Modulation of Dopamine Transporter Function by Tamoxifen

by

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"The mentality and behavior of drug addicts and alcoholics is wholly irrational until you understand that they are completely powerless over their addiction and unless they have structured help, they have no hope."

— Russell Brand

"Dopamine is the center of the universe"

— Margaret E. Gnegy, Ph.D.

DEDICATION

For Jay, who kept me sane

and

For Ken, you are missed

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ABSTRACT

The neurotransmitter dopamine is vital to motor coordination, reward, motivation, and cognition. Diseases of dopaminergic dysfunction include Parkinson's disease, addictions, and bipolar disorder, to name a few. The dopamine transporter is responsible for the reuptake of extracellular dopamine and as such plays a key role in modulating the strength and duration of the dopamine signal. Due to its role in dopamine regulation, the dopamine transporter is an attractive therapeutic target for pharmacological intervention in diseases of dopaminergic dysfunction. This dissertation presents results that support the repurposing of an old drug for a novel purpose. I demonstrate for the first time that the breast cancer therapeutic tamoxifen, widely investigated and prescribed for its role as a selective estrogen receptor modulator, is an atypical blocker of the dopamine transporter. Atypical dopamine transporter blockers are currently under investigation for the treatment of psychostimulant abuse due to their unique ability to antagonize the actions of cocaine and amphetamine at the dopamine transporter without exhibiting their own abuse liability. Here, I show that tamoxifen non-competitively inhibits dopamine uptake and amphetamine-stimulated dopamine efflux. This action at the transporter is independent of tamoxifen's effects on the estrogen receptors and appears to involve a direct interaction of tamoxifen with the S2 binding site of the dopamine transporter. Consistent with my assertion that tamoxifen is an atypical dopamine transporter blocker, I found that

tamoxifen attenuates amphetamine-stimulated hyperactivity, yet exerts no stimulant effects on its own. Further investigation into the active metabolites of tamoxifen revealed that 4-hydroxytamoxifen and endoxifen also impede dopamine uptake and amphetamine-stimulated dopamine efflux. Interestingly, these two compounds seem to act asymmetrically on the dopamine transporter, preferentially inhibiting dopamine uptake and amphetamine-stimulated dopamine efflux, respectively. I propose that tamoxifen, or a novel chemical based on its structure, may present a viable option for the treatment of psychostimulant abuse.

CHAPTER ONE. INTRODUCTION

The Dopaminergic System

The dopaminergic systems of the brain play a fundamental role in many vital neural processes including the regulation of movement, cognition, decision-making, learning, and reward processing (Beninger, 1983; Westbrook and Braver, 2016). Dysfunctions in the dopaminergic system can contribute to a number of pathological conditions, including Parkinson's disease and parkinsonism, schizophrenia, bipolar disorder, and addiction (Robinson and Berridge, 2008; Abi-Dargham, 2014; German et al., 2015; Whitton et al., 2015).

Neuroanatomy

The chief ascending dopaminergic pathways are the mesocorticolimbic and nigrostriatal pathways. The mesocorticolimbic pathway is composed of two parts, the mesolimbic pathway, which extends from the ventral tegmental area (VTA) to the nucleus accumbens, and the mesocortical pathway which projects from the VTA to the prefrontal cortex. The mesolimbic pathway is important in reward processing and the development of addiction (Pierce and Kumaresan, 2006), whereas the mesocortical pathway contributes to cognition and learning (Seamans and Yang, 2004). The nigrostriatal pathway extends from the substantia nigra *pars compacta* to the striatum. Loss of these neurons is a dominant characteristic of Parkinson's disease (Shadrina et al., 2010). Additionally, dopamine is

released from the arcuate nucleus of the hypothalamus into the portal vein where it interacts with the pituitary gland and regulates the secretion of prolactin (Figure 1-1).

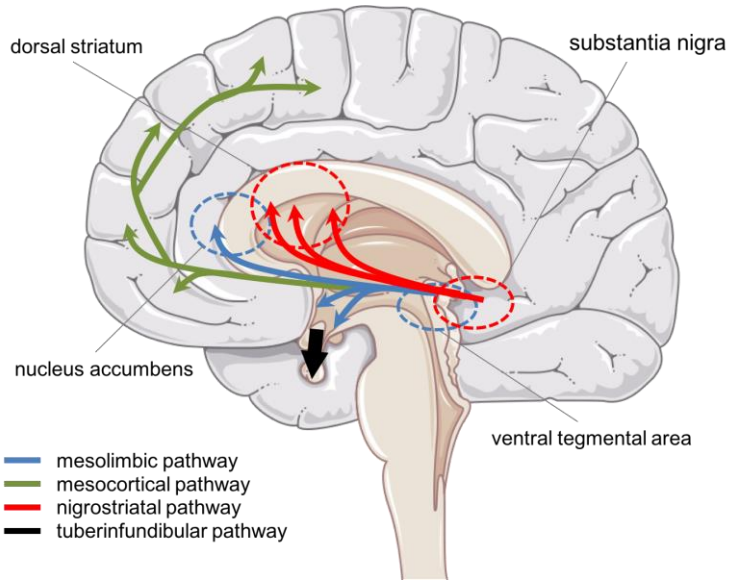

Figure 1-1 Dopaminergic pathways of the human brain.
Adapted from Brody et al. (1998)

Dopamine synthesis and metabolism

Dopamine signaling is tightly regulated at multiple levels beginning with production. The first step in the synthesis of dopamine is the conversion of tyrosine to 3,4-deoxyphenylacetic acid (DOPA) by tyrosine hydroxylase (Figure 1-2). Tyrosine hydroxylase performs the rate limiting step in dopamine production and is a site of dynamic regulation. Tyrosine hydroxylase gene expression can be up- or down-regulated for long term effects on dopamine levels, and enzyme activity can be more rapidly regulated by feedback inhibition. Catecholamines bind to tyrosine hydroxylase with very high affinity which inhibits the enzyme; this inhibition can only be removed by phosphorylation of tyrosine hydroxylase, most prominently at serine 40 (Ramsey and Fitzpatrick, 1998; Dickson and Briggs, 2013). Tyrosine hydroxylase is a substrate for various serine/threonine kinases,

including protein kinase A, protein kinase G, protein kinase C, calcium/calmodulin dependent kinase II (CAMKII), and extracellular regulated kinase (ERK), but only phosphorylation by protein kinase A, protein kinase G, or ERK leads to productive activation of the enzyme. Deactivation of tyrosine hydroxylase is restored by dephosphorylation by protein phosphatase 2A, which presents another target for regulation (Zhang et al., 2007). Following synthesis of DOPA, dopamine is formed by rapid decarboxylation by DOPA decarboxylase. Dopamine is rapidly metabolized by catechol-O-methyl transferase, expressed in glial cells (Myohanen et al., 2010; Zeng et al., 2010) and postsynaptic neurons, and by monoamine oxidase, which is expressed in the mitochondria of the synaptic neuron as well as in glia (Meiser et al., 2013).

Dopamine packaging and release

Once synthesized, DA is packaged into vesicles *via* the vesicular monoamine transporter 2 (VMAT2) and held in reserve until an action potential stimulates exocytotic release. Upon release, dopamine enters the extracellular space and binds to G-protein coupled dopamine receptors on both the pre- and post-synaptic neuron. Dopamine receptors on the postsynaptic neuron, which are either D1-like or D2-like propagate the downstream signal and are another site of regulation. Dopamine receptors on the presynaptic neuron, which are D2-like, exist extrasynaptically. When dopamine concentrations in the synapse are sufficient to produce an overflow of dopamine outside of the synaptic space, the D2 receptor is activated and creates a feedback inhibition to attenuate further dopamine release. Activation of this D2 autoreceptor leads to inhibition of calcium channels and activation of G-protein-coupled inwardly rectifying potassium channels (GIRKs) both of which cause a decrease in vesicle-fusion events following an action potential (Ford, 2014).

In addition to the rapid effects on exocytosis, D2 activation also leads to decreased dephosphorylation of tyrosine hydroxylase, and a consequent decrease in dopamine production in the neuron (Salah et al., 1989).

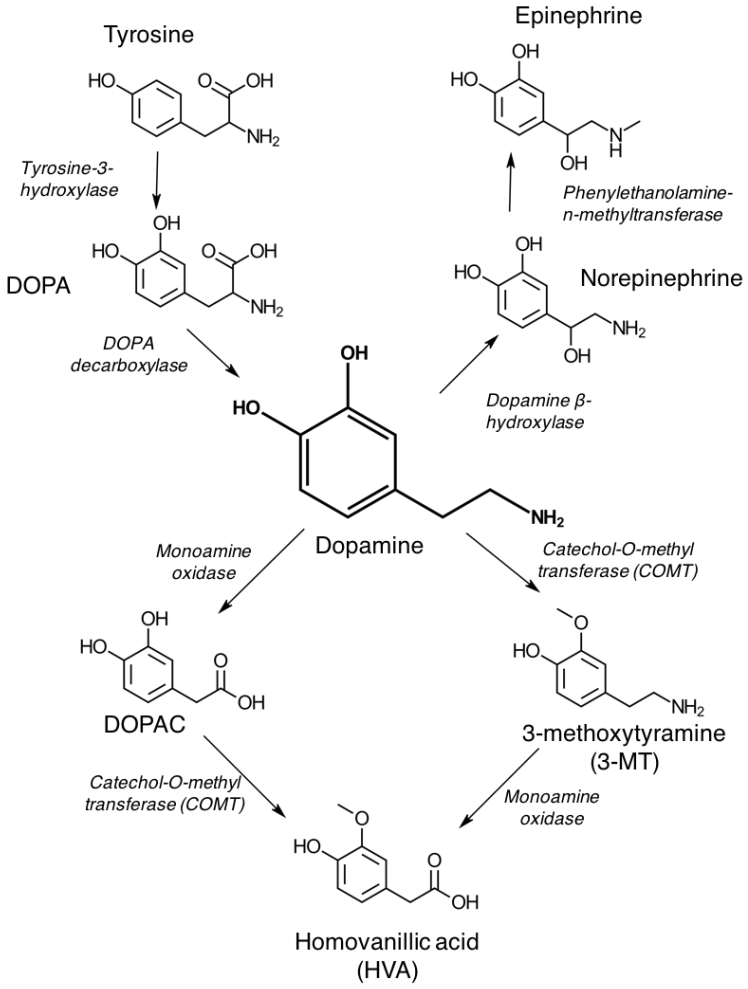

Figure 1-2. Synthesis and metabolism of the neurotransmitter dopamine.

Termination of the dopamine signal

The dopamine signal can be terminated in one of three ways: 1) diffusion into the extracellular space, 2) metabolism (described above), or 3) reuptake into the presynaptic neuron via the dopamine transporter. The dopamine transporter is the primary method by

which the dopamine signal is terminated, as evidenced by the fact that dopamine transporter knockout mice have significantly elevated extracellular dopamine levels in the brain and as a result exhibit a notable increase in locomotor activity.

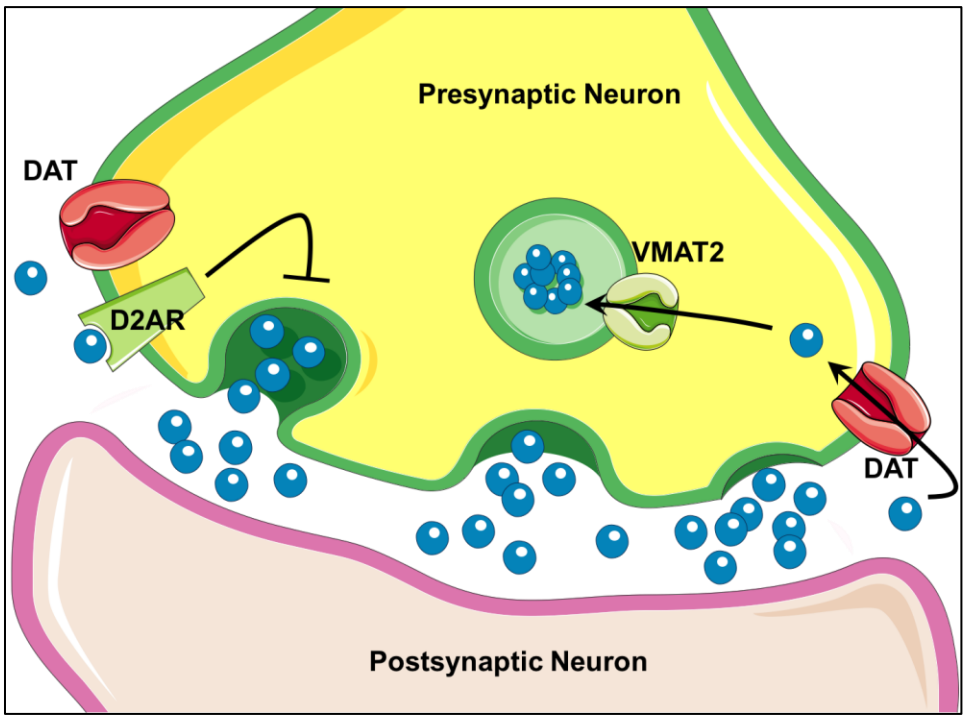

Figure 1-3. Presynaptic regulation of dopamine signaling.
Exocytotic release of dopamine floods the extracellular space with dopamine. Dopamine binds D2-like autoreceptors (D2AR) which initiate a negative feedback signal to decrease dopamine release. Dopamine is taken back up into the presynaptic neuron through the dopamine transporter (DAT) and repackaged into vesicles by the vesicular monoamine transporter (VMAT2) for future release.

The dopamine transporter is a 12-transmembrane protein of the SLC6 transporter family which includes the norepinephrine transporter and the serotonin transporter. Both the C-and N-termini of the protein rest on the cytoplasmic site of the membrane and serve as major sites for the dynamic regulation of the transporter. Dopamine transport is driven by the coupling of the inward movement of dopamine to the transport of two sodium ions down the electrochemical gradient into the neuron. One dopamine molecule is transported

for every two sodium ions and one chloride ion. Following uptake of dopamine into the neuron, it is repackaged into nearby vesicles through the vesicular monoamine transporter (VMAT) for further exocytotic release.

The dopamine transporter is also vital in mediating the mechanism of several important drugs of abuse including cocaine and amphetamine. Cocaine blocks the dopamine transporter while amphetamines, which are substrates for the dopamine transporter, deplete dopamine from vesicles into the cytoplasm, and stimulate the reverse transport of dopamine out of the neuron into the extracellular space. Both of these drugs stimulate a supraphysiological increase in extracellular dopamine levels, which are responsible for the majority, if not all, of these drugs' rewarding effects. The history of our understanding of the dopamine transporter will be presented in depth, followed by a review of current knowledge and controversies in its structure and regulation. Particular attention will be paid to the interaction between the transporter and amphetamine.

A history of catecholamine transport

Dopamine was originally believed to be merely a precursor for norepinephrine. Thus, much of the original work that would eventually lead to our understanding of the dopamine transporter was based around epinephrine and norepinephrine. Early attention was paid to the actions of "sympathomimetic" drugs, those compounds known to cause effects consistent with activation of the sympathetic nervous system, i.e. increased heart rate, elevated blood pressure, pupil dilation.

Early Indications

Perhaps the first indication of a catecholamine uptake mechanism was the demonstration in 1910 that cocaine, a popular anesthetic and stimulant whose use was becoming an

increasing matter for concern, potentiates the effects of adrenaline (Frölich and Loewi, 1910). We now understand that this potentiation resulted from blockade of the norepinephrine transporter, enabling a persistence of elevated levels of adrenaline. Catecholamine uptake was first proposed by Burn in 1932. He found that tyramine and ephedrine, both drugs that we now know increase the release of epinephrine, had only a minor vasoconstrictor effect on perfused tissue unless epinephrine was added to the bath and allowed to perfuse the tissue for a prolonged period. In hindsight, we now understand that this prolonged perfusion permitted the uptake of epinephrine into nerve terminals, whose original stores of epinephrine had been depleted due to experimental conditions, so that when tyramine or ephedrine were added, they would stimulate release (Burn, 1932). Decades later, the phenomenon described by Burn was still being investigated, though perhaps with improved models that were capable of retaining their original stores of norepinephrine. Several key finds were reported from the 1930s through the early 1960s. It was determined that functioning nerve terminals were required for the noradrenergic rescue of tyramine function, and cocaine would exert effects similar to total denervation. Notably, reserpine, which depletes norepinephrine stores, abrogated the effect of tyramine. However, infusion of noradrenaline following reserpine treatment restored the tyramine effect. Like cocaine, the effects of treatment with reserpine were similar to denervation (Fleckenstein and Bass, 1953; Fleckenstein and Stockle, 1955; Hertting et al., 1961). Julius Axelrod and colleagues continued these lines of research and found that epinephrine and norepinephrine were selectively taken up or bound by adrenal gland, heart, and spleen. Though metabolism by catechol-O-methyltransferase (an enzyme first described by Axelrod (Labrosse et al., 1958)) was detected, mostly in skeletal muscle, significant

amounts of unmodified catecholamine remained in other tissues. Additionally, accumulation of catecholamines in these tissues was inhibited by cocaine, indicating that cocaine was prolonging the effect of injected catecholamines by inhibiting its uptake, rather than impeding metabolism as had been proposed by some (Whitby et al., 1960; Whitby et al., 1961; Wolfe et al., 1962). This work was the first indication that uptake or sequestration was the predominant method for inactivation of the effects of circulating catecholamines and would contribute to Axelrod's share in the 1970 Nobel Prize in Physiology or Medicine. Axelrod and colleagues visually localized tritiated norepinephrine to sympathetic nerve axons by electromicroscropic autoradiography (Wolfe et al., 1962), and further determined its intracellular localization to small microsomes, which was one of the earliest indications of the packaging of catecholamines into vesicles for future release (Potter and Axelrod, 1962).

Dopamine vs. Norepinephrine

The concept of separate uptake mechanisms for norepinephrine and dopamine began with the observation that desipramine (now known to be a potent norepinephrine transporter inhibitor with little affinity for the DAT) prevented the accumulation of catecholamines in noradrenergic but not dopaminergic terminals (Hamberger, 1967). Though primarily noradrenergic regions of the brain efficiently took up both dopamine and norepinephrine (consistent with our current knowledge that dopamine has an even higher affinity for the norepinephrine transporter than the dopamine transporter), synaptosomes from the corpus striatum were significantly more efficient at accumulating dopamine than norepinephrine, with K_ms (the substrate concentration at which the reaction is half-

maximal) of 400 nM and 2 μM, respectively (Gfeller et al., 1968; Snyder et al., 1968a; Snyder et al., 1968b; Coyle and Snyder, 1969; Snyder and Coyle, 1969; Horn et al., 1971).

Identification of the dopamine transporter

During the following decade, compounds that seemed to bind to the "dopamine transport complex" with some specificity were identified and included cocaine, GBR 12935, mazindol, methylphenidate and nomifensine (Kennedy and Hanbauer, 1983; Javitch et al., 1984; Dubocovich and Zahniser, 1985; Janowsky et al., 1985; Scatton et al., 1985; Janowsky et al., 1986). Concurrently, the dopamine hypothesis of addiction was gaining traction. It was determined that drugs abused by humans selectively increased extracellular dopamine levels in the nucleus accumbens, while therapeutics without abuse potential had no such effect (Di Chiara and Imperato, 1988). Additionally, the potencies of cocaine-like drugs in producing self-administration correlated with their affinity for the dopamine transporter (Ritz et al., 1987), further indicating a role for the dopaminergic system in the reinforcing characteristics of such compounds. In 1989, Grigoriadis et al. successfully isolated the dopamine transporter using a ligand similar in structure to GBR 12935 (^{125}I-DEEP) which bound irreversibly to a protein following exposure to UV light. Selective blockade of the binding of ^{125}I-DEEP by known dopamine transporter blockers strongly indicated that the protein was indeed the dopamine transporter. The dopamine transporter was revealed to be a large glycoprotein with a molecular weight of 58 kilodaltons. The rat, bovine and human dopamine transporters were successfully cloned in rapid succession (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991; Usdin et al., 1991; Giros et al., 1992). The dopamine transporter was confirmed to be 12 membrane-spanning domain protein that is highly conserved between species (Figure 1-4). The human transporter has 92% and 84%

homology with rat and bovine transporters (Giros and Caron, 1993), respectively, and a 66% homology with the norepinephrine transporter, which was cloned nearly simultaneously with the dopamine transporter (Pacholczyk et al., 1991).

