to its hallucinogenic and empathogenic effects [29, 31, 32]. MDMA also inhibits tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of serotonin [33]. The combination of serotonin depletion and deficiency in 5-HT synthesis after MDMA use may lead to negative side effects such as irritability for days after ingestion. MDMA is metabolized primarily in the
liver by CYP2D6, with glucuronide and sulfate metabolites excreted in the urine [32]. Chronic use of MDMA may lead to permanent damage to serotonergic nerve terminals [34,35]. A single dose of MDMA leads to reduced 5-HT levels one week following use, and chronic use leads to a constitutive reduction in 5-HT as well as increased 5-HT₁A receptors in the postsynaptic cell in rats [36]. Chronic use has also been modeled in mice, where 4 days of repeated administration followed by 2 weeks of abstinence has been found to cause a reduction in SERT expression and 5-HT levels, with no effect on NE, NET, DA, or DAT levels [37]. Abstinent human users also have a reduction in serotonin markers [38].

MDMA use can lead to feelings of well-being, euphoria, and closeness to others, but also has risks of anxiety, elevated heart rate, sweating, and other unpleasant side effects. Alexander Shulgin coined the term empathogen to describe the experience of emotional connectedness that occurs due to MDMA use. Due to its empathogenic properties, MDMA was used as a psychotherapeutic tool in the 1970s and early 1980s [39,40]. The DEA added MDMA to Schedule I in 1985 due to apparent risk of addiction [20]. There was a resurgence of use in the 1990s club scene, quickly curbed by a public health campaign until a later resurgence in 2009. There is evidence of a low risk of dependence to MDMA, which may be in part due to the cultural use of drug in specific social settings [41]. MDMA is currently in Phase II clinical trials for use in the treatment of post-traumatic stress disorder (PTSD) and has been approved for Phase III clinical trials [42,43]. More research about MDMA and related compounds is crucial at a time when the drug may soon be available to millions of Americans by prescription.

**Synthetic Cathinones**

Closely related to amphetamines by structure are the cathinones, which contain a beta keto group (Figure 1.2). Within the past ten years, synthetic cathinones have emerged on the market and are being used recreationally. Many are currently
available as legal highs, skirting drug laws due to their unique structures; they may be labeled as “plant food,” “bath salts,” or “not for human consumption.” Due to the relative novelty of these compounds, much remains to be studied about their cellular targets as well as long-term effects. Cathinones are thought to have similar targets and effects to the structurally similar amphetamines. In 2010, the three most common cathinone drugs were methylone, mephedrone, and 3,4-methylenedioxypyrovalerone (MDPV) [27]. These three synthetic cathinones were banned by the United Kingdom in 2010, closely followed by a Schedule I classification by the DEA in 2011 [20].

**Methylone (MDMC)**

Methylone, or methylenedioxymethcathinone (MDMC) is the beta-keto analogue of MDMA (Figure 1.2); users experience similar energetic and empathogenic feelings [44]. Like other synthetic cathinones, methylone is a white powder that is taken orally in capsules or tablets, dissolved in water, or snorted [45]. Some users also report injecting, although this method is uncommon. MDMC, like MDMA, has similar selectivity for each of the monoamine transporters DAT, NET, and SERT [27]. Rats administered MDMA or MDMC have hyperactivity and comparably elevated 5-HT levels in the nucleus accumbens. Interestingly, rats administered MDMC had brain levels of DA, NE, and 5-HT similar to those of untreated rats after 2 weeks of chronic administration, while MDMA-treated rats had decreased 5-HT levels. Both rats and mice treated with a 4-day binge of MDMC showed no signs of depression, anxiety, or memory problems 2 weeks after cessation of treatment [46]. Treatment had no effect on neurotransmitter levels or number of monoamine transporters 2 weeks post-binge treatment. This contrasts to amphetamine treatment, which has been shown to cause widespread DA depletion in rodents [47,48].
Expanding the methylenedioxy ring of MDMA and MDMC to an ethylenedioxy ring makes ethylenedioxymethamphetamine (EDMA) and ethylenedioxy methcathinone (EDMC) (Figure 1.2) [44]. These compounds have lower potency at NET and DAT than MDMA or MDMC, but are more tolerated by SERT due to its larger and more flexible binding site [49,50].

**Mephedrone (4-MMC)**

Mephedrone, also known as 4-methylmethcathinone (4-MMC), first became available on the internet in 2007 [51]. 4-MMC acts similarly to MDMA and cocaine, which had declining purity and increasing costs at the time. The ease of purchasing mephedrone, legality, purity, and low price led to a rapid rise in its popularity. In 2010, the largest incidence of 4-MMC use was found to be in those age 16-24, 4.4%, which was equal to that of cocaine use [52]. 4-MMC targets DAT, NET, and SERT with approximately equal selectivity, enhances DA release, and is about twice as potent as MDMC [27,53]. 4-MMC is metabolized by reduction of the keto group, N-demethylation, and oxidation of the tolyl moiety [45]. Much like MDMC, rats administered 4-MMC had brain levels of DA, NE, and 5-HT similar to those of untreated rats after 2 weeks of chronic administration. Like MDMC, both rats and mice treated with a 4-day binge of 4-MMC showed no signs of depression or anxiety 2 weeks later [46]. Treatment had no effect on neurotransmitter levels or number of monoamine transporters 2 weeks post-treatment. However, mice showed reduced memory performance in the T-maze. Similarly, high-dose administration led to memory deficits in rats [45] In another study, high-dose administration of 4-MMC enhanced spatio-temporal learning in primates. Thus, the long-term effects of 4-MMC are complex and worthy of further study.

One reason 4-MMC may not have a long-term effect on neurotransmitter levels or depression is because amphetamine and MDMA have micromolar affinity for VMAT2 while the affinity of cathinones cannot be determined. Methamphetamine
and MDMA cause VMAT2 release of DA while the cathinones have <35% DA releasing effect at an EC$_{50}$ of >100 μM in in vitro studies [54]. Another contributing factor could be due to amphetamines, but not their beta-keto analogues, binding to trace amine-associated receptor 1 (TAAR1), a G-protein coupled receptor that regulates presynaptic dopaminergic neurotransmission, reuptake inhibition, and neurotransmitter efflux [55–57].

**MDPV**

Methylenedioxypyrovalerone (MDPV), another one of the first synthetic cathinones, was placed on the DEA Schedule I along with MDMC and 4-MMC in 2011. A closely related analogue with a pyrovalerone ring, α-pyrrolidinopentiophenone (α-PVP), was scheduled in 2013. MDPV and α-PVP are highly potent and selective blockers at DAT [45,58]. The bulky pyrrolidine ring makes MDPV an inhibitor, but not substrate, of DAT as compared to the synthetic cathinones discussed above. Selectivity for DAT is thought to be in large part due to the length of the α-carbon side chain in both structures. The R(-) enantiomer is significantly more potent, although MDPV is supplied as a racemic mixture [59]. MDPV is metabolized by CYP2D6, CYP2C19, and COMT, undergoing glucuronidation before renal excretion [45]. Similar to cocaine, it has been shown to be reinforcing and self-administered by rodents, as well as identified in drug discrimination tests.

The immediate effects of amphetamines and cathinones are due to their action at monoamine transporters. Blockade of transporters leads to accumulation of neurotransmitter and propagated signaling. Long-term potentiation of signaling can lead to permanent changes in neural structures. Better understanding of how psychostimulant drugs act at their primary target is needed to advance the field and aid in treatment or prevention of drug abuse and addiction.
1.3 Transporter Structure

DAT, NET, and SERT belong to the SLC6 family of neurotransmitter:sodium symporter (NSS) membrane proteins, controlling signaling by clearing neurotransmitters from the synapse and regulating neurotransmitter levels. Dysregulation of neurotransmitters plays a large role in depression, anxiety, and addiction. We can learn more about structure-function relationship between drugs and monoamine transporters by comparing the pharmacological effect a drug has to its mechanism of action and binding site(s).

Monoamine transporters are proteins composed of 12 transmembrane alpha helices. They can be found in neurons near the synapse, ready to reuptake dopamine, serotonin, or norepinephrine and return neurotransmitter to the pre-synaptic cell. Neurotransmitter:sodium symporters function according to the alternating access model, switching between an outward open conformation where they pick up substrate from the extracellular milieu, to an inward open conformation where the substrate binding site is exposed to the cytoplasm (Figure 1.3) [60–62]. The passage of substrate is coupled to the movement of sodium down its concentration gradient. Data from a bacterial NSS homolog, *A. aeolicus* leucine transporter (LeuT), provides information about intermediate transition states. Due to their familial relationship, LeuT and DAT have high structural similarity despite low sequence identity. High resolution structural information (up to 1.9 Å) is available for LeuT in the two conformations proposed by the alternating access model as well as an intermediate state, where substrate is occluded from both sides of the membrane [63,64].

Without substrate, the transporter is free to sample all conformations from inward open to outward open [65]. Binding of sodium stabilizes an outward facing conformation, facilitating substrate binding [66,67]. After substrate binds, transporter transitions to an occluded state. Small localized conformational changes in residues near the dopamine binding site, called gating residues, control access to
Figure 1.3.: Alternating access model for DAT.

Outward Open: Dopamine (DA) and sodium (Na\(^+\)) enter the transporter from the extracellular space. Occluded: Transporter shifts to hold DA occluded from access to either side of membrane. Inward Open: Transporter opens to intracellular space, allowing DA transport. DAT then returns to outward open, ready to transport another DA molecule.
each side of the cellular membrane. The extracellular gate closes and then release of an intracellular gate is associated with protein transition from occluded to inward open \cite{65,68}. The extracellular gate in dDAT is composed of R52 and D475.

Monoamine transporters have two proposed substrate binding sites \cite{66,69,70}. The central (S1) site is where endogenous neurotransmitter, selective serotonin reuptake inhibitors, cocaine, and amphetamines bind (Figure 1.4) \cite{63,71,72}. The allosteric S2 site is about 12 Å toward the extracellular side of the central binding site (Figure 1.5). Tricyclic antidepressants have been shown to bind at the S2 site LeuT and SERT \cite{72–75}. Computational studies on DAT suggest that binding of DA at the S2 site promotes translocation of DA from the S1 site \cite{76}. There are also a few allosteric modulator quinazolinamine compounds with currently unknown binding sites \cite{77}.

### 1.3.1 Experimental Structure

Crystallography on the leucine transporter provided an initial look at the structure of neurotransmitter:sodium symporters \cite{63}. Penmatsa et al. published the first crystal structure of the \textit{Drosophila melanogaster} dopamine transporter (dDAT) in 2013 \cite{71}. Since then, the Gouaux lab has investigated binding of antidepressants and cocaine analogues, leading to several high resolution crystal structures of dDAT in the outward-open state and one structure in an occluded state \cite{78,79}. The Gouaux lab has also revealed a crystal structure for the human serotonin transporter (hSERT) \cite{72}.