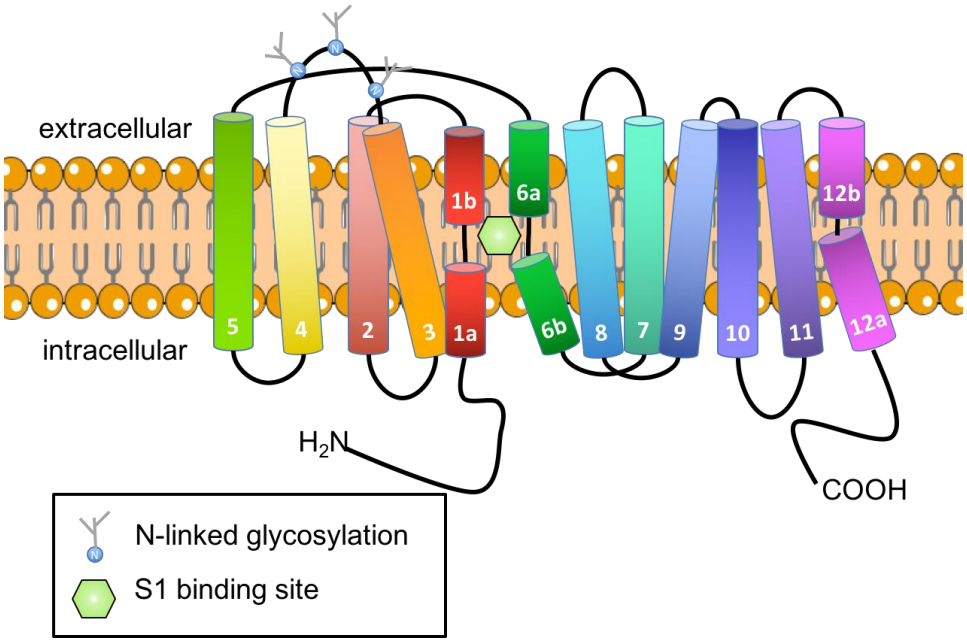

Figure 1-4 Topology of the dopamine transporter.
Adapted from Ng et al. (2014)

The dopamine transporter knockout mouse

Only a few years after the transporter was cloned, the Caron lab generated a mouse that lacked the dopamine transporter from birth. These mice exhibit profound hyperlocomotion and fast scan cyclic voltammetry demonstrates that dopamine persists in the extracellular space of the striatum 100 times longer than in the wild type animal. These results cleanly proved the importance of uptake as a mechanism of inactivation of dopamine in the brain (Giros et al., 1996). These mice were also indifferent to the locomotor activity stimulating effects of cocaine and amphetamine, indicating that the dopamine transporter is the primary site of action for these two psychostimulants and that amphetamine-stimulated dopamine release occurs through a dopamine transporter-dependent mechanism (Jones et

al., 1998). Several years of debate ensued when studies from both the Caron lab and others published conflicting evidence about the effects of psychostimulants on dopamine transporter knockout mice. It was found that dopamine transporter knockout mice would self-administer cocaine (Rocha et al., 1998) and developed conditioned place preference to cocaine and methylphenidate, another dopamine transporter blocker (Sora et al., 1998). Another group found that in dopamine transporter knockout mice, cocaine and amphetamine were still capable of increasing extracellular dopamine levels in the nucleus accumbens as measured by microdialysis (Carboni et al., 2001). In dopamine transporter knockout but not wild type mice a selective norepinephrine transporter blocker also increased extracellular dopamine in the nucleus accumbens, suggesting that a compensatory increase in expression of the norepinephrine transporter may have occurred in the nucleus accumbens of mice that lacked the dopamine transporter from birth (Carboni et al., 2001). However, the Jones lab was unable to reproduce these results through *in vitro* voltammetry and found no indication of compensatory expression of norepinephrine transporter or serotonin transporter within the accumbens: neither desipramine (a norepinephrine transporter blocker) nor fluoxetine (a serotonin transporter blocker) had any effect on dopamine levels in the nucleus accumbens of knockout mice (Budygin et al., 2002; Mateo et al., 2004). The problem was put to rest, however, when Rong Chen and colleagues generated a triple-mutated dopamine transporter that was insensitive to cocaine but still capable of dopamine uptake at approximately 50% of wild-type activity (Chen et al., 2005b). When this mutated dopamine transporter was "knocked in" to mice, the mice exhibited no conditioned place preference to cocaine (Chen et al., 2006) and would not self-administer cocaine intravenously

(Thomsen et al., 2009). This demonstrated once and for all that the interaction of cocaine with the dopamine transporter is necessary in order for the drug to exhibit its reinforcing effects.

The dopamine transporter: current knowledge

Subsequent work on the dopamine transporter pertinent to this thesis will be roughly divided into three groups: 1) research into the structure and physical mechanisms of transport; 2) pharmacology of the transporter; and 3) investigations into the various cellular mechanisms that regulate dopamine transporter function.

Structural considerations

There is a significant degree of homology among the different transporters of the solute carrier 6 family: dopamine transporter exhibits ~67% amino acid homology with norepinephrine transporter, a ~49% homology with serotonin transporter, and a ~45% homology with the GABA transporter. Additionally, the dopamine transporter is highly conserved across mammals; rat, bovine and mouse dopamine transporter exhibit 92%, 88%, and 93% amino acid homology with the human dopamine transporter, respectively (Chen and Reith, 2002). Pharmacologically, dopamine transporters from different species exhibit varied affinities for dopamine transporter ligands, with rat dopamine transporter most closely mimicking human dopamine transporter in its affinity for GBR 12909, mazindol, cocaine, and bupropion (Chen and Reith, 2002).

The current understanding of the mechanisms of dopamine transport indicates that the dopamine transporter functions through an "alternating access" mechanism (Jardetzky, 1966; Liang et al., 2009). That is, the transporter cycles through multiple conformations which can most simply be condensed to an outward facing conformation, where the

transporter binds extracellular sodium, chloride and dopamine, an occluded conformation, where the DA binding pocket is inaccessible to both the intracellular and extracellular space, and an inward facing conformation, where DA is released from the transporter and repackaged into nearby vesicles by the vesicular monoamine transporter 2 (Figure 1-5) (Rudnick, 2002). These differing conformations have been modeled with the aid of solved structures for the leucine transporter, LeuT, a bacterial homologue (Indarte et al., 2008; Gedeon et al., 2010; Shan et al., 2011; Loland, 2015; Penmatsa et al., 2015) and dopamine transporter from *Drosophila melanogaster* (Cheng and Bahar, 2015; Koldso et al., 2015; Wang et al., 2015). The recent solving of the human serotonin transporter structure is likely to provide new homology models in the near future (Coleman et al., 2016).

Here, the term 'conformational equilibrium' is used to describe the ratio of transporter in an outward facing conformation to inward facing conformation at a given point in time; in physiological conditions, most dopamine transporters will "prefer" to adopt the outward facing conformation. Alterations in conformational equilibrium are believed to be the predominant non-trafficking mechanism by which transport capacity is regulated.

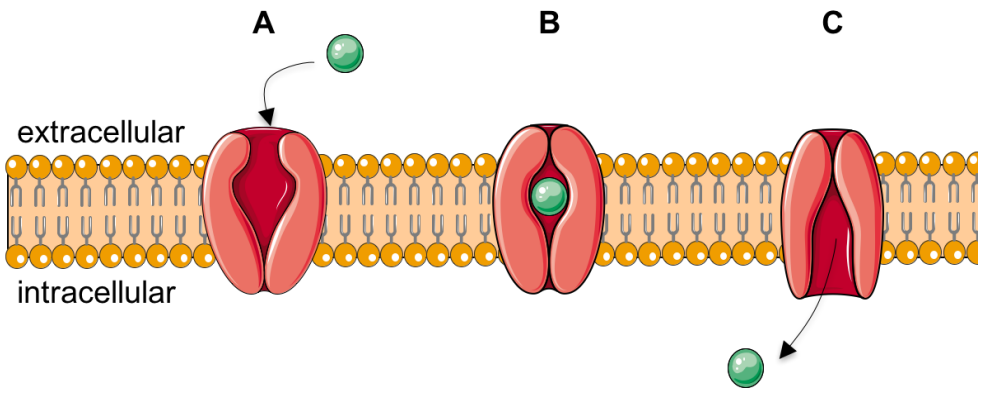

Figure 1-5. Alternating access model of dopamine transporter.

A. The outward facing conformation. B. The occluded conformation. C. The inward facing conformation.

For example, collapsing the Na^+ gradient with gramcidin (increasing intracellular sodium), leads to an increase of dopamine transporter in the inward facing conformation and a decrease in dopamine uptake (Chen and Reith, 2004). Unfortunately, the relationship between conformational preference and transport is not as clear-cut as one might hope. For example, zinc, which, like sodium, promotes the outward facing conformation of the dopamine transporter and decreases dopamine uptake, actually enhances reverse transport stimulated by amphetamine (Norregaard et al., 1998; Scholze et al., 2002). The complexity of conformational equilibrium and its effects on dopamine transporter function serve to highlight that the simplified "three conformation" model, though useful, provides an incomplete picture of dopamine transport.

One source of controversy for the neurotransmitter transporters resides in the putative existence of a second substrate binding site (S2) located extracellularly to the primary binding site (S1) (Shi et al., 2008). The S2 site could not be resolved with the LeuT structure alone (Piscitelli et al., 2010; Quick et al., 2012), but has been demonstrated in multiple homology models to be the likely site of action for several antidepressants at the serotonin transporter (Zhou et al., 2007). Additionally, putative occupation of this site by "allosteric" modulators slows dissociation of ligands from the primary site in multiple biogenic amine transporters including the dopamine transporter (Chen et al., 2005a; Pariser et al., 2008).

Kyle Schmitt of the Reith lab at New York University elegantly demonstrated the existence of a second agonist binding site by generating a family of bivalent dopamine transporter ligands that exhibited increased affinity at the dopamine transporter compared

to their monovalent components. This increase in affinity was particularly appreciable when the linker in the bivalent compound spanned 11-13 Å, the distance between the S1 and S2 sites. Computational modeling with the most potent compounds indicated simultaneous occupancy of two different substrate binding sites (Schmitt et al., 2010). Most recently, the solving of the human serotonin structure and the demonstration of an "allosteric" binding site analogous to the proposed S2 binding site, suggests that such a site may exist in the dopamine transporter. It is important to understand that the proponents of the S2 site in the dopamine transporter do not claim that a second molecule of dopamine is being transported; the stoichiometry of uptake, one dopamine for two sodium ions and one chloride ion, is well established and is not being questioned.

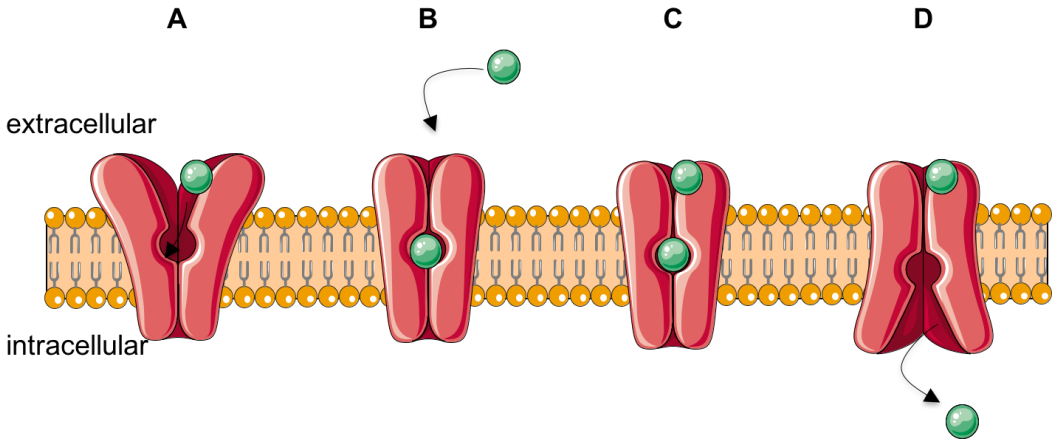

Figure 1-6 Modified dopamine transport cycle including the S2 site. Illustrating two potential mechanisms by which the S2 site facilitates dopamine transport. (A) Binding of dopamine to the S2 site facilitates permeation to the S1 site. Alternatively, (B) following binding of dopamine to the S1 site, (C) dopamine binds to the S2 site and (D) facilitates the opening of the intracellular gate so that dopamine can diffuse into the intracellular space.

Rather, it is proposed that the S2 site either represents an extracellular vestibule which aids in penetration of dopamine to the S1 site (Schmitt et al., 2010), or that substrate binding to the S2 site facilitates the conformational changes that allow for the release of the

primary substrate and sodium into the extracellular space (Figure 1-6) (Shi et al., 2008; Shan et al., 2011). Both of these hypotheses are supported by the observation that mutation of key binding residues in the S2 sites greatly impedes the uptake capacity of the dopamine transporter (Loland et al., 2004; Zhen and Reith, 2016).

Dopamine transporter pharmacology

Pharmaceutical agents acting on the dopamine transporter can be broadly classified as either transporter blockers or transporter substrates/releasers, with cocaine and amphetamine being the classic examples of each, respectively. Both of these drugs act upon the transporter to increase extracellular dopamine levels. This increase in extracellular dopamine is interpreted by the brain in such a way that drug taking behavior is reinforced, eventually leading to the psychological conditions of substance abuse and addiction.

DAT blockers prevent reuptake of dopamine, so that the dopamine released through exocytosis accumulates in the extracellular space. In recent decades, however, it has become clear that there is more to the reinforcing properties of a drug like cocaine than simple blockade of the dopamine transporters. A new class of compounds has been defined as "atypical dopamine uptake inhibitors." The characteristics of these compounds are many and varied, but atypical uptake inhibitors have in two features in common: 1) a blockade of dopamine uptake at the transporter; and 2) a lack of characteristic psychostimulant effects exhibited by a classical inhibitor such as cocaine. The reasons for the lack of psychostimulant effect in these drugs are as yet not understood, but due to the wide structural variety of compounds meeting these two criteria, it is unlikely that one explanation will fit all of the compounds. Hypotheses include muscarinic agonism, sigma receptor antagonism, pharmacokinetics, binding rate, and conformational effects on the

dopamine transporter. All have been demonstrated as possible explanations for some atypical uptake inhibitors, but no single unifying characteristic has been found across all atypical uptake inhibitors (Schmitt et al., 2013).

Atypical dopamine transporter blockers reduce the locomotor stimulating effects of cocaine and amphetamine (Velazquez-Sanchez et al., 2010). Some atypical dopamine transporter blockers have been shown to inhibit the self-administration and conditioned place preference of cocaine and amphetamine without exhibiting any reinforcing characteristics themselves (Ferragud et al., 2009; Hiranita et al., 2009; Ferragud et al., 2014). As a result of their lack of abuse potential (Schmitt et al., 2013), current research is focusing on atypical dopamine transporter inhibitors to develop a new therapeutic that might be useful in the treatment of amphetamine abuse (Tanda et al., 2009). Such a drug could either act as a substitution therapy, as methadone is used for heroin addiction, or an antagonist therapy, such as naloxone or naltrexone.

Substrates like amphetamine exert a more complex effect on the dopamine transporter. To begin with, as a substrate of the dopamine transporter, amphetamine will compete with dopamine for uptake, which will lead to increased extracellular dopamine levels. Following translocation into the cell, amphetamine exerts a number of effects including activation of several important signaling protein kinases, disruption of the vesicular proton gradient so that dopamine accumulates in the cytosol, and reversal of the transporter so that dopamine is transported out of the cell, a process known as efflux. Amphetamine appears to exert its effects on the dopamine transporter by taking advantage of many natural processes important to transporter regulation, thus the mechanism of

action of amphetamine will be addressed in greater length in the next section discussing regulation of the dopamine transporter.

Regulation of the Dopamine Transporter

Regulation of dopamine transporter function occurs at two levels, surface expression of the transporter and uptake capacity. Though diminished transport activity is frequently the result of reduced surface expression of the transporter, the dopamine transporter can demonstrate decreased dopamine uptake capacity independently of decreased surface expression levels. Both surface expression and uptake capacity are modulated by a number of potential post-translational modifications as well as interactions with the membrane environment in which the transporter resides.

Glycosylation

The dopamine transporter is a glycoprotein with N-glycosylation occurring at 3 extracellular sites (Vaughan et al., 1996; Li et al., 2004). Glycosylation of the dopamine transporter appears to increase with age (Patel et al., 1994). Additionally, dopamine transporter from the nucleus accumbens has a higher apparent molecular weight compared to that from striatum and this difference is due to glycosylation levels (Lew et al., 1992). Prevention of N-glycosylation reduced expression of dopamine transporter both on the surface and intracellularly, and non-glycosylated dopamine transporter mutants demonstrated decreased dopamine uptake efficiency in heterologous HEK293 cells as compared to wild type (Li et al., 2004). Interestingly, the expression pattern of N-glycosylated dopamine transporter correlates with those neurons which are most vulnerable to degradation in Parkinson's disease (Afonso-Oramas et al., 2009).

Phosphorylation

Phosphorylation is a ubiquitous post translational modification used to rapidly and dynamically regulate protein conformation, function, and interactions. The addition of a bulky phosphate group to a polar residue (most frequently serine and threonine) can drastically alter the hydrophobic and hydrophilic interactions which dictate protein structure, thus leading to conformational changes that alter protein function. The dopamine transporter is a target of regulation by numerous protein kinases (phosphorylating enzymes) including ERK, protein kinase B (PKB/Akt), CAMKII, and protein kinase C (Gnegy, 2003; Ramamoorthy et al., 2011). Many of these kinases are important not only for normal regulation of the dopamine transporter, but also vital to the induction of reverse transport by amphetamine. The following paragraphs will address the effects of these kinases on both transporter regulation and amphetamine action.