### 1.3.2 Computational Structure

Molecular modeling and protein-ligand docking software can be used to predict the position of small molecules noncovalently bound to macromolecular protein targets. Docking is especially important when predicting binding poses of drugs with known activity in order to establish the structural basis of a drug’s binding
The S1 binding site consists of amino acids F43, D46, A117, V120, D121, Y124, F319, F325, S421, and S422 interacting with dopamine. Adapted from [78], PDB code 4Xp1.
Figure 1.5.: Model of the S2 binding site in dDAT.

The S2 binding site consists of amino acids R52, Y123, I127, W130, F216, I220, L221, D312, Q316, P386, F471, and D475 forming an allosteric pocket. The extracellular gate is composed of a salt bridge between R52 and D475. Adapted from [78], PDB code 4Xp1.
affinity. Docking is also a key computational method used in structure-based drug
design for the screening of small molecule libraries in lead identification. One of the
most widely used and user-friendly docking programs available is AutoDock Vina [80,81].

1.3.3 Efflux and the External Gate

Forward translocation of substrate from the extracellular milieu to the cytoplasm
by monoamine transporters has been well studied. Drugs such as cocaine bind to
the substrate binding pocket of the transporter and stabilize the protein in an
extracellular-facing conformation [82,83]. Amphetamines and certain cathinones
can induce a reversal of the transport cycle and cause efflux of neurotransmitter out
of the cell. There are still unanswered questions about the mechanism of
transporter-mediated substrate efflux.

The N-terminus of DAT has been implicated in amphetamine-induced efflux.
Although truncation of the first 22 amino acids of hDAT does not change the
transporter's ability to uptake DA, it leads to an 80% reduction in
amphetamine-induced efflux in experiments measuring $[^{3}H]$ labeled DA levels [84].
Measuring amperometric current in voltage clamped cells with truncated hDAT
leads to a 91% reduction in amphetamine-induced efflux. The N-terminus contains
several serines available for phosphorylation. Mutations of the first five serine
residues in hDAT to alanine prevents phosphorylation; mutation does not effect DA
uptake but reduces voltage measured by amperometric experiments. Conversely,
mutation to the first five serines in hDAT to aspartate retains its ability to both
uptake and efflux. Interactions with syntaxin1A, protein kinase C (PKC) and
calcium/calmodulin-dependent protein kinase II ($\alpha$-CaMKII) enhance
amphetamine-induced efflux [26,85]. N-terminal phosphorylation of the dopamine
transporter is crucial to drug-induced efflux.
In the serotonin transporter, a mutation near the substrate recognition site allows binding, but not transport of amphetamines [86]. A charged pair of residues (D/E to R) at the extracellular gate is conserved across nearly all monoamine transporters (Figure 1.6(a)). This putative salt bridge is thought to prevent leak of 5-HT out of the cell by stabilizing the protein and reducing conformational flexibility in the inward open conformation. Drosophila SERT (dSERT) has an uncharged asparagine at the extracellular gate and is unable to efflux in the presence of amphetamines. The hSERT E493N mutant destabilizes the extracellular gate and is also unable to efflux. After aligning the sequences of SERT and DAT, the analogous mutation was found in dDAT and is predicted to have equivalent effects. I hypothesized that the D475N mutation would abolish amphetamine-induced efflux found in wild-type (WT) dDAT. This novel gating mutant may give structural insight into the function of transporters, as well as amphetamine action on transport proteins.

High sequence identity (52.4%) and similarity (69.2%) between hDAT and dDAT make the *Drosophila melanogaster* protein a useful tool in studying human DAT. Sequence similarity corresponds to structural similarities in DA recognition, uptake, and efflux. However, differences in the potency of amphetamines and other substrates between human and drosophila DAT have been observed [87], potentially due to a change in two residues near the S1 binding site (D121 and S426). Formation of a D121G mutant mimicking the residue found at this position in hDAT may restore dDAT functional selectivity to that of hDAT (Figure 1.6(b)).

The aim of this dissertation is to create a more comprehensive understanding of DAT and monoamine transporter efflux function by addressing structurally similar yet functionally diverse drug binding and pharmacological effects. The central hypothesis of this work is that the extracellular salt bridge in DAT is important to drug-mediated efflux. This was assessed by testing the effect of two separate mutations (D121G and D475N) on the pharmacology of drug-induced efflux at dDAT. I determined the binding poses and free energies of two binding sites for
(a) A highly conserved acidic residue across SLC6 family transporters forms part of the extracellular gate.

(b) The S1 binding site of human and drosophila DAT is highly conserved.

Figure 1.6.: Sequence alignments of select monoamine transporters.
various ligands using AutoDock Vina. By making mutations to DAT that change its sensitivity to amphetamine-induced efflux, we can better understand how the transporter functions and what exactly amphetamine is acting upon in the wild-type transporter to elicit its effect. Understanding how psychostimulants interact with DAT, where they bind, and how they block natural substrate passage, will tell more about drug and transporter mechanisms of action.
2. AMPHETAMINE AND CATHINONE PHARMACOLOGY AT DAT

2.1 Materials and Methods

2.1.1 Materials

HEK293 GripTite MSR cells stably expressing hDAT were generously provided by Keith Henry (University of North Dakota). $[^3]$H$\text{DA}$ (30 Ci/mmol) and $[^3]$H$\text{MPP}^+$ (80 Ci/mmol) were purchased from PerkinElmer. dDAT D121G mutagenesis was performed by GenScript. Mazindol, cocaine, methamphetamine, MDMC, 4-MMC, MDPV, and unlabeled MPP$^+$ were obtained from SigmaAldrich. MDMA was a gift from Dr. David Nichols (Purdue University).

2.1.2 dDAT D475N Construction

Wild-type dDAT DNA was obtained from Addgene. pBluescriptSKII vector and oocyte transcription vector containing dDAT were digested with KpnI and NotI; the resulting fragments were gel-purified, and complementary fragments were ligated to yield dDAT in a bacterial expression vector. Construction of a mutant insert was completed by GenScript, introducing the D475N mutation and a silent mutation to BamHI between BglII and Tth111I restriction sites in pUC57. Mutation was confirmed by restriction enzyme digestion. The mutant sequence was cloned into pBluescriptSKII.
2.1.3 Cell Culture

HEK293 cells stably expressing the dDAT WT and dDAT D475N genes were produced. cDNAs in pBluescriptSKII were digested with KpnI/NotI and subcloned into pcDNA3.1+. Wild type HEK293 cells were transfected with each gene using lipid-mediated transfer (Lipofectamine 2000; Invitrogen) as described by the manufacturer. Transfected cells were selected with 400 μg/mL genetecin (G418; Invitrogen). Selected colonies were characterized for [3H]DA uptake.

HEK293 cells stably expressing dDAT WT or dDAT D475N were maintained in Dulbecco’s modified Eagle’s medium with 5% dialyzed fetal bovine serum and 5% Fetal Clone I supplemented with penicillin, streptomycin, L-glutamine, and G418 (400 μg/mL). Cells were grown in a 37°C humidified environment with 5% CO2.

HEK293 GripTite MSR cells stably expressing hDAT were maintained in Dulbecco’s modified Eagle’s medium with 5% dialyzed fetal bovine serum and 5% Fetal Clone I supplemented with penicillin, streptomycin, L-glutamine, Geneticin (600 μg/mL) and G418 (400 μg/mL). Cells were grown in a 37°C humidified environment with 5% CO2.

2.1.4 [3H]DA Uptake Inhibition Assays

HEK293 cells stably transfected with dDAT WT, HEK293 cells stably transfected with dDAT D475N, or HEK293 cells transiently transfected with dDAT D121G using Lipofectamine 2000 (Invitrogen) were plated in 24-well plates precoated with poly-D-lysine (1*10^5 cells/well). The next day, cells were washed with KRH (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 10 mM HEPES, 2.2 mM CaCl2, pH 7.4) and then bathed in KRH with 5 mM glucose. Drugs (cocaine, methamphetamine, MDMC, 4-MMC, and MDPV) were added at increasing concentrations (1 nM-100 μM) and allowed to incubate for 10 minutes at 37°C. Three wells were left untreated to account for total uptake, while 10 μM mazindol was used to determine nonspecific uptake in an additional 3 wells. All
wells were treated in triplicate. 40 nM $[^3\text{H}]$DA with the antioxidant L-ascorbic acid (10 $\mu$M) and MAO inhibitor pargyline (10 $\mu$M) was added and incubated for an additional 10 minutes. Cells were washed twice with KRH and solubilized in MicroScint-20. The following day plates were read on a PerkinElmer TopCount NXT scintillation counter to measure $[^3\text{H}]$ accumulation.

### 2.1.5 $[^3\text{H}]$DA Uptake Kinetics Assays

HEK GripTite 293 MSR cells stably transfected with hDAT, HEK293 cells stably transfected with dDAT WT, or HEK293 cells stably transfected with dDAT D475N were plated in 24-well plates precoated with poly-D-lysine ($1*10^5$ cells/well). The next day, cells were washed with KRH (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 10 mM HEPES, 2.2 mM CaCl$_2$, pH 7.4) and then bathed in KRH with 5 mM glucose, 10 $\mu$M pargyline, and 10 $\mu$M ascorbic acid. Decreasing concentrations of $[^3\text{H}]$DA were added and allowed to incubate for 10 minutes at 37°C. 10 $\mu$M mazindol was used to determine nonspecific uptake. Cells were washed twice with KRH and solubilized in MicroScint-20. Plates were read on a PerkinElmer TopCount NXT scintillation counter to measure $[^3\text{H}]$ accumulation the next day.

### 2.1.6 $[^3\text{H}]$DA Efflux Rate Assays

HEK GripTite 293 MSR cells stably transfected with hDAT or HEK293 cells stably transfected with dDAT WT were plated in 24-well plates ($1*10^5$ cells/well). The next day, cells were washed with KRH (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 10 mM HEPES, 2.2 mM CaCl$_2$, pH 7.4) and bathed in KRH with 5 mM glucose. All work was completed in duplicate wells. Cells were preloaded with 40 nM $[^3\text{H}]$DA for 20 minutes at 37 °C, with four wells pretreated with 10 $\mu$M mazindol. Twelve wells were treated with 100 $\mu$M methamphetamine and twelve wells were treated with KRH. Efflux was terminated at 0, 1, 2, 3, and 5
minutes by washing twice in KRH. Cells were solubilized in MicroScint-20 and $[^3H]$ was quantified on a PerkinElmer TopCount NXT scintillation counter.