Protein kinase C is probably the most extensively characterized of the kinases known to regulate the dopamine transporter. Giambalvo (2004) first demonstrated that protein kinase C is activated by amphetamine in a calcium and phospholipase C/A2 dependent manner. Phorbol esters that activate protein kinase C were found to alter uptake activity of the dopamine transporter and even stimulate efflux (Cowell et al., 2000; Giambalvo, 2003). Activation of protein kinase C with the phorbol 12-myristate 13-acetate (PMA) has also been demonstrated to stimulate the internalization of the dopamine transporter (Pristupa et al., 1998; Daniels and Amara, 1999). The dopamine transporter possesses a serine rich N-terminus that is believed to be the target of phosphorylation by protein kinase C as well as several other kinases. Removal of the first 22 amino acids of the dopamine transporter N-terminus, which includes these serines, almost completely

eliminates phorbol ester-stimulated phosphorylation and attenuates amphetamine-stimulated reverse transport (Granas et al., 2003; Khoshbouei et al., 2004). However, the loss of these N-terminal serines has no effect on the phorbol ester-induced internalization of the dopamine transporter, indicating that protein kinase C is regulating the dopamine transporter through multiple mechanisms (Granas et al., 2003). Several accessory proteins that contribute to internalization of the dopamine transporter have been proposed to be the protein kinase C substrates that facilitate phorbol ester-induced dopamine transporter internalization, including flotillin (Cremona et al., 2011) and syntaxin A (Lee et al., 2004) though conflicting evidence exists for both (Cervinski et al., 2010; Sorkina et al., 2013). Similarly, inhibition of protein kinase C, and the β isoform in particular, has been found to decrease amphetamine-stimulated dopamine efflux and amphetamine-stimulated locomotor behavior without altering dopamine uptake (Browman et al., 1998; Kantor and Gnegy, 1998; Johnson et al., 2005b; Zestos et al., 2016). Protein kinase C β knockout mice also exhibit decreased amphetamine-stimulated locomotor activity, decreased amphetamine-stimulated dopamine efflux (Chen et al., 2009), and impaired development of condition place preference to amphetamine (Rong Chen, in preparation), indicating that loss of protein kinase C β activity not only impairs amphetamine-stimulated efflux, but also decreases the rewarding properties of the drug. Interestingly, experiments performed with enhanced temporal resolution have demonstrated that activation of protein kinase C by amphetamine induces a rapid increase in surface levels of the dopamine transporter, followed by a persistent and longer lasting down regulation of surface transporter levels (Johnson et al., 2005a; Furman et al., 2009). Protein kinase C modulation of the dopamine transporter is not entirely dependent on trafficking of the dopamine transporter. Inhibition

of endocytosis by either conconavalin A or sucrose only incompletely impairs protein kinase C-dependent down regulation of transporter activity despite a complete block of the decrease in surface DAT (Foster et al., 2008). This indicates that prolonged protein kinase C activation results in a two-pronged down regulation of the dopamine transporter: both decreasing surface expression of the dopamine transporter and decreasing transport activity of those transporters still expressed on the surface.

CAMKII is also important to the regulation of dopamine transporter activity. CAMKIIα binds to the C-terminus of the dopamine transporter (Steinkellner et al., 2012), resulting in its phosphorylation of several N-terminal serines. Removal of these serines or of the C-terminus impaired the stimulatory effects of CAMKIIα and amphetamine-stimulated dopamine efflux (Fog et al., 2006). CAMKIIα knockout mice exhibit reduced amphetamine-stimulated elevation in striatal dopamine and reduced amphetamine-stimulated hyperlocomotion and behavioral sensitization. Unlike the protein kinase C β knockout mice, amphetamine still evoked a conditioned placed preference in the CAMKIIα knockout mice, indicating that the rewarding properties of the drug were intact in the mutant mouse (Steinkellner et al., 2014). Additionally, inhibition of CAMKII in *Drosophila* larvae blocks amphetamine-stimulated hyperlocomotion (Pizzo et al., 2014).

Protein kinase B/Akt2, an important signaling molecule in the insulin-signaling pathway, also alters dopamine transporter surface expression. Basal Akt activity appears to support surface localization of the transporter, because inhibition of Akt leads to a decrease of surface dopamine transporter levels in heterologous cells (Speed et al., 2010). In this vein, rodents fed a high fat diet (diet-induced obesity) that exhibit an impairment in Akt activity, have less surface striatal dopamine transporter and thus exhibit reduced DA

clearance and reduced locomotion in response to amphetamine compared to their normal-diet counterparts. In addition, pharmacological inhibition of striatal Akt reduced both the behavioral response to intrastriatal amphetamine and amphetamine-stimulated reverse transport (as measured by amperometry) (Speed et al., 2011). It is as yet unclear whether Akt activation affects the phosphorylation state of the transporter, or whether it modulates the activity of other proteins involved in the regulation of surface dopamine transporter levels.

Mitogen activated protein kinase (MAPK/ERK) modulates dopamine transporter function at multiple levels. D2-like dopamine autoreceptors regulate surface expression levels of the dopamine transporter through activation of ERK (Bolan et al., 2007; Zapata et al., 2007), and ERK inhibitors reduce dopamine transport activity (Carvelli et al., 2002; Moron et al., 2003) and amphetamine-induced behaviors (Shi and McGinty, 2006). ERK directly phosphorylates the dopamine transporter at threonine 53 in the N terminus (Gorentla et al., 2009). Basal phosphorylation of T53 is approximately 50% and is increased by protein phosphatase inhibitors, indicating a sustained "phosphorylation tone" in the protein (Foster et al., 2012). As phorbol esters can also increase phosphorylation at T53, it is believed that activation of ERK, in this instance, occurs downstream of protein kinase C activation. Interestingly, the generation of dopamine transporter mutants that are non-phosphorylatable (threonine to alanine) or phosphomimetic (threonine to aspartic acid or glutamic acid) have the same phenotypic effect: both types of mutants exhibit a slight reduction in the Vmax of dopamine uptake, indicating a decrease in transport turnover rate, and a complete loss of the amphetamine-stimulated dopamine efflux (Foster et al., 2012). Loss of dopamine efflux with both mutants indicates that the

phosphorylation/dephosphorylation at T53 plays a role in the kinetic function of the dopamine transporter and is absolutely necessary for amphetamine-stimulated dopamine efflux.

Lipid Interactions

The dopamine transporter is affected by its interactions with local milieu. An interaction between the N-terminus of the dopamine transporter and the phosphatidylinositol 4,5 bisphosphate (PIP_2) has been demonstrated (Hamilton et al., 2014). Chinese hamster ovary (CHO) cells stably transfected to express the human dopamine transporter exhibited markedly less amphetamine-stimulated dopamine efflux following treatment of the cells with a PIP_2-sequestering peptide. Using a dopamine transporter K64A mutant, this group determined that K64 was the pertinent residue in the interaction between the dopamine transporter N-terminus and PIP_2. As one might expect from the experiments with PIP_2 sequestration, K64A-DAT exhibited decreased amphetamine-stimulated dopamine efflux as compared to wildtype, though dopamine uptake between wildtype and mutant dopamine transporter was unchanged. Even more compellingly, when the wildtype and mutant dopamine transporter were expressed in dopamine transporter knockout *Drosophila*, those flies expressing the mutant dopamine transporter exhibited significantly less amphetamine-stimulated hyperactivity (Hamilton et al., 2014).

The role for PIP_2 in the modulation of dopamine transporter function is particularly interesting when it is considered that breakdown of PIP_2 into diacyglyerol and inositol-triphosphate is a vital step in the activation of protein kinase C. Activation of protein kinase C would be expected to deplete PIP_2 from the membrane, which according to the results

discussed above, should result in a decrease in amphetamine-stimulated dopamine efflux. However, protein kinase C activation stimulates an increase in efflux (Cowell et al., 2000). Though no work has been done to indicate a connection between the interaction of PIP_2 with dopamine transporter and protein kinase C modulation of the transporter, it is an interesting coincidence that two such closely related signaling molecules both facilitate amphetamine-stimulated dopamine efflux.

Cholesterol plays an important role in the dopamine transporter conformation and function. The crystal structure of the *Drosophila melanogaster* dopamine transporter was solved with a cholesterol molecule bound within transmembrane helices 1a, 5, and 7 (Penmatsa et al., 2013). Other work has demonstrated that cholesterol binding to the dopamine transporter stabilizes the outward facing conformation (Hong and Amara, 2010).

Lipid interactions with the dopamine transporter have also been interrogated by studying the interaction of the dopamine transporter with lipid rafts. The transporter appears to exist in two populations which can be delineated by the presence of the transporter in or out of cholesterol rich microdomains (lipid rafts) (Adkins et al., 2007). It was originally postulated that association within a lipid raft would regulate transport capacity, because treatment with methyl-β-cyclodextrin, a cholesterol extracting and lipid raft-disrupting agent, decreased transport capacity (Adkins et al., 2007). However, work with more selective lipid raft disruptors that do not strip the cell of cholesterol demonstrated that cholesterol is vital for transporter function, but association in rafts does not appear to influence transport capacity (Jones et al., 2012). Depletion of cholesterol with methyl-□-cyclodextrin reduces both dopamine uptake and efflux. Disrupting lipid rafts without affecting membrane cholesterol levels with nystatin had no effect on efflux and

uptake. Repletion of cholesterol with desmosterol, which does not form lipid rafts, was sufficient to restore dopamine uptake and efflux transport rates, indicating that it is the direct interaction with cholesterol and not residence within a lipid raft that affects dopamine transporter functionality (Jones et al., 2012).

Other work has indicated that the two populations of transporter are differentially regulated by protein kinase C. In LLCPK1 cells stably expressing the human dopamine transporter, internalization specifically affected non-raft populations of the transporter. Protein kinase C-mediated decreases in dopamine transporter activity (independent of transporter internalization) occurred predominantly within lipid-raft localized populations (Foster et al., 2008). However, there remains some controversy in this area, as another group found that the protein kinase C-mediated internalization of the dopamine transporter occurs primarily in lipid raft populations through a non-traditional internalization mechanism involving flotillin in both HEK293 and HeLa cells transfected to express the dopamine transporter (Cremona et al., 2011). Contradictory to this, though, another group found that flotillin is not required for protein kinase C-stimulated dopamine transporter internalization and that internalization of dopamine transporter by protein kinase C in HEK293 cells is clathrin-mediated (Sorkina et al., 2013). Additionally, protein kinase C-stimulated internalization occurs through a different mechanism than constitutive dopamine transporter internalization. Biotinylation of mouse striatal slices revealed that while constitutive dopamine transporter internalization occurs independently of dynamin, protein kinase C-stimulated internalization of dopamine transporter is dynamin-dependent (Gabriel et al., 2013). Contradictory to Foster et al. (2008), protein kinase C-stimulated internalization of the transporter in neuronal cells appears to occur primarily from lipid

raft populations of the dopamine transporter, whereas constitutive internalization appears to occur equally from raft and non-raft populations (Gabriel et al., 2013). There is not a clear explanation for the discrepancy in these studies, but it is worth noting that association of dopamine transporter with lipid rafts appears to differ between non-neuronal and neuronal cell lines; in the non-neuronal HEK293 cell line, dopamine transporter appears to diffuse freely, while in the neuronal N2A cell line it associates with lipid rafts (Adkins et al., 2007). Of the studies discussed above, only those of Gabriel et al. (2013) were performed in a neuronal cell line, and their results were supported by biotinylation studies in mouse striatal slices.

Amphetamine abuse and rationale

The abuse of amphetamine-type stimulants presents a significant societal issue. Amphetamines, excluding ecstasy, are the world's second most widely abused drug type (UNODC, 2011) and emergency room visits due to amphetamines, including the non-medical use of prescription amphetamines, more than quadrupled from 2005 to 2011 (SAMHSA, 2011; SAMHSA, June 19, 2014). The treatment of drug abuse typically involves extensive behavioral therapy and, when available, pharmacological assistance. Though pharmacological interventions exist for opiate and alcohol abuse, and have been used with some success, no such therapeutic option exists for patients who wish to break free of the dangerous cycle of amphetamine abuse.

As we presented above, protein kinase C is important in the mechanism of action of amphetamine. As such, we set out to investigate the potential of a protein kinase C inhibitor and a therapeutic for amphetamine abuse. A successful therapeutic for this indication would have to cross the blood brain barrier. The only known protein kinase C inhibitor

capable of crossing the blood brain barrier is the breast cancer drug tamoxifen. A brief summary of tamoxifen pharmacology precedes an in depth review of the current literature concerning the protein kinase C inhibitory activity of tamoxifen and its known effects on the dopaminergic system.

Tamoxifen

Tamoxifen is a selective estrogen receptor modulator used in the treatment of breast cancer and the maintenance of remission. Tamoxifen is a lipophilic compound that accumulates in tissue (Lien et al., 1991a) giving it a high apparent volume of distribution of 50-60 liters (Lien et al., 1989). Despite the observation that tamoxifen and its metabolites are substrates for P-glycoprotein (Iusuf et al., 2011), tamoxifen readily crosses the blood brain barrier and concentrations in brain tissue can sometimes be 40-times higher than concentrations in serum (Lien et al., 1991b). There are currently three known active metabolites of tamoxifen that circulate at varying concentrations and demonstrate varying levels of efficacy. These metabolites are N-desmethyltamoxifen, 4-hydroxytamoxifen, and 4-hydroxy-N-desmethyltamoxifen, also known as endoxifen (Figure 1-7). Of these three metabolites, N-desmethyltamoxifen is the primary metabolite, circulating at concentrations 2-3 fold higher than tamoxifen (Lien et al., 1991a). 4-hydroxytamoxifen and endoxifen are present in much lower concentrations. The elimination half-life of tamoxifen in humans ranges from 7-10 days (de Vos et al., 1992).

In order to properly understand the context of our investigation into the dopamine modulating effects of tamoxifen, we must first understand the mechanisms of action of the drug. First of these is its role as an estrogen receptor modulator.

SERMS

The selective estrogen receptor modulators (SERMs) are part of a unique class of compounds that can act as both agonists and antagonists at the estrogen receptors, depending on the type of tissue in which they are acting. For example, tamoxifen acts as an antiestrogen in breast tissue, but has estrogenic effects in endometrial tissue, thus it is useful as a treatment in estrogen-sensitive breast cancers, but carries the possibility of increasing the risk of endometrial cancers (Ellis et al., 2015). Similarly, the drug raloxifene is estrogenic in bone tissue while being antiestrogenic in breast tissue, and as such has proven useful as a preventative treatment for osteoporosis in post-menopausal women that lacks the increased cancer risk and other unpleasant side effects of hormone replacement therapy (Dane et al., 2007; Pinkerton and Thomas, 2014).

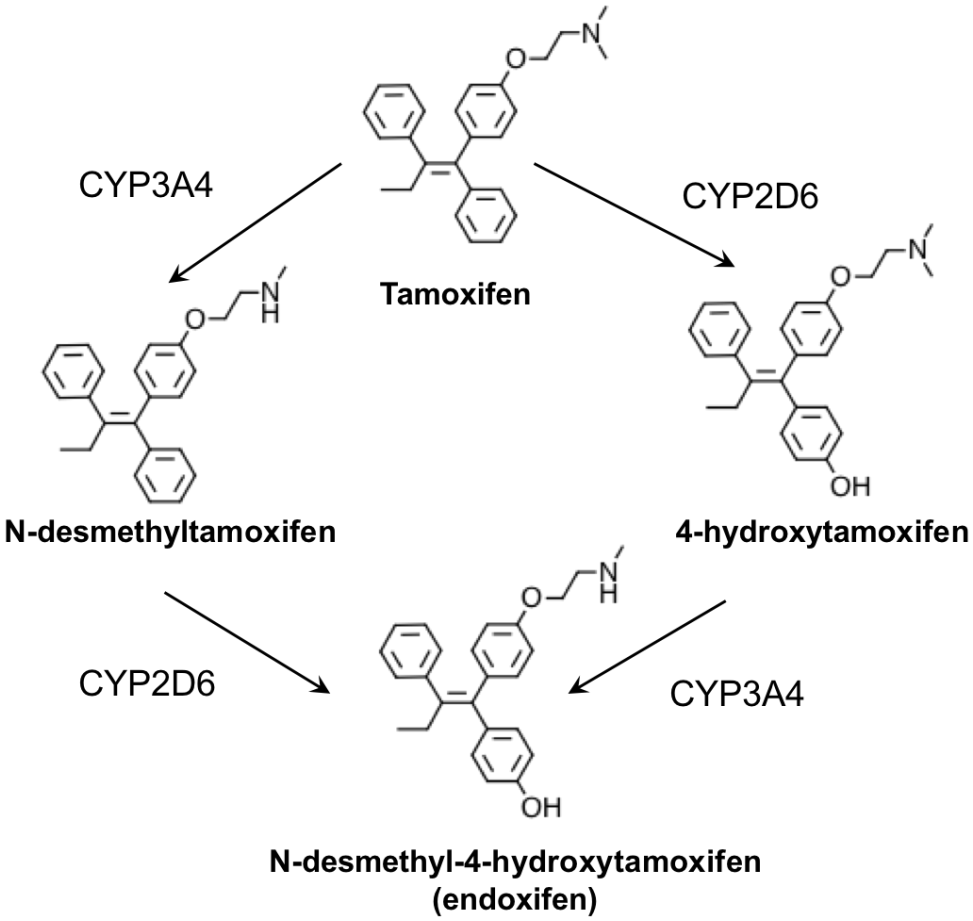

Figure 1-7. Metabolism of tamoxifen to its three active metabolites.

Neurological effects of estrogen

The steroid hormone estrogen is the primary sex hormone involved in the female reproductive system and development of secondary sex traits. Classically, estrogen exerts its effects by stimulating the translocation of estrogen receptors α or β into the nucleus where the receptors affect the expression of numerous genes. More recently, it has become clear that estrogen exerts a number of rapid, non-genomic effects, both through the classical estrogen receptors, which can translocate to the membrane to interact with membrane proteins, and through the G-protein coupled estrogen receptor (GPER1) originally known as GPR30 (Thomas et al., 2005). Estrogen appears to have a number of

effects on the dopaminergic system, including neuroprotective activity in many models of dopaminergic and general neurotoxicity, including models of stroke (Shao et al., 2012), Parkinson's disease (Litim et al., 2015), and methamphetamine-induced neurotoxicity (Dluzen and McDermott, 2006).

Effects of tamoxifen in the dopaminergic system: SERM or something else?

The investigation of the effects of tamoxifen on the dopaminergic system are complicated by the lack of knowledge concerning tamoxifen's specific effects on the estrogen receptor in the dopamine neuron. SERMs are known to act in either manner depending on the tissue and the specific response that is being measured. Additionally, tamoxifen has been identified as a potent agonist at GPER1, the G-protein coupled estrogen receptor (Thomas et al., 2005). Evidence that may clarify the SERM activity of tamoxifen in the dopamine neuron proves contradictory. Estrogen is known to increase amphetamine-stimulated dopamine efflux and tamoxifen, at concentrations where it would be expected to bind estrogen receptors, does not inhibit this effect (while the estrogen receptor antagonist fulvestrant does). This indicates that tamoxifen is not an antiestrogen in this system. It is also not estrogenic in this system, because tamoxifen by itself, at ER-occupying concentrations, has no effect on amphetamine-stimulated dopamine efflux (Xiao et al., 2003).

Tamoxifen is neuroprotective in some models of dopaminergic neurotoxicity. Though the neuroprotective properties of tamoxifen are beyond the scope of this thesis, a significant portion of the work addressing tamoxifen and the dopaminergic system has been conducted within this field and thus warrants a closer examination. The predominant models of dopaminergic toxicity utilize neurotoxic doses of MPTP (1-methyl-4-phenyl-

1,2,3,6-tetrahydrophridine), which is a prodrug for MPP^+ (1-methyl-4-phenylpyridinium). MPP^+ is taken up selectively by the dopamine transporter, where is interferes with oxidative phosphorylation and triggers cell death. In some models, MPP^+ is administered directly to the brain. Shortly after administration of MPTP or MPP^+, animals will begin to exhibit parkinsonism-like behaviors as dopaminergic neurons begin to degenerate. Methamphetamine-induced neurotoxicity is an additional model used in these studies, though the mechanisms of its neurotoxic effects are somewhat more complex than MPP^+ and likely involve a combination of oxidative stress, excitotoxicity, and neuroinflammation (Moratalla et al., 2015).