### 2.1.7 $[^3H]$MPP+ Efflux Assays

HEK GripTite 293 MSR cells stably transfected with hDAT, HEK293 cells stably transfected with dDAT WT, HEK293 cells stably transfected with dDAT D475N, or HEK293 cells transiently transfected with dDAT D121G using Lipofectamine 2000 (Invitrogen) were plated in 24-well plates ($1 \times 10^5$ cells/well). The next day, cells were washed with KRH (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 10 mM HEPES, 2.2 mM CaCl$_2$, pH 7.4) and bathed in KRH with 5 mM glucose. All work was completed in quadruplicate wells. The first four wells were treated with 10 μM mazindol and all cells were preloaded with 340 μM $[^3H]$-MPP+ for 20 minutes at 37 °C. The first eight wells were terminated to account for nonspecific and total uptake. Remaining wells were treated with KRH or a single concentration of drug (10 μM methamphetamine, 10 μM MDMA, 10 μM MDPV, 1 μM MDMC, 10 nM 4-MMC). Efflux was allowed for 10 minutes at 37 °C, then terminated by washing twice in KRH. Cells were solubilized in MicroScint-20 and $[^3H]$ was quantified on a PerkinElmer TopCount NXT scintillation counter. Drug-induced efflux was calculated by subtracting nonspecific uptake and normalizing to MPP+ remaining to the amount of $[^3H]$ remaining after 10 minutes of basal efflux.

### 2.1.8 Data Analysis

IC$_{50}$ values for drug-inhibited uptake were estimated using nonlinear curve-fitting analysis (GraphPad Prism 6 software, San Diego, CA). All results were expressed as mean ± standard error of the mean (SEM) for at least three experiments performed in triplicate unless otherwise noted.
Multiple t-tests were completed to compare basal and drug-induced efflux.

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

2.1.9 Protein-Ligand Docking

AutoDock Vina software was used to calculate binding free energy and poses of drugs on the 4Xp9 crystal structure of dDAT using methods outlined by Forli et al. [88]. Drugs docked were D-amphetamine, dopamine, methamphetamine, methylone (MDMC), mephedrone (4-MMC), MDPV, and MDMA. Receptor side chains at the S1 site (F43, D46, A117, V120, D121, Y124, F319, F325, S421, and S422) were treated as flexible during docking. Docking was also performed using a structure mutated to contain G121. Docking poses were inspected visually to ensure results were consistent with the binding pose of D-amphetamine shown in the 4Xp9 crystal structure. Search space was 16x16x16 Å along the binding site identified in the crystal structure.

Docking at the S2 binding site was performed with the same drugs, treating side chains at the S2 site (R52, Y123, I127, W130, F216, I220, L221, D312, Q316, P386, F471, and D475) as flexible. Docking was also performed using a structure containing N475. Docking poses were inspected visually to ensure ligand was docked within the binding site. Search space was 18x18x26 Å along the binding site identified in the LeuT crystal structure.

2.2 Results

2.2.1 Uptake Inhibition Results

The Barker lab previously engineered a series of SERT mutants demonstrating the importance of a charge in the outer gate to MDMA-induced efflux [86]. A highly conserved structure, the salt bridge has been shown to be essential to drug-induced efflux. The extracellular gate of dDAT was shown in Figure 1.5. A
Mazindol inhibition of DA uptake in HEK-293 cells stably transfected with wild type and D475N DAT or transiently transfected with dDAT D121G. [³H]DA uptake assays were performed as described in Materials and Methods. Data were plotted as percentage of total DA uptake. Results shown represent mean ± standard errors of triplicate determination and are representative of at least three independent experiments.

Summary of IC₅₀ values (95% confidence interval): ○WT IC₅₀ = 35 nM (20-61 nM), □D475N IC₅₀ = 18 nM (4.9-66 nM), ◇D121G IC₅₀ = 5.1 nM (3.1-8.4 nM).
Drug inhibition of DA uptake in HEK-293 cells stably transfected with wild type and D475N DAT. [³H]DA uptake assays were performed as described in Materials and Methods. Nonspecific uptake was determined with 10 μM mazindol. Data were plotted as a percentage of specific DA uptake. Results shown represent mean ± standard errors of triplicate determination and are representative of at least three independent experiments.
dDAT mutant (D475N) was designed to test if presence of a charged residue at the extracellular gate was also important to efflux function in DAT. To investigate the role of D121 in uptake and efflux, a D121G mutant was made to mimic the residue found at this position in hDAT. Uptake inhibition experiments were first performed with the stimulant drug mazindol to ensure that cells were expressing dDAT and able to uptake DA (2.1(a)). Mazindol was an especially potent inhibitor of the D121G mutant, with an IC$_{50}$ of 5 nM, nearly ten times less than that of wild-type DAT.

Uptake inhibition experiments by psychostimulant drugs showed only slight differences in uptake between dDAT WT and dDAT D475N (Figure 2.2). Table 2.1 summarizes the IC$_{50}$ values established by uptake inhibition experiments. These findings revealed that amphetamines and synthetic cathinones remain capable of binding dDAT with either aspartic acid or asparagine at the outer gate.

Uptake kinetics experiments revealed differences between hDAT, dDAT, dDAT D475N. hDAT had lowest maximal velocity (V$_{\text{max}}$) and K$_{m}$ (the substrate concentration at which the reaction rate is half of V$_{\text{max}}$) for uptake. The dDAT D475N mutant slowed DA uptake rate compared to wild-type dDAT, not reaching V$_{\text{max}}$ within the DA concentrations tested.

Table 2.1: Summary of DA uptake inhibition IC$_{50}$ Values

<table>
<thead>
<tr>
<th></th>
<th>Mazindol</th>
<th>MDPV</th>
<th>Meth</th>
<th>MDMC</th>
<th>4-MMC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>-7.5±0.1</td>
<td>-4.3±0.2</td>
<td>-5.4±0.1</td>
<td>-4.3±0.7</td>
<td>-4.0±0.2</td>
</tr>
<tr>
<td><strong>D475N</strong></td>
<td>-7.7±0.3</td>
<td>-4.8±0.3</td>
<td>-6.5±0.5</td>
<td>-4.0±0.1</td>
<td>-4.2±0.2</td>
</tr>
</tbody>
</table>

Estimated IC$_{50}$ values (log M) for inhibition of [$^3$H]DA uptake in stably transfected HEK-293 cells. IC$_{50}$ values represent mean ± standard errors for at least three independent experiments.
2.2.2 Efflux Results

I attempted to assess drug-induced efflux of DA. Although efflux occurred, there was no difference between the amount with and without amphetamine drugs. Hypothesizing that there is a high amount of basal efflux, I tested the rate of efflux with 100 μM methamphetamine treated and vehicle treated cells (Figure 2.3). Due to the high rate of basal efflux, there was no apparent drug-induced efflux in human or drosophila DAT. Dopamine is a highly reactive molecule that is subject to oxidation, which could lead to a decrease in signal over time. It is also somewhat polar, making the ligand able to diffuse through plasma membranes.

1-methyl-4-phenylpyridinium (MPP+) is a positively charged substrate of DAT which will not oxidize or cross the membrane as easily. Leaky cells allowed an excess of MPP+ to cross back through the membrane, but not to the same magnitude as DA (Figure 2.4). While dDAT D475N had overall lower raw counts per minute of tritium, both dDAT mutants exhibited similar levels of basal efflux compared to WT.

Drug-induced reversal of MPP+ transport was tested in hDAT, dDAT, dDAT D475N, and dDAT D121G. Both methamphetamine and MDMA induced a change over basal efflux in hDAT and dDAT D121G (p≤0.05) (Figure 2.5). The cathinone 4-MMC induced efflux in hDAT but not dDAT or any dDAT mutants at the 10 nM concentration. MDPV and MDMC did not induce efflux in any of the experiments. While methamphetamine and 4-MMC may have induced some efflux in dDAT WT, drug-induced efflux did not reach statistically significant levels, which is likely due to high basal efflux relative to total uptake. Cells expressing dDAT D475N had no notable drug-induced efflux.

2.2.3 Docking Results

Docking simulations using AutoDock Vina were completed to investigate drug binding at the S1 and S2 sites and see if a difference in binding modes could
Effects of 100 μM methamphetamine or vehicle on the efflux of [3H]DA in cells stably expressing dDAT or hDAT. Cells were loaded with [3H]DA (40 μM) for 20 minutes and efflux induced as described in Materials and Methods. There is no difference in efflux rate between methamphetamine and untreated cells.
Figure 2.4.: Basal DA and MPP\(^+\) Efflux

Total uptake and basal efflux of \(^3\)H\(\text{MPP}^+\) and \(^3\)H\(\text{DA}\) in cells stably expressing dDAT. Cells were loaded with \(^3\)H substrate for 20 minutes and immediately terminated or allowed to incubate for an additional 10 minutes as described in Materials and Methods. Basal efflux of DA was greater than basal efflux of MPP\(^+\). Results shown represent mean ± SEM and are representative of five independent experiments.
Figure 2.5.: Drug-induced MPP⁺ Efflux

(Continued on the following page.)
Figure 2.5.: Effects of drug on the efflux of $[^3$H]MPP$^+$ in cells expressing hDAT, dDAT, dDAT D475N, or dDAT D121G. Cells were loaded with $[^3$H]MPP$^+$ (340 $\mu$M) for 20 minutes and efflux induced by drug as described in Materials and Methods. Drug-induced efflux occurred in hDAT and dDAT D121G. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Results shown represent mean ± SEM of quadruplicate determination and are representative of three independent experiments. Basal efflux was calculated as the amount of $[^3$H] remaining after 10 minutes without drug treatment.
account for the difference in uptake and efflux between WT and the dDAT mutants. To investigate the role of D121 at the S1 site, docking was performed on WT and a D121G mutant. This mutation is expected to enhance human-like receptor recognition at the site and increase apparent drug potency, represented by higher drug affinity. Calculations demonstrated similar poses (Figure 2.6, 2.7) and scores (Table 2.2) between the site variants. The aromatic ring of 4-MMC shifts positions from perpendicular to Y124 in dDAT WT to nearly parallel to Y124 in the dDAT D121G mutant. MDMC was actually 0.6 kcal/mol more affine to the dDAT WT S1 binding site than dDAT D121G, suggesting that the binding pose is a more accurate fit for drosophila than human DAT.

In order to verify predictive accuracy at the S1 site, the calculated docking pose of D-amphetamine and D-amphetamine density in the amphetamine bound LeuT crystal structure (PDB ID 4XP9) were compared. All-atom RMSD between the computational and experimental ligand was calculated to be 1.087 Å. Accuracy at the S2 site is more difficult to determine because there is no crystal interaction with which to compare. The approved pose was chosen as the highest affinity dock within the proposed binding site that had at least one similar calculated pose.