Estrogen is neuroprotective in multiple models of dopaminergic neurotoxicity, and SERMS have been investigated as potential neuroprotective agents that lack some of the wide spread side effects of estrogen. Dluzen et al. (2001a) demonstrated that tamoxifen was neuroprotective against methamphetamine-induced neurotoxicity in males and females, though estrogen was neuroprotective in females only. Though at first glance this may seem to indicate an estrogenic role for tamoxifen in the dopamine neuron, the fact that tamoxifen was effective in males, where estrogen was not, may be the first indication of a non-estrogenic mechanism for the neuroprotective effects seen with tamoxifen. The same group later found that tamoxifen antagonized the neuroprotective action of estrogen in methamphetamine-induced neurotoxicity (Gao and Dluzen, 2001) and MPTP-induced neurotoxicity (Dluzen et al., 2001b), indicating an antiestrogenic role for tamoxifen. In another study, tamoxifen prevented the formation of hydroxyl radicals following administration of MPP^+ to rat striatum by retrodialysis. Interestingly, in this study tamoxifen was administered after the initial MPP^+ insult, indicating that tamoxifen was

affecting the downstream neurotoxic pathways triggered by MPP^+ (Obata and Kubota, 2001). The antioxidant properties of tamoxifen provide a potential explanation for the observation made by Dluzen et al. (2001a) that tamoxifen was neuroprotective in male mice. Because estrogen is known to affect the dopaminergic neuron, all work with the drug, with regard to the dopaminergic system, should take in to consideration its effects at the estrogen receptors.

Therapeutic potential of tamoxifen: bipolar mania

An interest in tamoxifen as a therapeutic for bipolar disorder was sparked by the observation that valproate (Chen et al., 1994) and lithium (Wang and Friedman, 1989; Lenox et al., 1992; Manji et al., 1993), the only two drugs approved at the time for the treatment of mania, were both found to inhibit protein kinase C, despite being structurally unrelated. Furthermore, the brains of bipolar patients exhibit enhanced protein kinase C activity (Wang and Friedman, 1996). A preliminary clinical study in patients exhibiting mania found that tamoxifen, at doses ranging from 20-80 mg per day, reduced symptoms of mania (as measured by the Young Mania Rating Scale (YMRS)) by 50% in 5 out of 7 patients, and that the drug was well tolerated (Bebchuk et al., 2000). Further studies with larger sample sizes have been conducted. In one, 40 patients were give lithium supplemented with 80 mg/day of tamoxifen or placebo for a 6 week double blind study; tamoxifen with lithium was significantly more effective in reducing scores on the YMRS compared to lithium alone. Again, tamoxifen was generally well tolerated (Amrollahi et al., 2011). In a third trial, 66 inpatients were given tamoxifen or a placebo for up to 3 weeks. Tamoxifen significantly lowered scores on both the YMRS and the Clinical Global Impressions Mania scale compared to placebo and patients taking tamoxifen required less

sedation with lorazepam as the trial progressed. Interestingly, tamoxifen also caused a greater decrease in depressive symptoms compared to placebo, as measured by the Hamilton Depression Rating Scale and the Montgomery-Åsberg Depression Rating Scale, though these results did not reach significance (Yildiz et al., 2008). Three additional trials have been carried out to test the effectiveness of tamoxifen either as an adjunct- or mono-therapy. A meta-analysis of these three trials as well as the two described above concluded that tamoxifen is an effective treatment for bipolar mania, but that further studies utilizing a larger sample size and a longer follow up duration will be necessary to make any conclusions as to its safety and efficacy (Talaei et al., 2016).

Although these early clinical results were encouraging, it was difficult to determine in human subjects whether the success observed with tamoxifen could be attributed to its activity as a selective estrogen receptor modulator or as a protein kinase C inhibitor. Preclinical studies in rodents have attempted to clarify this issue.

The dominant models of bipolar mania in rodents typically involve repeated administration of amphetamine or amphetamine-like psychostimulants. Relatively small doses of tamoxifen (1-5 mg/kg) attenuate amphetamine-stimulated hyperactivity, risk taking behavior, and appetitive vocalization in rats (Einat et al., 2007; Cechinel-Recco et al., 2012; Abrial et al., 2013; Pereira et al., 2014). On a cellular level, tamoxifen prevented and reversed amphetamine-induced mitochondrial dysfunction, oxidative damage, and changes in neuronal signaling activity (Moretti et al., 2011; Cechinel-Recco et al., 2012; Steckert et al., 2012; Valvassori et al., 2014). Interestingly, tamoxifen has proven equally effective in alternative models of bipolar mania, including paradoxical sleep deprivation in rats, albeit with a much higher dose of tamoxifen (80 mg/kg) (Abrial et al., 2015), sleep deprivation in

mice (Armani et al., 2012), and methylphenidate-induced hyperactivity in mice (Pereira et al., 2011). The effect of tamoxifen in models of bipolar mania is hypothesized to be the result not of its SERM activity, but its effects as a protein kinase C inhibitor. This assumption is supported by the observation that lithium, the most common treatment for mania, inhibits protein kinase C (Chen et al., 2000) and that clomiphene, a SERM that lacks protein kinase C inhibitory activity, and medroxyprogesterone, a progestin with indirect antiestrogenic activity, have no effect on methylphenidate-induced hyperactivity in mice (Pereira et al., 2011). Surprisingly, though all of these models of bipolar mania are dependent on dysfunction of the dopaminergic system, and most of them specifically on the dopamine transporter, no work has been done to determine the effects of tamoxifen on dopamine transporter function. It is worth noting that a preliminary investigation into serotonin transporter function demonstrated that tamoxifen inhibited serotonin uptake with an IC_{50} of approximately 17 μM (Chang and Chang, 1999).

Dissertation Summary

The aim of this dissertation is to better understand the mechanisms by which tamoxifen affects the dopaminergic system. Tamoxifen's actions on the dopaminergic system have clinical implications both with regard to the side effects experienced by breast cancer patients taking the drug, as well as in the potential repurposing of tamoxifen to treat diseases of dopaminergic dysfunction. Therefore, a better understanding of how tamoxifen modulates dopaminergic signaling may reveal new possibilities for its use in the clinic.

As detailed in Chapter 2, we demonstrate for the first time that tamoxifen inhibits dopamine transporter function. Furthermore, our studies indicate that this is likely through a direct interaction on the dopamine transporter, as tamoxifen decreases binding of the

cocaine analog [^{3}H]WIN 35,428 in rat striatal membranes, alters the conformational equilibrium of the transporter, and has no effect on surface expression levels of the transporter. A comparison of *ex vivo* and *in vivo* studies indicate that tamoxifen may represent a previously unrecognized atypical dopamine uptake inhibitor, and the potential clinical implications of this finding are explored.

In Chapter 3, we compare the effects of tamoxifen and its metabolites on the dopamine transporter between an *ex vivo* tissue preparation (rat striatal synaptosomes) and a cell culture model (hDAT-N2A mouse neuroblastoma cells). Though we do find some inconsistencies between the two model systems, we are able to utilize the cell culture model to demonstrate that tamoxifen and its metabolites affect the dopamine transporter independently of their actions on the estrogen receptor. Additionally, our results suggest that the effects of tamoxifen on amphetamine-stimulated dopamine efflux, in this model, are due to an inhibition of amphetamine uptake, rather than a selective attenuation of amphetamine-stimulated dopamine efflux. Potential explanations for the discrepancies between synaptosomes and cells are explored.

The final chapter of this dissertation summarizes the evidence for a direct interaction between tamoxifen and the dopamine transporter. I also explore the possibility of a dual action of tamoxifen on the dopamine transporter: as an atypical blocker and as a protein kinase C inhibitor. I present preliminary results that support a further investigation into tamoxifen's modulation of the dopamine transporter through direct and indirect mechanisms. I also discuss several questions that were raised by my results and propose future studies to address those concerns. Finally, I present the evidence supporting the use of tamoxifen, or a related compound, in the treatment of psychostimulant abuse.

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Chemical structures were produced in ChemDoodle (www.chemdoodle.com). All original illustrations were created using Servier medical art (Creative Commons Attribution 3.0 Unported License), www.servier.com.

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CHAPTER TWO. TAMOXIFEN INHIBITS DOPAMINE TRANSPORTER FUNCTION.

Abstract

The dopaminergic systems of the brain are important in the regulation of a number of processes, including motor control and reward processing. Psychostimulants such as amphetamine and cocaine take advantage of these systems to exert their stimulant and reinforcing effects. The selective estrogen receptor modulator tamoxifen has been demonstrated to increase extracellular dopamine *in vivo* and act as a neuroprotectant in models of dopamine neurotoxicity. However, the nature and mechanism of these effects remains to be clarified. We investigated the effect of tamoxifen on dopamine transporter-mediated dopamine uptake, dopamine efflux, and [^{3}H]WIN 35,428 binding in rat striatal tissue. We found that tamoxifen dose dependently blocks [^{3}H]dopamine uptake (38% reduction at 10 μM) and efflux (63% reduction at 10 μM) in synaptosomes. It also effects a small but significant reduction in [^{3}H]WIN 35,428 binding in striatal membranes, indicating a weak interaction with the WIN 35,428 binding site. Biotinylation and cysteine accessibility studies indicate that tamoxifen stabilizes the outward-facing conformation of the dopamine transporter in a cocaine-like manner and does not affect surface expression of the dopamine transporter. Additional studies with S2-site dopamine transporter mutants indicate a direct interaction between tamoxifen and the secondary substrate binding site of the transporter. Locomotor studies indicate that tamoxifen attenuates amphetamine-stimulated hyperactivity in rats, but has no effect on locomotor activity in

the absence of amphetamine. These results suggest a complex mechanism of action for tamoxifen as a regulator of the dopamine transporter. We believe that this activity of tamoxifen makes the tamoxifen structure an excellent starting point for a structure-based drug design program to develop a pharmacological therapeutic for psychostimulant abuse.

Introduction

Dopamine plays a significant role in multiple neural processes, including motor control and reward processing. Dysfunction of the dopaminergic system can lead to diverse disorders such as addiction, Parkinson's disease, and schizophrenia (Abi-Dargham, 2014; German et al., 2015). Tamoxifen is a widely prescribed selective estrogen receptor modulator (SERM) used in the treatment and prevention of estrogen receptor positive breast cancer (Jordan, 2014). It has long been known that the drug tamoxifen affects the dopaminergic system, yet the mechanisms by which it does so have remained unclear. Tamoxifen has been found to cause a slight but significant increase in extracellular dopamine levels in the nucleus accumbens following systemic administration (Chaurasia et al., 1998). Tamoxifen also inhibits amphetamine-stimulated hyperactivity (Einat et al., 2007; Cechinel-Recco et al., 2012; Pereira et al., 2014), which relies on elevation of extracellular dopamine levels in the striatum (French, 1986). Tamoxifen has been investigated as a neuroprotective agent against MPP^+ and MPTP-induced neurotoxicity in a mouse model of Parkinson's disease (Obata and Kubota, 2001; Bourque et al., 2007; Obata and Aomine, 2009), and against methamphetamine-induced neurotoxicity (D'Astous et al., 2005). Both models of neurotoxicity selectively damage dopaminergic neurons and depend upon uptake of the neurotoxin by the dopamine transporter to exert their deleterious effects. While the effects on amphetamine-stimulated hyperactivity are believed to be the

result of protein kinase C inhibition (O'Brian et al., 1985; Einat et al., 2007), the neuroprotective effects of tamoxifen have largely been assumed to be the result of tamoxifen's activity as a SERM. However, tamoxifen has many alternative mechanisms of action, including but not limited to binding to calmodulin (O'Brian et al., 1990), and the D2-like dopamine receptor (Hiemke and Ghraf, 1984; Toney and Katzenellenbogen, 1987). It is unclear whether any of these mechanisms are responsible for the effects of tamoxifen on the dopaminergic system. Therefore we set out to better characterize the dopaminergic effects of tamoxifen and determine the mechanism by which it exerts these effects.

A logical query is the effect of tamoxifen on dopamine transporter function. Amphetamine, MPP^+ and MPTP are all substrates for the dopamine transporter and require the transporter in order to enter the cell and exert their effects. The dopamine transporter is responsible for clearing the extracellular space of dopamine following release and in this capacity is a crucial mechanism for regulating dopaminergic signaling (Jaber et al., 1997). Although tamoxifen has been demonstrated to block amphetamine-stimulated locomotor behavior, no one has systematically examined the effect of tamoxifen on dopamine transporter function.

The dopamine transporter facilitates the movement of dopamine across the plasma membrane through a series of conformational changes that can be simplified into the "alternating access" model of transport (Jardetzky, 1966). In this model, the dopamine transporter alternates between an outward-facing conformation, where dopamine binds the primary binding site, a series of occluded conformations, through which dopamine is transporter across the membrane, and an inward-facing conformation, forming the transition between the two (Shi et al., 2008; Shan et al., 2011). More recently, a secondary

site (S2) has been proposed to play a role in transporter function. The existence of the site had been demonstrated with bivalent dopamine transporter ligands that demonstrate a much increased affinity compared to their monovalent counterparts, indicating the presence of two binding sites on the dopamine transporter (Schmitt et al., 2010). This secondary site has been indicated as an allosteric binding site for modulation of dopamine transporter conformation and dopamine transport (Shan et al., 2011).

Here we demonstrate that tamoxifen directly interacts with the dopamine transporter. We find that tamoxifen non-competitively inhibits dopamine uptake and blocks amphetamine-stimulated dopamine efflux. We utilize cysteine accessibility assays and a newly characterized "S2-defective" dopamine transporter mutant (Zhen and Reith, 2016) to demonstrate that tamoxifen is stabilizing the outward-facing conformation of the dopamine transporter, apparently through an interaction with the S2 domain. Finally, we demonstrate that tamoxifen inhibits amphetamine-stimulated hyperactivity, yet exhibits no stimulant effects of its own. Our results demonstrate a heretofore unrecognized mechanism of action for tamoxifen.

Materials and Methods

Materials

Tamoxifen citrate and amphetamine hemi-sulfate were obtained from Sigma-Aldrich. For *in vitro* experiments, tamoxifen was dissolved in DMSO to produce a 50 mM stock. Amphetamine was dissolved in aqueous buffer or water to produce a 10 mM stock. Cocaine hydrochloride was provided by NIDA and dissolved in aqueous buffer or water to produce a 10 mM stock. [^{3}H]WIN 35,428 and [^{3}H]dopamine were purchased from Perkin Elmer. All other chemicals were obtained from Sigma Aldrich unless otherwise noted.

Animals

All animal use procedures were approved by the University Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines. Male Sprague-Dawley rats from 7-12 weeks of age were obtained from Harlan Laboratories. Rats were maintained under standard conditions on a 12-h light dark cycle and were housed in groups of two or three.

Synaptosome preparation

Rat striata were gently homogenized in 10 volumes of homogenization buffer (0.32 M sucrose, 1 mM EDTA, pH 7.4). Homogenates were centrifuged at 1000 x g for 10 min and the supernatant was transferred to a fresh vial. The supernatant fraction was centrifuged at 15,000 x g for 15 minutes. The resulting pellet was resuspended in the appropriate buffer for subsequent use.

Suprafusion

The pellet containing the synaptosomes was resuspended in Kreb's Ringer Buffer (KRB, 145 mM NaCl, 2.7 mM KCl, 1.2 mM KH2PO4, 1.0 mM MgCl2, 10 mM glucose, 24.9 mM NaHCO3, 0.05 mM ascorbic acid, 0.05 mM pargyline, pH 7.4). Synaptosomes were then loaded into the reaction chambers of a Brandel Suprafusion Apparatus (Brandel Inc., Gaithersburg, MD) and washed with KRB (with or without drug) for one hour at 37 ˚C at 800 µl/min to allow for adequate treatment time and to reach a steady baseline. Following the wash, 1 minute fractions were collected for a total of 14 fractions, with 10 µM amphetamine sulfate added to the buffer during fractions 7 and 8. Vehicle or tamoxifen were included in the buffer throughout fraction collection. An internal standard solution (ISS, final concentration 50 mM perchloric acid, 25 µM EDTA, 10 nM 2-aminophenol) was

added to each sample in an approximately 1:20 dilution and samples were analyzed for dopamine content by HPLC coupled with electrochemical detection (Thermo Scientific/ESA, Sunnyvale, CA). A small aliquot of synaptosomes was reserved and diluted 1:50 in ISS. After 30 min incubation at 4 ˚C, the solution was centrifuged for 20 min at 15,000 x g. The supernatant was diluted 1:20 in KRB and measured for dopamine content. This value was used to calculate the total dopamine content of the synaptosomes.

[^{3}H]Dopamine Uptake

Synaptosomes were resuspended in KRB as described above, aliquoted into 13x100 mm borosilicate glass test tubes, and incubated with tamoxifen or vehicle for 60 minutes at 37 ˚C. Cocaine (100 μM) was used to measure nonspecific [^{3}H]dopamine uptake. Unlabeled dopamine (20-300 nM) supplemented with 10 nM [^{3}H]dopamine was added to the synaptosomes. The reaction was stopped at 3 min for saturation experiments and 30 seconds for kinetic experiments by the addition of 3 ml of cold KRB followed by filtration through glass fiber filters (GF/C, Fisher Scientific) and washed twice more with cold KRB. Filters were dried and transferred to scintillation vials and radioactivity was counted in 5 ml of ScintiVerse cocktail (ThermoFisher Scientific, Waltham, MA) using a Beckman LS5801 scintillation counter (Beckman Coulter, Brea, CA).

[^{3}H]WIN 35,428 binding in membranes

Membranes were prepared by resuspending synaptosomes in buffer containing 30 mM sodium phosphate, 0.32 M sucrose, pH 7.4 and homogenizing the suspension with a polytron tissue homogenizer. This solution was then aliquoted into 13x100 mm glass test tubes containing tamoxifen or vehicle and [^{3}H]WIN 35,428. Membranes were incubated for 2 hours at 4˚C to allow the binding reaction to reach equilibrium, then filtered, washed, and

counted as described above. Nonspecific binding was determined by incubation with 30 μM nomifensine.

[^{3}H]dopamine uptake in LLCPK1 cells

LLCPK-1 cells stably transfected to express WT and mutant human dopamine transporters were grown to confluency in a 24-well plate for 2-3days. To enhance dopamine transporter mutant expression, D476A- and I159A-human dopamine transporter-transfected cells were treated for 16 hr with 100 mM sodium butyrate prior to experiment. Cells were washed 3x with phosphate-buffered saline (PBS) and preincubated with vehicle, 10 μM tamoxifen prepared in 240 μl of uptake buffer supplemented with 1mM ascorbic acid (122 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 15 mM Na_2HPO_4, 10 mM glucose and 1 mM $CaCl_2$) for 1hr at room temperature. [^{3}H]Dopamine uptake assays were initiated by addition of 30 μl of varying concentrations of unlabeled dopamine (final concentration ranging from 0-10 μM) followed quickly by addition of 30 μl of 6–11 nM [^{3}H]dopamine for a final per-well reaction volume of 300 μl. Nonspecific uptake was determined using 100 μM cocaine. Assays were conducted in 24-well plates for 5 minutes (WT cells) and 7 minutes (mutant cells) at 25 ˚C followed by extensive washing (3x) in ice-cold PBS. Cells were lysed with 5% ice-cold trichloroacetic acid for 30 minutes at 4 ˚C, and measured by liquid scintillation counting.