Consistent with previous findings, the S1 site has much higher affinity for dopamine and psychostimulant drugs than the S2 site. In the case of all drugs studied here, drugs had higher affinity for the binding sites than endogenous dopamine (Table 2.2). This is supportive of their role as potent competitive inhibitors, out-competing DA for its binding site. Docking poses at WT and D475N S2 binding site are shown in Figures 2.8 and 2.9. There were only small differences between calculated affinities for drug binding at the S2 site between WT and D475N protein. Low affinity scores suggest that confidence in the docking poses at the S2 binding site was low. dDAT crystal structures have not yet been solved in the inward open conformation, where the extracellular vestibule is closed. Residues surrounding the S2 site in the amphetamine bound LeuT crystal structure (PDB
ID 4XP9) may have been too far apart to accurately predict ligand binding when protein is in this conformation.

Figure 2.6.: Model of drugs docked to the S1 binding site of wild-type dDAT

All ligands adopt a similar pose with the positively charged side chain pointing toward D46 and the ring moiety pointing into the binding pocket toward V120 and D121.
Figure 2.7.: Model of drugs docked to the S1 binding site of dDAT D121G

Ligands adopt a similar pose with the positively charged side chain pointing toward D46 and the ring moiety pointing into the binding pocket toward V120. The tolyl ring of 4-MMC is rotated further into the binding pocket compared to the other ligands.
Figure 2.8.: Model of drugs docked to the S2 binding site of wild-type dDAT

Ligands interact with the extracellular gate R52 and D475. MDPV is unique because the pyrovalerone ring interacts with R52, while other ligands interact along their carbon side-chains.
Figure 2.9.: Model of drugs docked to the S2 binding site of dDAT D475N

Ligands interact with R52 but are docked further outside the proposed binding site, interrupting the extracellular gate when compared to the wild-type protein.
Table 2.2: Computationally determined binding affinities (kcal/mol) for ligands at dDAT S1 and S2 binding sites

<table>
<thead>
<tr>
<th>Ligand</th>
<th>WT S1</th>
<th>D121G S1</th>
<th>WT S2</th>
<th>D475N S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>-6.1</td>
<td>-6.2</td>
<td>-4.4</td>
<td>-4.5</td>
</tr>
<tr>
<td>MDPV</td>
<td>-9.0</td>
<td>-9.4</td>
<td>-6.6</td>
<td>-6.0</td>
</tr>
<tr>
<td>Meth</td>
<td>-6.3</td>
<td>-6.2</td>
<td>-4.8</td>
<td>-4.8</td>
</tr>
<tr>
<td>MDMC</td>
<td>-7.9</td>
<td>-7.3</td>
<td>-5.4</td>
<td>-5.2</td>
</tr>
<tr>
<td>4-MMC</td>
<td>-7.1</td>
<td>-7.1</td>
<td>-5.4</td>
<td>-5.5</td>
</tr>
<tr>
<td>MDMA</td>
<td>-7.7</td>
<td>-7.5</td>
<td>-5.1</td>
<td>-5.1</td>
</tr>
</tbody>
</table>

Binding affinities (kcal/mol) calculated in AutoDock Vina. Ligands were docked to the S1 binding site of wild-type and humanized D121G dDAT structure. Ligands were also docked to a model of the S2 binding site with both wild-type dDAT and D475N gating mutant.
3. DISCUSSION

3.1 Discussion of Results

Amphetamines and synthetic cathinones have limited medical use and a high potential for dependence and abuse. Addictive properties are caused largely by their action on DAT and the monoamine transporters leading to increased concentrations of DA in the synapse and activation of brain reward pathways [6].

Different regions of DAT have been implicated for substrate recognition and transport. The substrate binding (S1) site was identified in a crystal structure of dDAT (PDB ID 4XP1), and computational studies on DAT, as well as crystal structures of the homologous LeuT (PDB ID 2Q6H) and hSERT (PDB ID 5I6X) proteins suggest an allosteric S2 site. Although many studies have explored the inward and outward transport of substrate in DAT and other SLC6 transporters, the molecular mechanisms of transporter efflux is not fully understood. A point mutation in hSERT (D493N) abolishes MDMA-induced efflux [86]. Here the analogous mutant dDAT D475N was used to further explore the role of the extracellular gate in amphetamine-induced efflux. Three relatively new cathinone analogue drugs were utilized to investigate the interaction of structurally similar yet functionally diverse drugs on DAT.

Cells expressing dDAT, dDAT D121G, and dDAT D475N, were all able to translocate DA and MPP⁺. DA uptake was inhibited by drugs at similar potencies, demonstrating that the D475N gating mutant does not interfere with DA uptake. Amara and colleagues showed that dDAT is less selective for cocaine and amphetamines than hDAT [87]. Consistent with this trend, methylone, mephedrone, and MDPV were had IC_{50} values at least tenfold less potent at dDAT than hDAT [53].
Both dDAT and hDAT exhibit basal efflux of DA and MPP+. Previous experiments in the Barker lab demonstrate that dSERT also exhibits basal efflux of 5-HT, while hSERT does not [86]. This was hypothesized to be due to the absence of the salt-bridge. The data shown herein suggest that basal efflux is also due to additional residues not present in hSERT. Basal efflux of \[^3H\]DA may be due to DA degradation, but \[^3H\]MPP+ is not as susceptible to oxidation. Differences in transfection efficiency between the two genes could also contribute to discrepancies. The amount of cell surface expression was not determined and could contribute to differences in measured efflux.

hDAT and dDAT D121G exhibited drug-induced efflux with methamphetamine and MDMA, while dDAT WT and dDAT D475N did not. Consistent with its action as a cocaine-like inhibitor, MDPV did not induce efflux in any of the experiments. Interestingly, MDMC prevented basal efflux through the leaky hDAT and dDAT WT transporters. Experiments in hDAT showed no statistically significant release of neurotransmitter by MDMC even at >100 μM concentrations [53]. Docking experiments showed that MDMC had high affinity for the S1 site, second only to MDPV. It is possible that MDMC spends more time at the S1 site and prevents transporter reversal on the timescale tested. My results suggest that the D121 residue in the S1 site of dDAT and perhaps further unidentified residues make dDAT less sensitive to efflux than hDAT. BLAST sequence alignment between hDAT and dDAT show very low sequence similarity at the N-terminus [89]. N-terminus phosphorylation has been demonstrated to be critical in amphetamine-induced efflux [84,90]. The shortened dDAT N-terminus could also contribute to the lack of measurable efflux in dDAT.