Biotinylation to determine surface levels of the dopamine transporter and cysteine accessibility

Biotinylation assays were adapted from Hong and Amara (2010). Synaptosomes were incubated with vehicle, 10 μM tamoxifen or 100 μM cocaine in KRB for 1 hour at 37 ˚C. The reactions were transferred to ice and washed with cold KRB. The vehicle- and tamoxifen-

treated samples were divided in two. One set (vehicle-, cocaine- and tamoxifen-treated) was further incubated with 5 mg/ml maleimide-PEG_2–biotin (Thermo Scientific) in PBSCM (PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$) pH 7.1, for 45 min at 4 ˚C in the continued presence of vehicle, cocaine, or tamoxifen where indicated. These samples were used to assess cysteine accessibility on the dopamine transporter. The other set (vehicle- and tamoxifen- treated) was incubated with 2 mg/ml sulfo-NHS-biotin (Thermo Scientific) in PBSCM, pH 7.4 under the same conditions. These samples were used to assess surface levels of the dopamine transporter following treatment with tamoxifen. The remaining maleimide-PEG_2 –biotin or sulfo-NHS-biotin was quenched by adding 500 mM cysteine or 1 M glycine, respectively, in PBSCM at 4 ˚C for 15 min, respectively. Synaptosomes were centrifuged for 10 min at 16000 x g and washed once more with the quenching solution. After centrifugation for 10 min at 16000 x g, maleimide-PEG_2-biotin treated synaptosomes were resuspended in TNE lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.5 containing protease inhibitors – Roche Applied Science). Sulfo-NHS-biotin treated samples were resuspended in solubilization buffer (50 mM Tris, 150 mM NaCl, 1% Triton x 100, pH 7.4, containing protease inhibitors) and lysed for 1 hr at 4 ˚C followed by 10 min centrifugation at 12000 x g. The supernatant was incubated with a 50% slurry of streptavidin agarose beads (Thermo Scientific) overnight at 4 ˚C. The beads were washed once with 400 µl respective buffers and twice with 600 µl PBS. Biotinylated proteins were eluted with SDS-PAGE sample buffer and a 20 µl sample of lysate was prepared for electrophoresis as a control. All samples were heated at 70 ˚C for 10 min and separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-dopamine

transporter antibody, mab 16, (Roxanne Vaughan, University of Nebraska), HRP-conjugated mouse secondary antibody and developed using chemiluminescence.

Locomotor assays

Locomotor activity was evaluated in testing chambers (41 cm x 25.4 cm x 20.3 cm) equipped with a photocell beam array. Activity was quantified as the number of beam breaks in a designated period of time. Animals were allowed to acclimate in the chamber for two hours prior to administration of 5 mg/kg tamoxifen citrate or vehicle (i.p., 2 mg/ml in 3.5% DMSO and 10% Tween-80 in saline) and monitored for an additional three hours. This procedure was repeated the next day. On the third day, animals were allowed to acclimate in the chambers for two hours prior to administration of 1 mg/kg amphetamine (i.p. 1 mg/ml in saline) or the equivalent volume of saline and activity was monitored for an additional three hours.

Statistical Analysis

All statistical analyses were carried out using Graphpad Prism 6 software (San Diego, CA). Data are plotted as mean ± S.E.M. Significance was set at $p<0.05$. Comparisons between multiple groups were made with one- or two- way ANOVA, with *post hoc* Dunnett's multiple comparison test. Where only two groups were analyzed, unpaired or paired, two-tailed Student's *t*-tests were used. In kinetic uptake and saturation binding assays, non-linear regression was used to determine the appropriate parameters. Calculated K_d/K_m and B_{max}/V_{max} were compared across experiments by a paired, two-tailed Student's *t*-test.

Results

Tamoxifen non-competitively inhibits [^{3}H]dopamine uptake in rat striatal synaptosomes

Because amphetamine and methamphetamine are substrates for the dopamine transporter, and are competitive with dopamine, we examined whether tamoxifen might impede the effects of amphetamine by reducing the transporter's capacity to take up substrate. Rat striatal synaptosomes were pretreated with 0.3 μM to 10 μM tamoxifen or vehicle prior to initiation of uptake with 310 nM [^{3}H]dopamine. RM one-way ANOVA indicated a significant treatment effect for tamoxifen [$F(3,12) = 27.49$, $p<0.0001$]. Post-hoc Dunnett's multiple comparison indicated that tamoxifen significantly decreased [^{3}H]dopamine uptake at 3 μM and 10 μM (Figure 2-1A). Further kinetic analysis demonstrated that this blockade was the result of non-competitive inhibition of dopamine uptake, as 10 μM tamoxifen significantly decreased the V_{max} of [^{3}H]dopamine uptake but not the K_m (Figure 2-1B, Table 2-1).

Tamoxifen attenuates amphetamine-stimulated dopamine efflux

We next examined the dose-dependent effect of tamoxifen on amphetamine-stimulated dopamine efflux using suprafusion of rat striatal synaptosomes to determine whether tamoxifen would affect efflux to a similar degree that it affects uptake. A one-way ANOVA indicated a significant treatment effect [$F(3,9)=9.686$,$p<0.0035$] for tamoxifen. Pretreatment with 1 μM to 10 μM tamoxifen had no effect on baseline dopamine levels (Table 2-2), yet tamoxifen dose-dependently inhibited amphetamine-stimulated dopamine efflux with statistically significant decreases at 3 μM (34% reduction, $p<0.05$ vs. vehicle) and 10 μM (63% reduction, $p<0.01$ vs vehicle) tamoxifen (Figure 2-2).

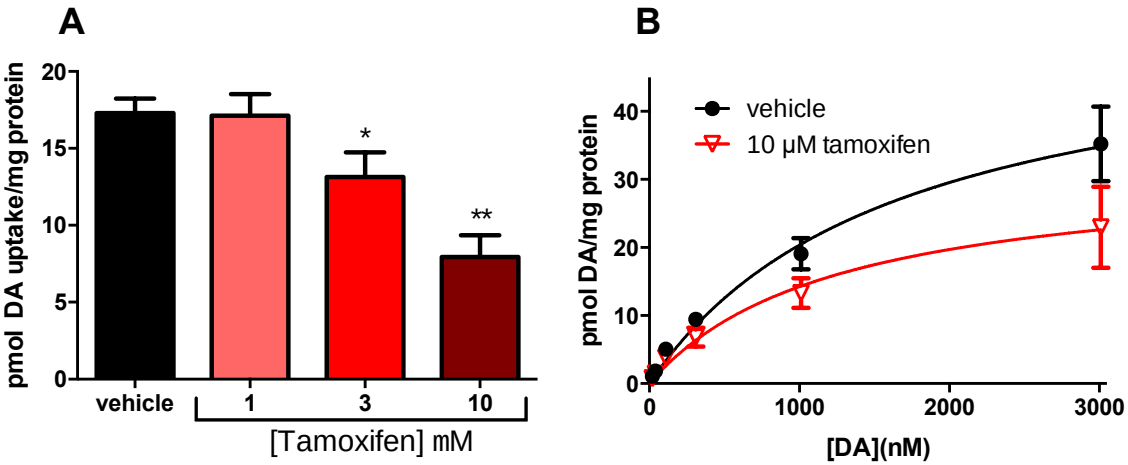

Figure 2-1. Tamoxifen impairs dopamine uptake.

Striatal synaptosomes from male Sprague Dawley rats were incubated for one hour at 37°C with vehicle or indicated concentrations of tamoxifen, then treated with 310 nM (A) or indicated concentrations of [^{3}H]dopamine and incubated for an additional 3 min (A) or 30 seconds (B). **A**. Repeated measures one-way ANOVA indicated a significant treatment effect for tamoxifen. Post-hoc Dunnet's multiple comparison indicate a significant reduction in dopamine uptake at 3 µM ($p<0.05$) and 10 µM tamoxifen ($p<0.01$) compared to vehicle. Data are represented as mean ± S.E.M. n=5. **B.** K_m and V_{max} were calculated as averages from 4 experiments and are displayed in table 1. Paired two-tailed t-test indicated a significant effect of tamoxifen on V_{max} ($p<0.001$) but not K_m. Data are represented as mean ± S.E.M. n=4

	Vehicle	10 µM TMX
K_m (nM)	1576 ± 263.3	1531 ± 643.7
V_{max} (nM)	48.5 ± 13.6	34.0 ± 13.5*

Table 2-1. Kinetics of [^{3}H]dopamine uptake

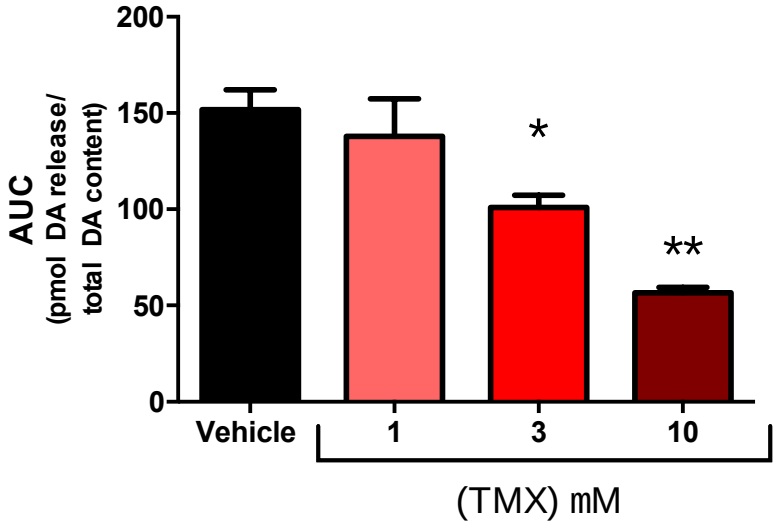

Figure 2-2. Tamoxifen attenuates amphetamine-stimulated dopamine efflux.
Striatal synaptosomes were perfused for one hour at 37°C with vehicle or various concentrations of tamoxifen at 800 µL/min before collection of 1 minute fractions. 10 µM amphetamine was included in the perfusate during fractions 7 and 8. Data were calculated as the area under the curve following treatment with amphetamine. A one-way ANOVA indicated a significant treatment effect for tamoxifen. Post-hoc Dunnet's multiple comparison indicate a significant reduction in amphetamine-stimulated dopamine release at 3 µM tamoxifen ($p<0.05$) and 10 µM tamoxifen ($p<0.01$) compared to vehicle. Data are represented as mean ± S.E.M. N=2-4

	Vehicle	1 µM TMX	3 µM TMX	10 µM TMX
Baseline (pmol DA/tDA)	6.8 ± 2.0	7.1 ± 1.8	8.9 ± 1.2	4.9 ± 0.3
dopamine release (AUC)	151.7 ± 10.3	137.9 ± 19.5	100.9 ± 6.4*	56.5 ± 2.9**

Table 2-2. Basal and amphetamine-stimulated dopamine release
(normalized to total dopamine (tDA) content of synaptosomes)

Tamoxifen does not affect surface expression of the dopamine transporter.

In order to determine whether tamoxifen was reducing dopamine uptake and efflux by decreasing surface dopamine transporter levels, we utilized a surface biotinylation assay. Synaptosomes were incubated for one hour with 10 µM tamoxifen or vehicle at 37 °C followed by incubation with NHS-sulfo-biotin. Following biotin pulldown, dopamine transporter levels in the biotinylated fraction and total lysate were quantified by western blotting. Dopamine transporter levels in the biotinylated fraction were normalized to lysate. We found no change in biotinylated dopamine transporter levels in synaptosomes treated with tamoxifen compared to vehicle (Figure 2-3).

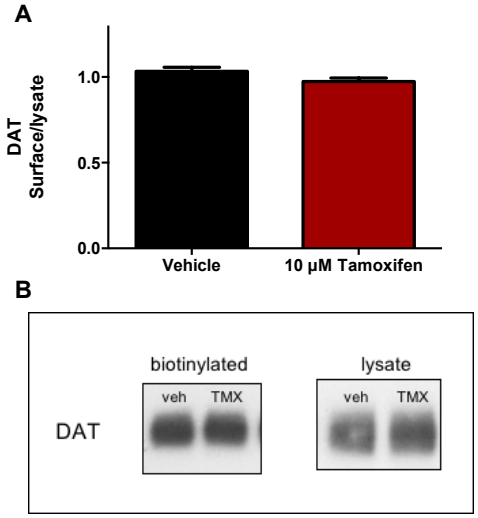

Figure 2-3. Tamoxifen does not affect surface expression of the dopamine transporter.

Rat striatal synaptosomes were incubated for one hour with 10 µM tamoxifen or vehicle prior to biotinylation of surface proteins with sulfo-NHS-biotin. Following avidin-biotin pulldown, dopamine transporter content in biotinylated fractions and lysates was quantified by western blotting. **A**. Tamoxifen did not affect surface expression of the dopamine transporter. **B**. Representative western blot showing the biotinylated dopamine transporter protein and its corresponding total lysate. N=3

Tamoxifen inhibits binding of [^{3}H]WIN 35,428 to the dopamine transporter

We next determined whether the effects of tamoxifen on dopamine uptake and efflux were the result of a direct interaction with the dopamine transporter, such as that which would be seen with a typical dopamine transporter blocker like cocaine. To test this we looked at the effect of tamoxifen on the binding of [^{3}H]WIN 35,428, a cocaine analogue, to the dopamine transporter in rat striatal membranes. In a competition binding assay, a repeated measures one-way ANOVA indicated a significant effect of treatment [$F(4, 16) = 8.571$, $p=0.0007$]. Post-hoc Dunnett's multiple comparison test revealed that 3 and 10 μM tamoxifen significantly decreased binding of 4 nM [^{3}H]WIN 35,428 (Figure 2-4a). In a saturation binding assay comparing membranes pretreated with or without 10 μM tamoxifen, tamoxifen significantly decreased [^{3}H]WIN 35,428 binding to the dopamine transporter ($p<0.0001$, Figure 2-4b). Further analysis indicated that tamoxifen significantly increased the K_d of WIN for the transporter (veh vs. TMX, 9.3±0.6 vs. 15.4±1 nM, paired t-test, $p<0.05$), but had no effect on B_{max} compared to vehicle (veh vs. TMX, 3.5±0.2 vs. 3.4±0.2) (Figure 2-4b). These results indicate a weak, competitive interaction with the WIN 35,428 binding site.

Tamoxifen increases biotinylation of extracellular cysteines in a cocaine-like manner.

We next determined whether tamoxifen is affecting dopamine transporter conformation. In the alternating access model of dopamine transport, the dopamine transporter alternates between the outward facing conformation, where it binds extracellular dopamine, and the inward facing conformation, where dopamine is released into the cytosol. Previous work demonstrated that the availability of cysteine 306 in the dopamine transporter for modification by maleimide-PEG2 biotin is enhanced by cocaine, and that this correlates to

an increase in the outward facing conformation of the dopamine transporter. We found that incubation with 10 μM tamoxifen increases biotinylation of extracellular cysteines similar to cocaine (Figure 2-5), though whether this increased biotinylation is occurring at cysteine 306 remains unknown without further study.

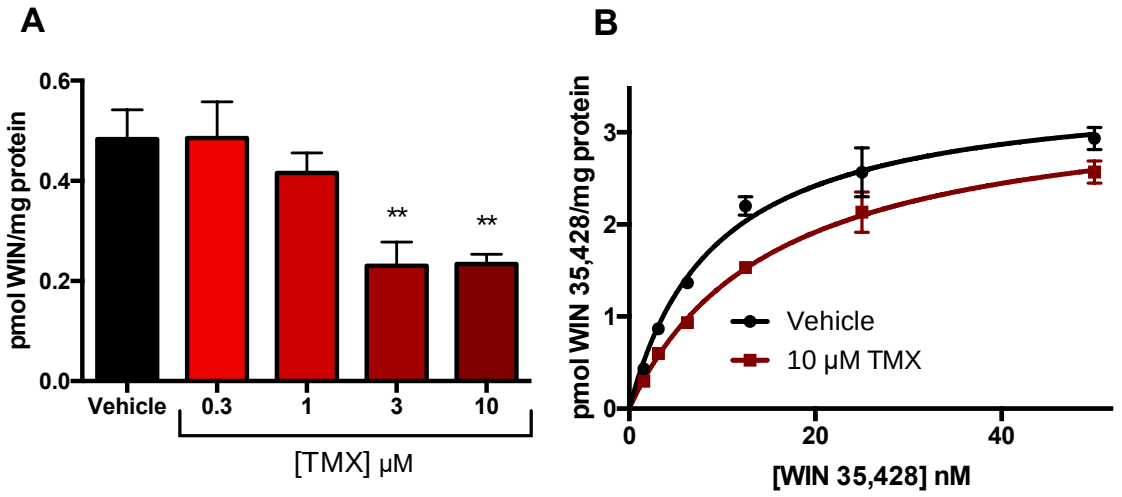

Figure 2-4. Tamoxifen inhibits [^{3}H]WIN 35,428 binding to the dopamine transporter in rat striatal membranes.
In a series of binding assays, rat striatal membranes were incubated with [^{3}H]WIN 35,428 and tamoxifen or vehicle for 3 hours at 4 °C. Non-specific binding was determined with 30 μM nomifensine. **A**. In a competition binding assay, membranes were incubated with 4 nM [^{3}H]WIN 35,428 and various concentrations of tamoxifen. A repeated measures one-way ANOVA indicated a significant effect of treatment on WIN binding. Tamoxifen at 3 and 10 μM significantly decreased [^{3}H]WIN 35,428 binding compared to the vehicle control ($p<0.01$, *post hoc* Dunnett's multiple comparisons test). n=4 **B**. In a saturation binding assay, membranes were incubated with 10 μM tamoxifen and various concentrations of [^{3}H]WIN 35,428 to equilibrium. Comparison of fits in a non-linear regression demonstrated a significant effect of tamoxifen on [^{3}H]WIN 35,428 binding compared to vehicle ($p<0.0001$). N=3. Further analysis indicated that tamoxifen significantly increased K_d but had no effect on B_{max} (paired *t*-test, $p<0.05$). Data represented as mean ± S.E.M.

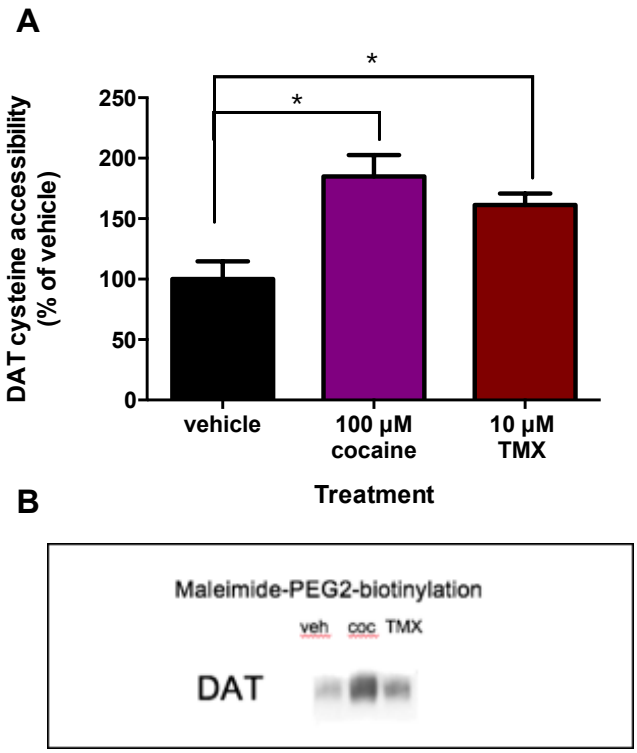

Figure 2-5. Tamoxifen stabilizes the outward facing conformation of the dopamine transporter in a cocaine-like manner.
Rat striatal synaptosomes were incubated for one hour with 100 µM cocaine, 10 µM tamoxifen, or vehicle prior to biotinylation of surface cysteines with maleimide-PEG_2-biotin. Following avidin-biotin pulldown, dopamine transporter content in biotinylated fractions were quantified by western blotting. An ordinary one-way ANOVA found a significant effect of treatment ($p<0.05$) and *post hoc* analysis with Dunnett's multiple comparisons test indicated that both tamoxifen ($p<0.05$) and cocaine ($p<0.05$) significantly increased the availability of extracellular cysteines on the dopamine transporter to biotinylation. N=3

Mutation of the S2 site of the dopamine transporter eliminates the effect of tamoxifen on dopamine uptake.