Models can be used to make predictions about ligand binding to transporter proteins like DAT in computational docking studies, and should be cross-validated with biological studies [91,92]. Binding modes predicted by ligand docking display insights into the affinity and selectivity of ligands for dDAT. While algorithms are good at scoring binding poses, they should not be used to compare the relative
affinity of a series of ligands. The empirical scoring algorithm employed by AutoDock Vina does not fully account for factors such as hydrophobic contribution, explicit electrostatic interactions, and hydrogen bonding, making the software inaccurate at estimating binding free energy [80]. Scores are, however, useful for assessing confidence in the pose, where a reduction in calculated affinity after mutation could mean the pose is more questionable in the mutant.

In this dissertation, I report models of amphetamine and several cathinone analogues bound to dDAT. Transporter conformation used in docking should be physically realistic and represent functional states relevant to binding. Residues around the binding sites were treated as flexible to provide a small amount of conformational diversity and allow the docking algorithm to yield accurate binding poses. I hypothesized that ligands bind to dDAT in an open-to-out conformation in the S1 site for both wild-type and D121G transporter. I hypothesized that the binding pose of ligands to the D475N mutant would differ from wild-type, with lower binding affinity. Figures 2.7 and 2.8 include poses for ligands for both WT and D121G S1 sites using AutoDock Vina, while Figures 2.9 and 2.10 show poses for ligands at the S2 site for WT and D475N. The poses and calculated binding affinities were nearly identical at the S1 site, while ligand binding was shifted more through the extracellular gate in the D475N mutant at the S2 site. Similar poses at both the WT and mutant S1 site suggest D121 may be involved in the conformational changes necessary for drug-induced efflux. The crystal structure of D-amphetamine in complex with dDAT provides experimental validation of the pose for amphetamine in this model [78].

Together, these data contribute to a better model for substrate-induced efflux of the monoamine transporters. Future research on the dopamine transporter will be useful in treating addiction and toxicity resulting from the use of amphetamine and its analogues.
3.2 Future Directions

Previous studies have demonstrated a channel mode of operation for monoamine transporters [93–95]. Electrophysiological studies with two-electrode voltage clamp in oocytes expressing DAT could be used to assess whether drugs induce an inward current comparable to that observed for DA and are thus acting as substrates.

hDAT and dDAT may differ in transport turnover rate for inward transport versus outward transport/efflux. The D475-R52 extracellular gate charge interaction may influence the reorientation of substrate containing dDAT back to outward open. Additionally, residues around the S1 site may interact with other transmembrane domains to stabilize a specific conformation that is favorable for outward transport.

Structural biology experiments can look directly at the static structure of DAT; crystallography has been used to capture the protein in various drug-bound states. Another possible method of studying structure is using cryogenic electron microscopy (cryo-EM) to bypass some of the challenges associated with X-ray crystallography. Double electron-electron resonance (DEER) or fluorescence resonance energy transfer (FRET) look at dynamics of protein movement. Cysteine crosslinking MTS assays could be used to assess pore accessibility changes by the D475N mutant, and a shift from open to occluded conformations.

Structural studies require pure protein to carry out experiments. I have expressed and purified dDAT WT and dDAT D475N protein that may be used in future experiments. First attempts at overexpression were done in bacteria based on the expression of LeuT used for its crystal structure. Although bacterial expression proved unsuccessful, I was able to express DAT in High5 insect cells. This differs from the expression of dDATmfc in human embryonic kidney (HEK) cells, used in the first DAT crystal structure [71].

Although X-ray crystallography provides valuable information about the structure of proteins, it is unable to reveal much about the dynamics of protein movement. DAT is believed to adopt three different conformations with a possible
fourth channel-like state, but the transition between each state is still a topic of study. Molecular dynamics (MD) simulations allow further understanding of how these transitions occur. Molecular dynamics simulations using a model of DAT based on LeuT could be used to predict the transition between conformations, and identify other residues that are important in reversal of transport. By performing MD simulations on models of dDAT docked with psychostimulants, we can observe and predict how the drugs act to inhibit conformational change and better understand drug mechanisms. The structure of LeuT has been used as a template for computational modeling of SERT, and similar techniques can be used to model the structural dynamics of DAT [86]. Docking could be verified in other programs or used to explore other amphetamine and cathinone derivatives. Docking results should also be validated through structural biology experiments such as crystallography or cryo-EM.

The results presented in this dissertation show that the presence of a salt bridge at the external gate of DAT, as well as a hydrophobic environment within the DA binding site, are important for drug-induced DA efflux. Better understanding of the molecular mechanisms underlying drug-induced substrate efflux could lead to better treatments for drug addiction.
LIST OF REFERENCES
LIST OF REFERENCES


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