The atypical nature of tamoxifen's effects on the dopamine transporter led us to hypothesize that tamoxifen may be interacting with the putative S2 site of the dopamine transporter. Evidence suggests that D476A and I159A dopamine transporter mutants have an impaired S2 site (Zhen and Reith, 2016). These mutants exhibit an increase K_m and a

decrease V_{max}, but are still capable of taking up dopamine, bind WIN 35,428, and can be inhibited by cocaine. Thus we utilized these mutants, which were stably expressed in porcine kidney LLCPK-1 cells, to determine whether an intact S2 site is necessary in order for tamoxifen to inhibit uptake. 10 μM tamoxifen significantly reduced the V_{max} of [^{3}H]dopamine uptake in wild-type transporter compared to vehicle, but had no effect in either of the S2 mutants (Table 2-3). Tamoxifen did not affect the K_m of dopamine uptake in the wild-type or mutant dopamine transporter, consistent with our previous observations of non-competitive inhibition of dopamine uptake by tamoxifen.

	WT DAT		D476A DAT		I159A DAT	
treatment	Vehicle	10 μM TMX	Vehicle	10 μM TMX	Vehicle	10 μM TMX
K_m (μM)	0.7 ± 0.06	0.6 ± 0.09	4.2 ± 0.5	4.3 ± 0.3	1.3 ± 0.2	1.3 ± 0.1
V_{max} (pmol/mg/min)	8.1 ± 0.7	3.4 ± 0.4***	1.5 ± 0.2	1.3 ± 0.1	0.2 ± 0.02	0.1 ± 0.02

Table 2-3 Effect of tamoxifen on dopamine uptake kinetics in wild-type and S2 mutant dopamine transporter (DAT)
Unpaired t-test, veh vs. TMX. ***p<0.001, data expressed as mean ± S.E.M. , n=5-6

Tamoxifen decreases amphetamine-stimulated locomotor activity but not basal locomotion

The decrease in dopamine uptake capacity caused by tamoxifen might be expected to lead to an increase in extracellular dopamine levels, and in fact, tamoxifen has been demonstrated to increase extracellular dopamine levels by a small but significant amount *in vivo* via microdialysis (Chaurasia et al., 1998). This increase in extracellular dopamine could cause tamoxifen to have stimulant effects on its own. To test whether this would occur, we followed the tamoxifen dosing protocol established by Einat et al. (2007).

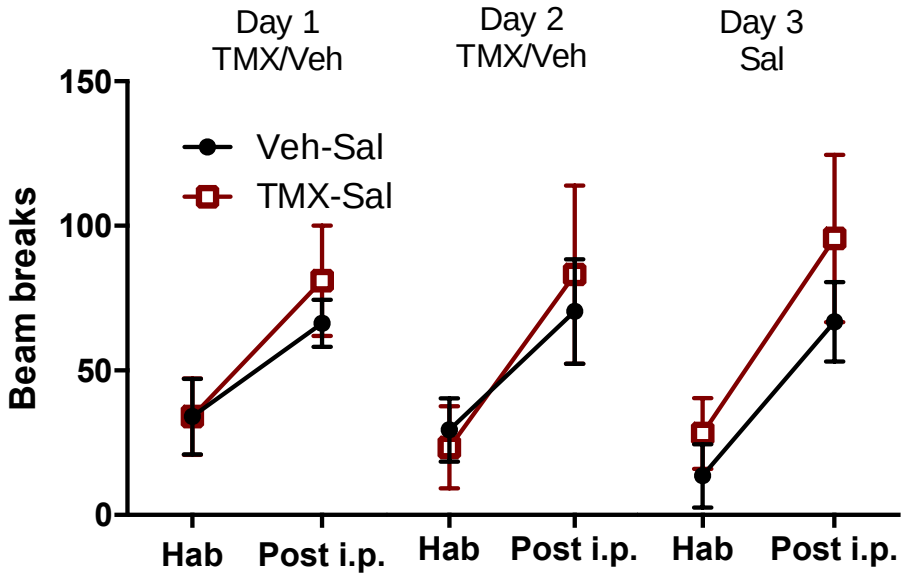

Figure 2-6. Tamoxifen does not affect normal locomotor activity in rats. Male Sprague-Dawley rats (n=8) were allowed to habituate for three hours in a beam break apparatus before administration of an i.p. injection. Locomotor activity was monitored throughout habituation and two hours following i.p. injection. On Days 1 and 2, animals received either 5 mg/kg tamoxifen citrate (2 mg/ml tamoxifen, 3.5% DMSO, 10% Tween-80 in saline) or an equivalent volume of vehicle. On day 3 all animals received saline. Though locomotor activity following i.p. injection with drug or saline was significantly higher than activity during habituation in both groups and across all days, there was no significant difference in locomotor activity between tamoxifen and vehicle treated animals. Values represented are the total beam breaks in the two hours preceding (habituation) and the two hours following (post i.p.) injection.

Animals were given an intraperitoneal injection of 5 mg/kg tamoxifen citrate or vehicle once each day for two days while acclimating to locomotor beam break boxes. On the third day, animals were given an injection of saline. Locomotor activity was measured as the number of beam breaks in the two-hour period preceding and following each injection. Though locomotor activity following i.p. injection with saline or drug was significantly higher than activity during habituation in both groups and across all days, there was no

significant difference in locomotor activity between tamoxifen- and vehicle-treated animals (Figure 2-6). Following the same experimental protocol, rats were pretreated with tamoxifen over two days, however, on day three they received an injection of amphetamine (1 mg/kg, i.p.). In contrast to the lack of effect of tamoxifen on basal locomotor activity, a repeated measures 2-way ANOVA indicated a significant effect for pretreatment on amphetamine-stimulated hyperactivity [$F(1, 18) = 7.26, p=0.0148$], time [$F(23, 414) = 15.67, p<0.0001$], and interaction [$F(23, 414) = 2.543, p=0.0001$] (Figure 2-7).

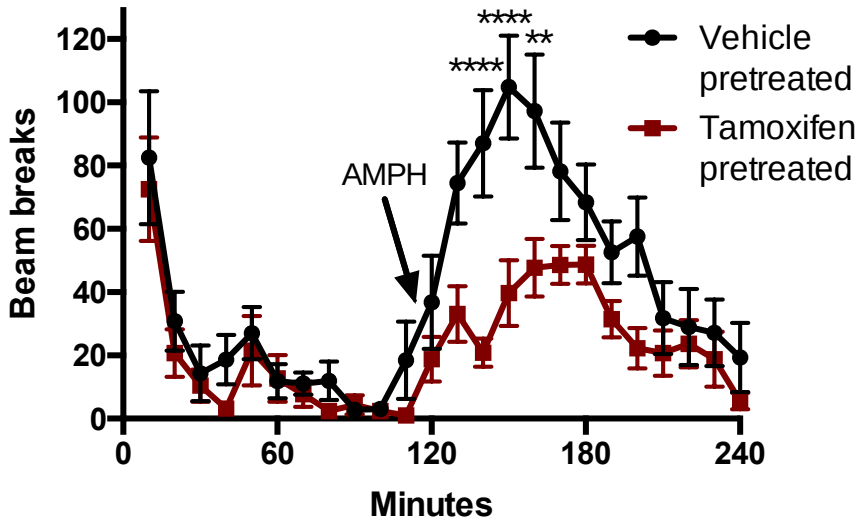

Figure 2-7. Tamoxifen pretreatment attenuates amphetamine-stimulated hyperactivity.

Male Sprague Dawley rats (n=10) were pretreated with 3 mg/kg tamoxifen or vehicle 48 and 24 hours prior to administration of amphetamine (1 mg/kg). Basal locomotor activity was collected and amphetamine was administered (i.p. 1mg/kg) at time point 100, as indicated by the arrow. Pretreatment with tamoxifen significantly reduced amphetamine-stimulated hyperactivity. A repeated measures 2-way ANOVA indicated a significant effect for pretreatment. *Post-hoc* Sidak's multiple comparisons test indicate a significant difference between vehicle and tamoxifen treated animals at 140 minutes ($p<0.0001$), 150 minutes ($p<0.0001$), and 160 minutes ($p<0.01$). Data are represented as the mean ± S.E.M.

Discussion

In this study, we demonstrate for the first time that tamoxifen inhibits normal function of the dopamine transporter in an atypical manner, as opposed to the typical inhibition seen with drugs like cocaine. These conclusions are based on our results demonstrating that tamoxifen attenuates both dopamine uptake and amphetamine-stimulated dopamine efflux *in vitro* and hyperactivity *in vivo*, yet fails to induce hyperlocomotion *in vivo* on its own. Tamoxifen's inhibition of dopamine uptake is non-competitive, and independent of any changes in surface dopamine transporter levels, further indicating the atypicality of tamoxifen's effects on the dopamine transporter. Interestingly, though this is the first demonstration of tamoxifen inhibiting dopamine transporter function, it is not the first demonstration of inhibition of a neurotransmitter transporter by tamoxifen; Chang and Chang (1999) found that tamoxifen inhibited uptake through the serotonin transporter with an IC_{50} of approximate 17 μM. Unfortunately, this observation was never fully explored.

The fact that tamoxifen inhibits dopamine uptake without increasing locomotor activity is reminiscent of the effects of atypical dopamine transporter blockers. An atypical dopamine transporter blocker is defined as a compound that inhibits dopamine uptake, has no stimulant effects of its own (Reith et al., 2015), and reduces the locomotor stimulating effects of cocaine and amphetamine (Velazquez-Sanchez et al., 2010). Some atypical dopamine transporter blockers also inhibit the self-administration and conditioned place preference of cocaine and amphetamine without exhibiting any reinforcing characteristics themselves (Ferragud et al., 2009; Hiranita et al., 2009; Ferragud et al., 2014). There is a

hope that the atypical dopamine transporter blockers may serve as effective therapies for psychostimulant abuse (Tanda et al., 2009) due to their ability to antagonize psychostimulant action without exhibiting their own abuse liability (Schmitt et al., 2013). Because tamoxifen exhibits similar blockade of dopamine transporter function without psychostimulant properties of its own, there is a potential that many of the beneficial effects of atypical blockers may be seen with tamoxifen as well.

Further examination of the effects of tamoxifen on the dopamine transporter indicate that tamoxifen stabilizes the outward facing conformation of the dopamine transporter, similar to cocaine. If tamoxifen were allosterically stabilizing the outward facing conformation of dopamine transporter, we would expect to see an increase in [^{3}H]WIN 35,428 binding, as was observed with cholesterol (Hong and Amara, 2010). However, we find that tamoxifen attenuates [^{3}H]WIN 35,428 binding to dopamine transporter in striatal membranes, albeit weakly, indicating that tamoxifen is somehow interfering with [^{3}H]WIN35,428 binding at the dopamine transporter. It is possible that the effect of tamoxifen on dopamine transporter function that we see is the result of binding to the putative S2 site of the transporter. This site is hypothesized to facilitate binding of substrate to the primary substrate site (Nyola et al., 2010) and possibly even drive the translocation of the dopamine molecule across the membrane (Shan et al., 2011). The existence of the S2 site in the dopamine transporter has been hypothesized from homology models with the bacterial leucine transporter, whose own S2 site is a matter of some controversy (Nyola et al., 2010; Piscitelli et al., 2010). However, the recent solving of the human serotonin transporter structure (Coleman et al., 2016) and the discovery of an allosteric (S2) site homologous to the proposed S2 site in dopamine transporter, promises

to shine a new light on this line of investigation. Pharmacologically, the existence of the S2 site on the dopamine transporter was demonstrated utilizing bivalent ligands. These interesting compounds, constructed from two dopamine transporter ligands connected by a poly-carbon linker, exhibited a significant increase in affinity for the dopamine transporter compared to their monovalent counterparts, indicating concurrent binding to multiple sites on the transporter (Schmitt et al., 2010). More recently, our collaborators in the Reith lab at New York University have developed a pair of S2 site dopamine transporter mutants, D467A and I159A, which possess a disrupted S2 site (Zhen and Reith, 2016). Though these mutants exhibit a much decreased affinity for WIN 35,428 and decreased uptake capacity, they are still vulnerable to uptake inhibition by typical DAT blockers such as cocaine. Excitingly, these mutants are completely resistant to the effects of tamoxifen on dopamine uptake, indicating that these residues are important to the interaction of tamoxifen with the dopamine transporter. These results further support our assertion that tamoxifen is interacting directly with the dopamine transporter.

Tamoxifen is well established as an inhibitor of protein kinase C and other labs have demonstrated that treatment with tamoxifen at therapeutically comparable doses can inhibit amphetamine-stimulated phosphorylation of the protein kinase C substrate GAP43 *in vivo* (Einat et al., 2007). Our lab and others have demonstrated that activation of protein kinase C by amphetamine contributes to amphetamine-stimulated dopamine efflux. Conversely, inhibition of protein kinase C, and the β isoform in particular, decreases amphetamine-stimulated dopamine efflux (Giambalvo, 1992b; Giambalvo, 1992a; Kantor and Gnegy, 1998; Johnson et al., 2005; Zestos et al., 2016) and amphetamine-stimulated locomotor activity (Browman et al., 1998; Zestos et al., 2016). Protein kinase C exhibits

other roles in the regulation of the dopaminergic neuron. Inhibition of protein kinase C potentiates D2-like autoreceptor regulation of exocytotic dopamine release (Luderman et al., 2015). Activation of accumbens protein kinase C promotes the reinstatement of cocaine seeking behavior (Schmidt et al., 2013; Ortinski et al., 2015; Schmidt et al., 2015), while blockade of protein kinase C signaling in the nucleus accumbens prevents the development of amphetamine conditioned place preference (Aujla and Beninger, 2003). As a result of the extensive work connecting psychostimulant abuse and protein kinase C, we have proposed that protein kinase C may present a viable target for the treatment of psychostimulant abuse.

With the shared mechanisms of protein kinase C inhibition and "atypical-like" dopamine transporter blockade in mind, it is possible that tamoxifen may present a two-pronged approach to the modulation of the dopamine systems of the brain. A drug like tamoxifen may be able to prevent the acute actions of drugs like amphetamine and cocaine both through protein kinase C inhibition and blockade of the dopamine transporter, while also inhibiting some of the processes that may lead to relapse in recovering addicts. Tamoxifen is CNS permeant and has been used clinically for many years, yielding a well-defined safety profile. Furthermore, the structure activity relationship of tamoxifen is sufficiently understood (Poirot et al., 2000; de Medina et al., 2004) that the tamoxifen scaffold could be used as a basis for a compound with both atypical dopamine transporter blocker activity and protein kinase C inhibition, but which lacks the estrogen receptor-mediated side effects that make tamoxifen a less than ideal therapeutic.

The discovery that tamoxifen blocks the dopamine transporter shines a new light on work done in years past. For example, tamoxifen has been demonstrated to act as a

neuroprotectant in methamphetamine and MPP^+ induced dopaminergic neurotoxicity. This was largely believed to be due to the estrogen receptor-modulating effects of tamoxifen, since estrogen is neuroprotective as well. However, though estrogen is neuroprotective against methamphetamine-induced neurotoxicity in female mice only, tamoxifen is neuroprotective in both male and female mice (Bourque et al., 2007), indicating the presence of an alternative non-estrogenic-mediated mechanism. Furthermore, tamoxifen will antagonize the neuroprotective effects of estrogen in methamphetamine-induced dopaminergic neurotoxicity (D'Astous et al., 2005), indicating that, in these cases, tamoxifen is likely not estrogenic. Both methamphetamine and MPP^+ require uptake through the dopamine transporter to induce dopamine neurotoxicity, thus, in light of our results here, the "neuroprotective" effects of tamoxifen could simply be the result of decreased dopamine transporter-mediated uptake.

It's important to acknowledge that the concentrations of tamoxifen used in these studies and the IC_{50}s calculated are quite high. However, published data on tamoxifen pharmacokinetics and brain tissue disposition indicate that concentrations near 1 mg/mg of tissue and higher are readily achieved in human brain at steady state (assuming 1 mg tissue = 1 µl of volume, this is estimated at ~2-10 µM and higher) (Lien et al., 1991a; Lien et al., 1991b; Kisanga et al., 2003). Furthermore, though we have found that tamoxifen has a similarly high IC_{50} for inhibition of protein kinase C (1 µM) in a cell based assay, data not shown), the dosing regimen used here was sufficient to decrease amphetamine-stimulated, protein kinase C-mediated phosphorylation of GAP-43 in rat striatum *in vivo* (Einat et al., 2007), indicating that tamoxifen can reach sufficient levels in the brain *in vivo* to be comparable to our *in vitro* studies.

It is also important to consider tamoxifen's SERM activity in the context of the above results. It is still unclear whether the effects of tamoxifen on the estrogen receptor in a dopamine neuron are predominantly estrogenic or antiestrogenic. Estrogen increases amphetamine-stimulated dopamine efflux in striatal tissue (Becker, 1990). However, at concentrations where it binds the estrogen receptor, tamoxifen is unable to block this effect of estrogen on amphetamine-stimulated efflux, nor affect efflux on its own, indicating that it does not have anti-estrogenic effects in the dopamine neuron (Xiao et al., 2003). Furthermore, our studies utilized male animals, which generally are considerably less responsive to estrogenic effects (Becker, 1990; Cummings et al., 2014). In addition, although tamoxifen inhibits breast cancer cell growth at 100 nM through estrogen receptor-dependent mechanisms (Coezy et al., 1982), we fail to see any effect of tamoxifen on dopaminergic processes until we approach concentrations 10-100 times higher. Though micromolar concentrations of estrogen have been found to inhibit dopamine uptake (Disshon et al., 1998), this effect follows a pattern of competitive inhibition (increased K_m, unchanged V_{max}), while we find that tamoxifen noncompetitively inhibits dopamine uptake. Taken together, these results strongly indicate a division between estrogen and tamoxifen and their effects on the dopamine transporter. Still, future work in our lab will more closely examine the role of the estrogen receptors in the effects of tamoxifen on the dopamine transporter.

Something of interest in our experiments was that, contrary to some previously published work (Pereira et al., 2014), we were unable to see an effect of tamoxifen on amphetamine-stimulated hyperactivity unless the animals were treated over two days (data not shown). These results are especially puzzling when taken into consideration with

the observation that one-hour treatment with tamoxifen *in vitro* is sufficient to produce a robust inhibition of the dopamine transporter. Given that tamoxifen was capable of producing an increase in striatal extracellular dopamine *in vivo* within an hour of systemic administration (Chaurasia et al., 1998), the need for an extended incubation time in order for the effects of tamoxifen on behavior to become apparent indicates that there may be alternative mechanisms at work in regards to the effects of tamoxifen on amphetamine-stimulated behaviors. It is also possible that higher doses of tamoxifen may have a more acute effect on amphetamine-stimulated hyperactivity. It may be worth noting that ongoing work in our lab has found that protein kinase C inhibitors administered to the intracerebral ventricular space require an extended incubation time (12-24 hours) before a measurable inhibition of amphetamine-stimulated behaviors is obtained (publication forthcoming).

In conclusion, we demonstrated that tamoxifen significantly impairs dopamine transporter function *in vitro. In vivo,* however, this effect on dopamine transporter functionality appears to only have behavioral relevance in the presence of a non-physiological stimulus such as amphetamine. This effect provides an explanation for many of the results seen previously in studies of tamoxifen and dopaminergic signaling. Additionally, we believe that the combined protein kinase C inhibition and dopamine transporter modulating functions of tamoxifen exhibit potential for a two pronged approach to the pharmacological treatment of psychostimulant abuse and that a further investigation of the tamoxifen structure is warranted.

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CHAPTER THREE. TAMOXIFEN AND ITS METABOLITES INHIBIT DOPAMINE TRANSPORTER FUNCTION INDEPENDENTLY OF THE ESTROGEN RECEPTORS

Abstract

As one of the primary mechanisms by which dopamine signaling is regulated, the dopamine transporter is an attractive pharmacological target for the treatment of diseases based in dopaminergic dysfunction. Previously, we demonstrated that the breast cancer therapeutic, tamoxifen, exhibits characteristics of an atypical inhibitor of dopamine uptake. Here, we further characterize the effects of tamoxifen and two of its metabolites, 4-hydroxytamoxifen and endoxifen, on the dopamine transporter. We found that tamoxifen non-competitively inhibits amphetamine-stimulated dopamine efflux in hDAT-N2A cells and that the effects of tamoxifen on dopamine transporter function in this model are comparable to those seen previously in synaptosomes. The metabolites 4-hydroxytamoxifen and endoxifen prove more complex in their modulation of the dopamine transporter than tamoxifen. Endoxifen asymmetrically inhibits dopamine transporter function in hDAT-N2A cells, showing a preference for the inhibition of amphetamine-stimulated dopamine efflux. Conversely, 4-hydroxytamoxifen exhibited no difference between inhibition of dopamine uptake and inhibition of amphetamine-stimulated dopamine efflux in cells, yet demonstrated a preference for inhibition of uptake in rat striatal synaptosomes. Additionally, we demonstrate that the effects of tamoxifen on the dopamine transporter occur independently of its activity as a selective estrogen receptor

modulator, further supporting our assertion that tamoxifen inhibits dopamine transporter function though a previously unidentified mechanism

Introduction

The dopamine transporter is the chief mechanism by which extracellular dopamine is removed from the synapse following its release, and as such plays a vital role in the regulation of dopamine signaling. Pharmacological modulation of the dopamine transporter, therefore, is a source of great interest for the treatment of multiple dopaminergic diseases.

Previously, we demonstrated that the breast cancer therapeutic tamoxifen exhibits the characteristics of an atypical dopamine uptake inhibitor (Chapter 2). Tamoxifen inhibits dopamine uptake, amphetamine-stimulated dopamine efflux, and amphetamine-stimulated hyperlocomotion, yet fails on its own to elicit the hyperlocomotion characteristic of "typical" dopamine transporter blockers such as cocaine and methylphenidate. We also demonstrated that tamoxifen stabilizes the outward facing conformation of the dopamine transporter in a striatal membrane preparation, indicating a direct interaction between tamoxifen and the transporter.

Tamoxifen is metabolized into three active metabolites that reach appreciable levels in the brain (See Figure 1-7): 4-hydroxytamoxifen, N-desmethyltamoxifen, and 4-hydroxy-N-desmethyltamoxifen (known as endoxifen). 4-hydroxytamoxifen and endoxifen are considered the active metabolites of tamoxifen, because their potency as SERMs is 100-fold higher than the parent compound (Lien et al., 1991). Any combination of these metabolites could be responsible for the effects of tamoxifen on dopamine-regulated behaviors.

Though our previous results indicated a direct interaction between tamoxifen and the dopamine transporter, we have not yet ruled out the possibility that tamoxifen is exerting its effects on the dopamine transporter through its well-established activity as a selective estrogen receptor modulator. Estrogen is well known to affect dopamine transporter function; it stimulates dopamine efflux on its own and enhances amphetamine-stimulated dopamine efflux (Becker, 1990; Xiao et al., 2003). There are three known estrogens receptors: ERα, ERβ, and the G-protein coupled estrogen receptor (GPER1). All three receptors have been demonstrated to modulate dopamine transporter function to varying degrees (Alyea et al., 2008; Alyea and Watson, 2009). Beyond its SERM activity at ERα and ERβ, tamoxifen is also an agonist at GPER1 (Thomas et al., 2005).

Investigation of direct effects of tamoxifen on the dopamine transporter would be facilitated by the use of cell culture models. In this study, therefore, we characterize the effects of tamoxifen on dopamine transporter function in a mouse neuroblastoma cell line stably expressing the dopamine transporter (hDAT-N2A) and compare these results to those achieved in rat striatal tissue. We determined that tamoxifen and two of its three major metabolites, 4-hydroxytamoxifen and endoxifen, directly inhibit dopamine uptake and amphetamine-stimulated dopamine efflux to varying degrees in hDAT-N2A cells. N2A mouse neuroblastoma cells have been demonstrated to express all three known estrogen receptors (Mendez and Garcia-Segura, 2006; Manthey et al., 2010; Su et al., 2012), and as such are useful in determining whether tamoxifen's SERM activity contributes to its effects on the dopamine transporter. We demonstrate that tamoxifen and its metabolites are not exerting their effects *via* activation of the estrogen receptors. Therefore, antagonism of the

estrogen receptors is not sufficient to explain the effects of tamoxifen or its metabolites on dopamine transporter function.

Methods and materials

Materials

All chemicals were obtained from Sigma Aldrich with the exception of [^{3}H]dopamine, which was purchased from Perkin Elmer, and G36, which was purchased from Tocris. Tamoxifen citrate, endoxifen, 4-hydroxytamoxifen, and G36 were prepared as a 50 mM stock in dimethylsulfoxide (DMSO). Fulvestrant was dissolved in DMSO as a 10 mM stock. The stock solutions were then diluted in the buffers appropriate for the assay. Final concentration of DMSO in all solutions ranged from 0.01-0.03% and were kept constant across all conditions within an experiment.

Cell culture

N2a mouse neuroblastoma cells stably expressing the human dopamine transporter (hDAT-N2a) cells were grown in Optimem reduced serum media (Gibco) supplemented with fetal bovine serum (10%), penicillin-strepavidin (1%), and G418 (100 μg/ml, Gibco) at 37 °C in 5% CO_2. For experiments involving estrogen receptor antagonists, cells were maintained under the same conditions and in a similar media formulation except that the Optimem was free of phenol-red and was supplemented with a charcoal-stripped bovine serum (kindly provided by Dr. James Rae, University of Michigan) in order to limit exposure of the cells to exogenous estrogens. For dopamine uptake and amphetamine-stimulated dopamine efflux assays cells were trypsinized 1-2 days before the experiment and seeded on 24-well plates (50,000 to 100,000 cells per well) such that at the time of the

experiment, cell density would be approaching confluency with approximately 200,000 cells per well.

[^{3}H]dopamine uptake – cell based assay

Cells were grown in 24-well plates. Media was aspirated from the wells and cells were washed three times with Krebs Ringer HEPES buffer (KRH, pH 7.4, 25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 50 µM pargyline, and 50 µM ascorbic acid) before the addition of 400 µl of drug treatment or vehicle in KRH. Cells were kept in treatment throughout the duration of the experiment. After a one-hour incubation, cocaine was added to non-specific wells, and dopamine uptake was initiated 10 minutes later. For the dopamine uptake concentration response curves, dopamine uptake was initiated by the addition of 10 nM [^{3}H]dopamine supplemented with 300 nM unlabeled dopamine and the reaction was terminated after 10 minutes. In the kinetic assay, dopamine uptake was initiated with the addition of 10 nM [^{3}H]dopamine supplemented with 10 nM to 3 µM unlabeled dopamine and the reaction was terminated after 5 minutes. Dopamine uptake was terminated by aspiration of the dopamine solution followed by three rapid washes with ice cold KRH. Cells in each well were lysed with 400 µl of 2 M perchloric acid and transferred to scintillation vials with 5 mls of Scintiverse Scintillation Cocktail (Fisher Scientific) and counted for 2 minutes each on a Beckman scintillation counter.

[^{3}H]dopamine efflux - cell based assay

Media was aspirated from wells and cells were washed three times with KRH before the addition of 200 µl of 50 nM [^{3}H] dopamine supplemented with 5 µM unlabeled dopamine in KRH and incubation at 37°C for 40 min. After dopamine loading, each well was rapidly

washed with KRH three times. KRH plus drugs or vehicle was added to each well. A stable baseline was established by removing and replacing the solution in the cells every 10 minutes for a total of 50 minutes. Beginning at 50 minutes, three 10 minute fractions were collected. 20 μM amphetamine was added during the second fraction. Baseline was defined as the counts per minute in the fraction immediately preceding the addition of amphetamine. Following removal of the final fraction, cells were lysed with 2 M perchloric acid to quantify total remaining dopamine content. In each experiment, treatment conditions were carried out in triplicate and an additional well was reserved for each treatment condition which received no amphetamine in order to quantify non-specific dopamine release. Dopamine efflux was quantified as the percentage of the dopamine content released during the efflux fraction divided by the total dopamine present in the cells.

Animals

All animal use procedures were approved by the University Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines. Male Sprague-Dawley rats (Harlan laboratories) from 7-12 weeks of age were maintained under standard conditions on a 12-hour light-dark cycle and were housed in groups of two or three.

Synaptosome preparation

Following decapitation of the rat, striata were isolated and homogenized in 10 volumes of 0.32 M sucrose with 1 mM EDTA (pH 7.4). Homogenates were centrifuged at 1000 x g for 10 minutes. The supernatant was centrifuged again at 15,000 x g for 15 minutes and the resulting pellet was gently resuspended in Kreb's Ringer Buffer for subsequent use (KRB,

145 mM NaCl, 2.7 mM KCl, 1.2 mM KH2PO4, 1.0 mM MgCl2, 10 mM glucose, 24.9 mM NaHCO3, 0.05 mM ascorbic acid, 0.05 mM pargyline, pH 7.4).

Dopamine efflux assay – suprafusion

The entirety of the suprafusion assay was carried out in KRB. Suprafusion was carried out as described previously (chapter 2). Briefly, following a one hour wash (800 µl/min) with vehicle or drug on a Brandel Suprafusion Apparatus (Brandel Inc, Gaithersburg, MD), 1 min fractions were collected for a total of 14 fractions and 10 µM amphetamine sulfate was added to the wash during fractions 7 and 8. An internal standard solution was added to each sample in an approximately 1:20 dilution (ISS, final concentration 50 mM perchloric acid, 25 µM EDTA, 10 nM 2-aminophenol). Samples were analyzed for dopamine content by HPLC coupled to electrochemical detection (Thermo Scientific/ESA, Sunnyvale, CA). A small aliquot of synaptosomes was reserved for quantification of total dopamine content.

[^{3}H]Dopamine Uptake - synaptosomes

Uptake assays were carried out as described previously (Chapter 2). Briefly, synaptosomes were incubated with drug or vehicle for 60 minutes at 37 °C. Unlabeled dopamine (300 nM) supplemented with 10 nM [^{3}H]dopamine was added to the synaptosomes and the reaction was stopped at 3 min with the addition of 3 ml of cold KRB. 100 µM cocaine was added to select vials to determine nonspecific uptake. Specific [^{3}H]dopamine uptake was determined as the [^{3}H]dopamine content following filtration minus the [^{3}H]dopamine content in the nonspecific samples. Samples were filtered through glass fiber filters (GF/C, Fisher Scientific) and washed twice more with cold KRB. Filters were dried and transferred to scintillation vials and radioactivity was counted in 5 ml of ScintiVerse cocktail (ThermoFisher Scientific, Waltham, MA) using a Beckman LS5801 scintillation counter

(Beckman Coulter, Brea, CA).

Statistical analysis

All statistical analyses were carried out in Graphpad Prism 6 (San Diego, CA). Data are plotted as mean ± S.E.M. Significance was set at $p<0.05$. Comparisons between multiple groups were made with one-way ANOVA, with *post hoc* Dunnett's multiple comparison test. Non-linear regression was used to determine IC_{50}, V_{max}, and K_m values. IC_{50} values were calculated as the average of those calculated for each experiment. When concentration response curves were compared, comparison of fits in non-linear regression was used to determine whether curves differed from each other. Calculated K_m and V_{max} were compared across experiments by a one-way ANOVA.

Results

Tamoxifen and dopamine uptake kinetics

We previously found that tamoxifen inhibits dopamine uptake in rat striatal synaptosomes in a non-competitive manner (Chapter 2). To validate the results in heterologous cultured cells, we examined the potency and kinetic effects of tamoxifen on dopamine uptake in the hDAT-N2A cells. Confirming our result in synaptosomes, we found that tamoxifen significantly decreases the V_{max} of dopamine uptake at 3 and 10 μM. In contrast to the results in synaptosomes, 10 μM tamoxifen significantly decreases the K_m of [^{3}H]dopamine uptake. (Figure 3-1, Table 3-1).

Best-fit values	**Vehicle** (n=6)	**3 μM TMX** (n=3)	**10 μM TMX** (n=3)
V_{max} (± S.E.M.)	34.5 ± 2.8	13.8 ± 2.1**	4.9 ± 0.9****
K_m (± S.E.M.)	430.9 ± 57.2	232.6 ± 33.7	148.6 ± 53.9*

Table 3-1. Best fit Michaelis-Menten values for dopamine uptake
*p<0.05, **p<0.01, ****p<0.0001 vs. vehicle, One-way ANOVA with Dunnett's *post hoc* multiple comparisons.

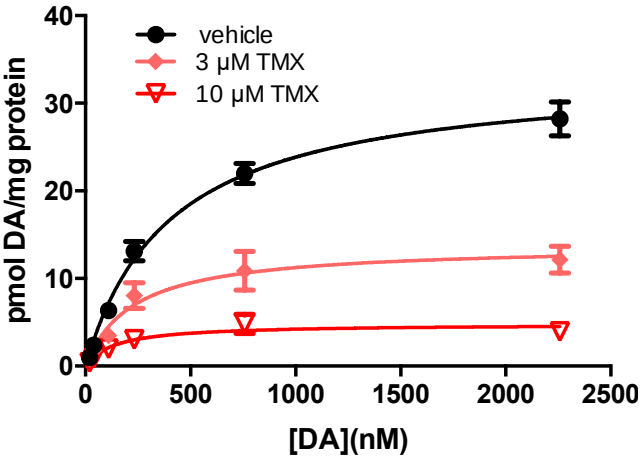

Figure 3-1. Kinetic analysis of [^{3}H]dopamine uptake following pretreatment of hDAT-N2A cells with tamoxifen.
hDAT-N2A cells seeded on a 24-well plate were treated for one hour at room temperature with vehicle, 3 µM, or 10 µM tamoxifen. [^{3}H]Dopamine uptake was initiated with the addition of 10 nM [^{3}H]dopamine supplemented with 10 nM to 3 µM unlabeled dopamine and allowed to precede for 5 minutes prior to halting the reaction with cold wash buffer, lysis of cells with 2 M perchloric acid, and quantification of tritium content by scintillation counting. Comparison of fits indicated a significant effect of both concentrations of tamoxifen on V_{max} but not K_m compared to vehicle ($p<0.0001$). Data are represented as mean ± S.E.M. n=3

Effect of tamoxifen on amphetamine-stimulated dopamine efflux

We next probed the dose-dependent effects of tamoxifen on [^{3}H]dopamine efflux stimulated by a wide range of amphetamine concentrations. hDAT-N2A cells were treated with 1 µM, 3µM, and 10 µM tamoxifen and efflux was stimulated by 30 nM to 3 µM amphetamine. As the tamoxifen concentration increases we see no significant change in EC_{50} of amphetamine (range across tamoxifen doses 156-209 nM, mean 172 ± 12 nM), but we do see a significant reduction in maximal effect for 3 µM and 10 µM (comparison of fits, $p<0.05$), indicating that tamoxifen is affecting amphetamine-stimulated dopamine efflux through a non-competitive mechanism (Figure 3-2).

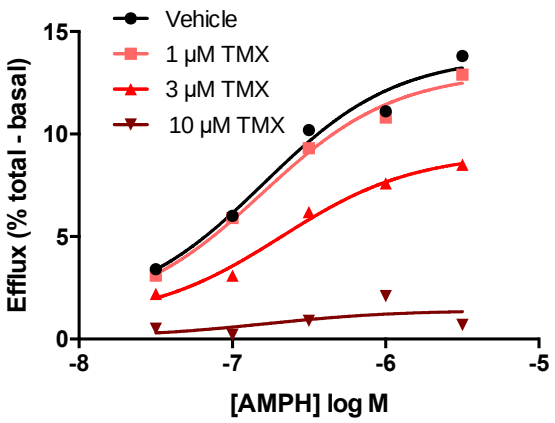

Figure 3-2. Inhibition of amphetamine-stimulated dopamine efflux by tamoxifen.

Following loading with 5 μM [^{3}H]dopamine, hDAT-N2A cells were incubated with vehicle or tamoxifen at the indicated concentrations. Following one hour of incubation amphetamine was added to the wells and the supernatant was collected after 10 minutes for quantification via scintillation counting. Efflux represents the amount of dopamine in the supernatant relative to the total dopamine content of the well, minus the dopamine in the supernatant of the prior unstimulated well (basal dopamine release, no amphetamine).

Tamoxifen inhibits amphetamine-stimulated dopamine efflux and uptake with similar potency in hDAT-N2A cells.

In order to characterize the effects of tamoxifen on dopamine transporter function, we quantified dopamine uptake and amphetamine-stimulated dopamine efflux in hDAT-N2A cells following 1-hour pretreatment with the increasing concentrations of tamoxifen (Figure 3-3a). One-way repeated measures ANOVA indicated a significant effect of treatment on dopamine uptake ($F(5, 20) = 32.74$, $p<0.0001$) and efflux ($F(5, 20) = 40.19$, $p<0.0001$). *Post hoc* Dunnett's multiple comparisons test revealed a significant decrease in uptake for 3 μM ($p<0.001$) and 10 μM ($p<0.0001$) tamoxifen and a significant decrease in efflux for 0.3 μM ($p<0.05$), 1 μM ($p<0.001$), 3 μM ($p<0.01$) and 10 μM ($p<0.0001$) tamoxifen compared to vehicle. There was no significant difference between the concentration

response curves generated for uptake and amphetamine-stimulated efflux in hDAT-N2A cells. See table 3-2 for IC_{50} values.

For comparison, I have included an alternate representation of the data presented in Figures 2-1a and 2-2. The IC_{50} values for tamoxifen were 10.6 ± 2.4 and 8.3 ± 3.0 µM for dopamine uptake and amphetamine-stimulated dopamine efflux, respectively, with no significant difference between the two curves (Figure 3-3b). A repeated measures one-way ANOVA found a significant effect of tamoxifen treatment on uptake ($F(3,12)=27.5$, $p<0.0001$). A one-way ANOVA found a significant effect of tamoxifen treatment on efflux ($F(3,9)=9.7$, $p<0.01$). Significant reductions in dopamine uptake were seen at 3 µM ($p<0.05$) and 10 µM ($p<0.0001$). Significant reductions in amphetamine stimulate dopamine efflux were seen at 3 µM ($p<0.05$) and 10 µM ($p<0.01$).

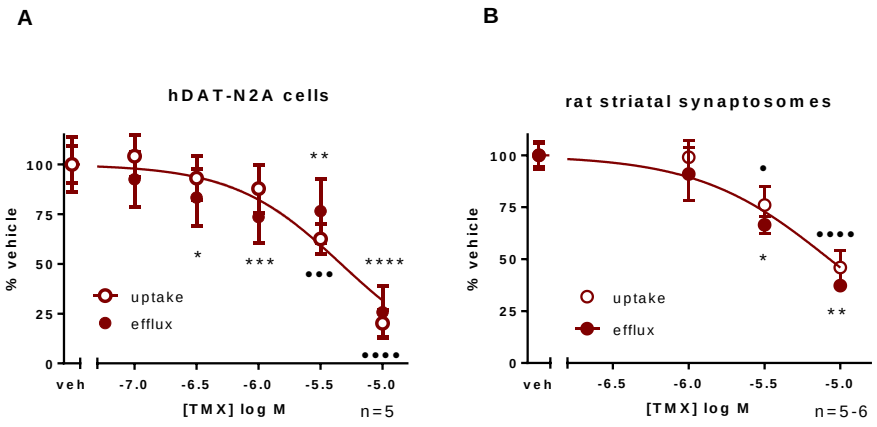

Figure 3-3. Tamoxifen inhibits the dopamine transporter.
Inhibition of [^{3}H]dopamine uptake and amphetamine-stimulated [^{3}H]dopamine efflux in (**A**) hDAT-N2A cells and (**B**) rat striatal synaptosomes (from chapter 2) following a 1-hour treatment with tamoxifen. Comparison of fits for non-linear regression indicated no difference between the concentration response curves for uptake vs. efflux in either systems. The solubility limitations of tamoxifen prevented the testing of higher concentrations. *efflux, $p<0.05$ vs vehicle; **efflux, $p<0.01$ vs vehicle; ***efflux, $p<0.001$ vs vehicle; ****efflux, $p<0.0001$ vs vehicle; • uptake, $p<0.05$ vs vehicle; ••• uptake, $p<0.001$; •••• uptake, $p<0.0001$.

Effects of tamoxifen metabolites on dopamine transporter function.

We next characterized the effects of the active metabolites of tamoxifen on the dopamine transporter. N-desmethyltamoxifen had no effect on [^{3}H]dopamine uptake or efflux through the dopamine transporter at concentrations up to 3 μM, but was cytotoxic at higher concentrations as measured by PrestoBlue (ThermoFisher, data not shown). Our focus was then on the two remaining metabolites, 4-hydroxytamoxifen and endoxifen.

In hDAT-N2A cells, a repeated measures one-way ANOVA indicated a significant effect of 4-hydroxytamoxifen treatment on [^{3}H]dopamine uptake ($F(5, 15) = 33.68$, $p<0.0001$) and efflux ($F(5,10) = 31.63$, $p<0.0001$). *Post hoc* Dunnett's multiple comparisons test revealed a significant decrease in [^{3}H]dopamine uptake 1 μM ($p<0.05$), 3 μM ($p<0.0001$) and 10 μM ($p<0.0001$) 4-hydroxytamoxifen and a significant decrease in [^{3}H]dopamine efflux for 3 μM ($p<0.001$), and 10 μM ($p<0.0001$) 4-hydroxytamoxifen compared to vehicle. The concentration response curves generated by these two data sets were not significantly different from each other. See table 3-2 for IC_{50} values.

In rat striatal synaptosomes, a one-way ANOVA indicated a significant effect of 4-hydroxytamoxifen treatment on dopamine uptake ($F(4,9) = 15.68$, $p<0.001$) and efflux ($F(3, 11) = 5.769$, $p<0.05$). *Post hoc* Dunnett's multiple comparisons test revealed a significant decrease in dopamine uptake for 1 μM ($p<0.05$), 3 μM ($p<0.01$) and 10 μM (0.001) 4-hydroxytamoxifen and a significant decrease in efflux for 10 μM ($p<0.05$) 4-hydroxytamoxifen compared to vehicle. Unlike in cells, the concentration response curves generated by these data sets were significantly different from each other ($p<0.001$) see table 3-2 for IC_{50}s.

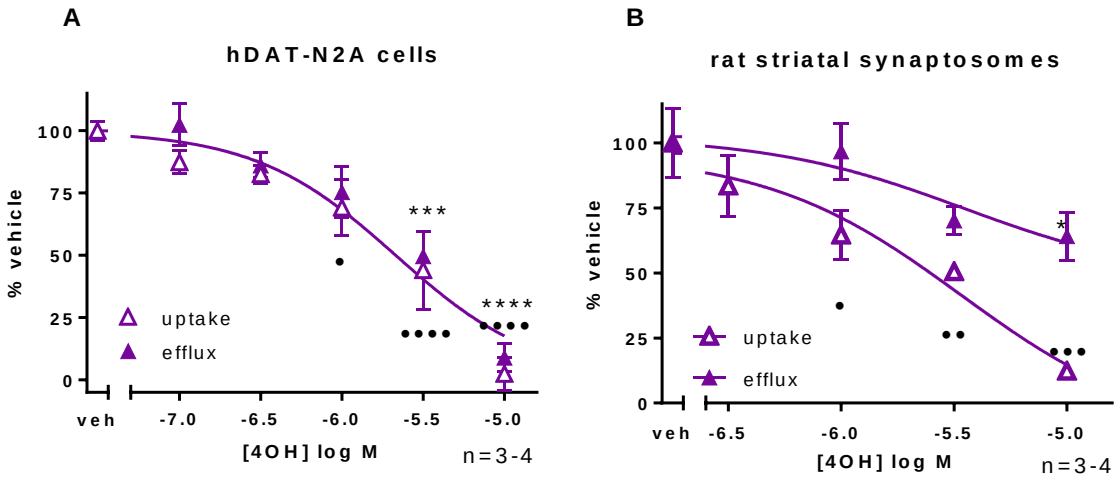

Figure 3-4. 4-hydroxytamoxifen inhibits the dopamine transporter. Inhibition of dopamine uptake and amphetamine-stimulated dopamine efflux in hDAT-N2A cells and rat striatal synaptosomes following 1 hour treatment with 4-hydroxtamoxifen. Comparison of fits for non-linear regression indicated no difference between the concentration response curves for dopamine uptake vs. amphetamine-stimulated dopamine efflux in cells (**A**) but a significant difference between potency (lower IC_{50} for efflux, $p<0.01$) and efficacy (greater efficacy for uptake, $p<0.01$) in synaptosomes (**B**). Note: in cells, efflux is stimulated with 20 µM amphetamine, whereas in synaptosomes efflux is stimulated by 10 µM amphetamine. *efflux, $p<0.05$ vs vehicle; **efflux, $p<0.01$ vs vehicle; ***efflux, $p<0.001$ vs vehicle; ****efflux, $p<0.0001$ vs vehicle; • uptake, $p<0.05$; •• uptake, $p<0.01$; ••• uptake, $p<0.001$; •••• uptake, $p<0.0001$.

In hDAT-N2A cells, a one-way repeated measures ANOVA indicated a significant effect of endoxifen treatment on [^{3}H]dopamine uptake ($F(4,8) = 4.1$, $p<0.05$) and amphetamine-stimulated [^{3}H]dopamine efflux ($F(4,8) = 36.4$, $p<0.001$). *Post hoc* Dunnett's multiple comparisons test revealed a significant decrease in [^{3}H]dopamine uptake at 3 µM ($p<0.05$) endoxifen and a significant decrease in amphetamine-stimulated [^{3}H]dopamine efflux at 1 µM ($p<0.01$) and 3 µM ($p<0.0001$) endoxifen compared to vehicle. The concentration response curves generated by these two data sets were significantly different from each other ($p<0.01$) though the calculated IC_{50}s were not significant different. In rat striatal

synaptosomes, we have yet to obtain efflux data for endoxifen; however, a repeated measures one-way ANOVA indicated an effect of endoxifen on uptake ($F(3,9) = 17.06$, $p<0.001$) with a significant reduction at 3 μM ($p<0.001$) in synaptosomes (Figure 3-5).

Effect of estrogen antagonists

Because tamoxifen and its metabolites are selective estrogen receptor modulators, we sought to determine whether their actions on the dopamine transporter might be attributable to their activity at any of the three estrogen receptors. The hDAT-N2A cells used in these experiments were maintained in medium supplemented with charcoal stripped fetal bovine serum in order to preclude complications that might arise from the presence of exogenous estrogens. Using the ERα and ERβ antagonist fulvestrant (3 μM), and the GPER1 antagonist G36 (10 μM), we found that blockade of the estrogen receptors had no significant effect on basal dopamine uptake or efflux. Moreover, neither fulvestrant nor G36 inhibited the effects of tamoxifen or its metabolites on [^{3}H]dopamine uptake or amphetamine-stimulated [^{3}H]dopamine efflux (Figure 3-6).

IC_{50}	Tamoxifen	4-hydroxytamoxifen	Endoxifen
hDAT-N2A cells			
Uptake	**5.1 μM ± 1.5** (n=5)	**2.6 μM ± 1.0** (n=4)	**4.1 μM ± 0.6** (n=3)
Efflux	**14.1 μM ± 10.0** (n=5)	**2.5 μM ± 0.2** (n=3)	**3.0 μM ± 1.0** ¥ (n=3)
synaptosomes			
Uptake	**13.4 μM ± 5.7** ‡ (n=5)	**2.3 μM ± 0.5*** (n=3)	**10.0 μM ± 5.4** (n=5)
Efflux	**8.4 μM ± 2.6** ‡ (n=5)	**13.1 μM ± 2.8** (n=4)	*n.d.*

Table 3-2 – IC_{50}s for uptake and efflux in hDAT-N2A cells and rat striatal synaptosomes. (±S.E.M.).
*p<.01 vs. IC_{50} for efflux, ‡ from Chapter 2, reanalyzed for concentration response curve comparison, ¥ p=0.054 vs. uptake, statistical trend.

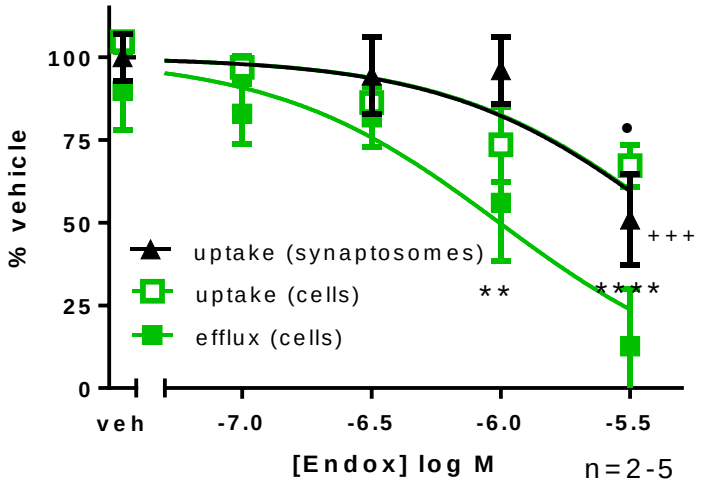

Figure 3-5. Endoxifen inhibits the dopamine transporter.
Inhibition of dopamine uptake and amphetamine-stimulated dopamine efflux in hDAT-N2A cells and rat striatal synaptosomes following 1-hour treatment with endoxifen (endox). Comparison of fits for non-linear regression indicated a significant difference between the concentration response curves for uptake (open squares) *vs* efflux (20 μM amphetamine, closed squares) in hDAT-N2A cells. **efflux (cells), $p<0.01$ vs vehicle; ****efflux (cells), $p<0.0001$ vs vehicle; • uptake (cells), $p<0.05$; +++ uptake (synaptosomes), $p<0.001$ vs vehicle.

Discussion

In this study we demonstrate that tamoxifen and two of its active metabolites, 4-hydroxytamoxifen and endoxifen, impair dopamine transporter function independently of their actions at the estrogen receptors. We also demonstrate that in cells, tamoxifen impairs both dopamine uptake and amphetamine-stimulated dopamine efflux through a non-competitive mechanism. Due to the similarities between the concentration response curves for tamoxifen in inhibition of dopamine uptake and amphetamine-stimulated

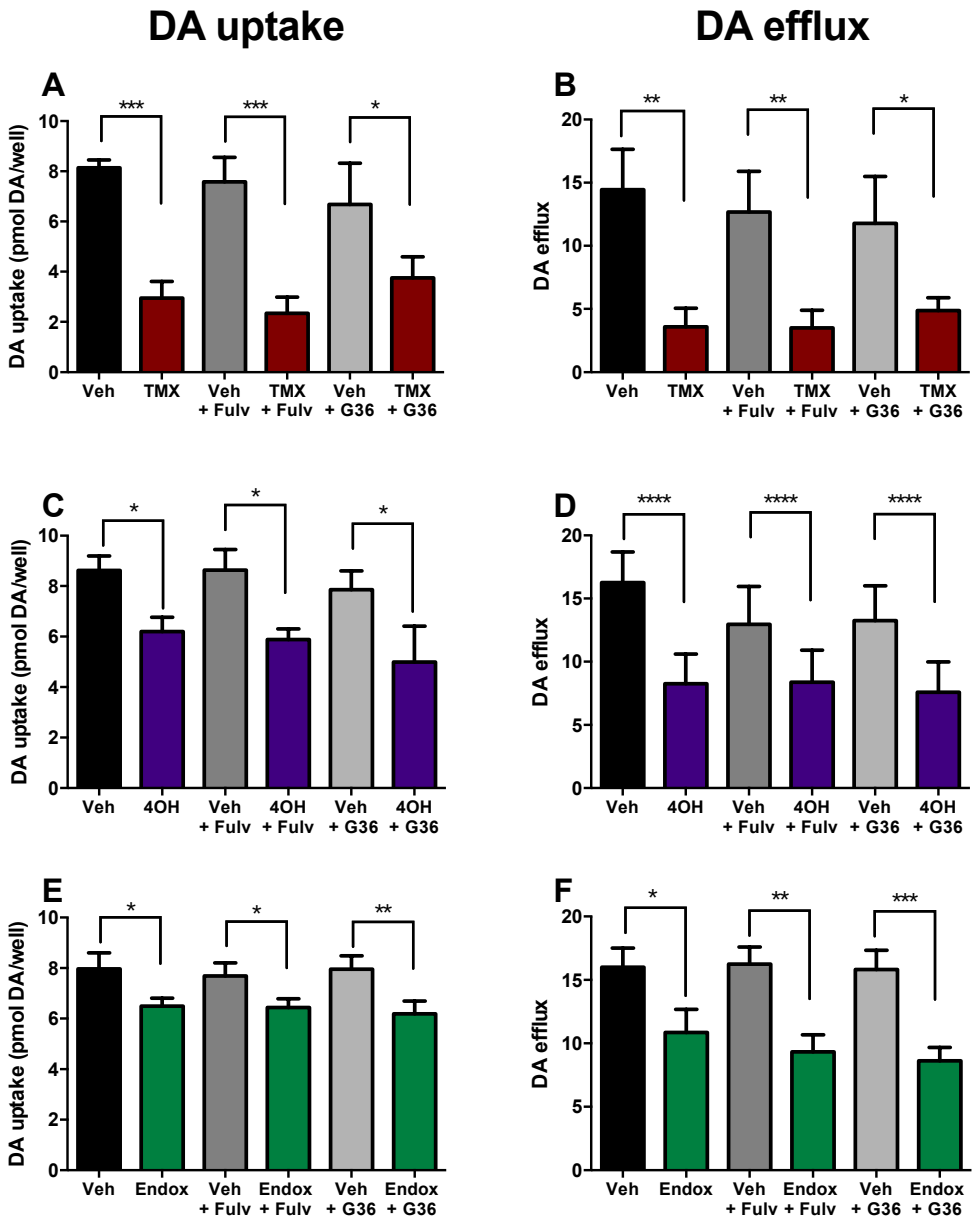

Figure 3-6. Effects of estrogen receptor antagonists on [^{3}H]dopamine uptake and amphetamine-stimulated [^{3}H]dopamine release.
Inhibition of dopamine uptake (A, C, E) and amphetamine-stimulated dopamine efflux (20 μM amphetamine, B, D, F) by tamoxifen (10 μM, A, B), 4-hydroxytamoxifen (3 μM, C, D), and endoxifen (3 μM, E, F) were measured in the presence and absence of the ERα/ERβ antagonist fulvestrant (3 μM) or the GPER1 antagonist G36 (10 μM). The presence of the estrogen receptor antagonists had no significant on amphetamine-stimulated [^{3}H]dopamine efflux or [^{3}H]dopamine uptake and failed to affect the ability of tamoxifen and its metabolites to inhibit these activities.

dopamine efflux, we hypothesize that the effect of tamoxifen on amphetamine-stimulated dopamine efflux is the result of an impairment of amphetamine uptake.

Interestingly, we find that some discrepancies exist for the effect of tamoxifen metabolites on dopamine uptake and amphetamine-stimulated dopamine efflux between the hDAT-N2A cell model and the striatal synaptosomes. Though tamoxifen exhibits similar inhibition of uptake and efflux in both cells and synaptosomes, modulation of transporter activity by the metabolites appears somewhat more complex. We find that in cells, 4-hydroxytamoxifen inhibits dopamine uptake and amphetamine-stimulated dopamine efflux with similar potency and efficacy, yet in synaptosomes, 4-hydroxytamoxifen is significantly less efficacious in the inhibition of efflux as compared to uptake. Though we have yet to obtain efflux data in synaptosomes for endoxifen, we find that endoxifen appears to be *more* efficacious and potent as an inhibitor of amphetamine-stimulated dopamine efflux as compared to [^{3}H]dopamine uptake in hDAT-N2A cells.

Before the implications of these discrepancies can be discussed, it is important to consider the differences between the assays used for cells *vs* synaptosomes. All cell-based efflux assays were performed with adherent cells plated in 24-well plates with exogenously-loaded [^{3}H]dopamine, while amphetamine-stimulated dopamine efflux from synaptosomes is carried out in a suprafusion apparatus with buffer rapidly perfusing the tissue preparation, with only endogenous dopamine contributing to efflux. The rapid perfusion of the synaptosomes ensures that as dopamine is transported into the extra-synaptosomal space, it is washed away before any significant amount of reuptake can take place. However, in a plated cell based assay, the efflux that we are measuring is in reality a measure of the amount of dopamine released through amphetamine-stimulated dopamine

efflux minus the amount of dopamine taken back up in to the cell. In this setting, an inhibitor of dopamine uptake might be expected to appear to have less of an effect on amphetamine-stimulated dopamine efflux, as the blockade of reuptake might cancel out any reduction in efflux. Thus, it is difficult for us to make a truly accurate comparison between uptake and efflux in the plated cell-based assay.

It is worth considering that the stationary condition of the cell-based efflux assay is likely more comparable to the *in situ* environment of the dopamine neuron, yet it is still not an ideal model. Previously, we have seen that in every other measure of efflux used in our lab, including *in vivo* microdialysis (Zestos et al., 2016), suprafusion with striatal tissue (Kantor and Gnegy, 1998; Johnson et al., 2005), and even suprafusion with cells (unpublished data), protein kinase C inhibitors significantly attenuate amphetamine-stimulated dopamine efflux for concentrations of amphetamine reaching as high as 20 μM. In the cell based assay, however, we find that protein kinase C inhibitors have a minimal and unreliable effect on amphetamine-stimulated dopamine efflux, and then only when very small concentrations of amphetamine are used (< 1 μM, data not shown). The reasons for this incongruence eludes us, as well as whether it is attributable to the assay (plated vs. suprafusion) or the model (cells vs. synaptosomes), but it serves to highlight an important caveat in the cell-based efflux assay.

Returning to the discrepancies observed between dopamine uptake and amphetamine-stimulated efflux in the tamoxifen metabolites, both 4-hydroxytamoxifen and endoxifen appear to be asymmetrically affecting the function of the dopamine transporter. While 4-hydroxytamoxifen appears to exhibit a “preference” for inhibition of dopamine uptake, at least in synaptosomes, endoxifen preferentially inhibits efflux in cells.

The difference between the effects of endoxifen on dopamine uptake and amphetamine-stimulated dopamine efflux in the cells is intriguing. Endoxifen is a known inhibitor of protein kinase C, and, based on our previous studies (Kantor and Gnegy, 1998; Johnson et al., 2005), our initial thought upon observing the effect of endoxifen on amphetamine-stimulated dopamine efflux was that it may be exerting its effects through inhibition of protein kinase C. However, the above mentioned caveats with the cell-based assay preclude this hypothesis, as we might not expect to see an effect of protein kinase C inhibition on amphetamine-stimulated dopamine efflux. As yet, we are unable to speculate as to the means by which endoxifen and 4-hydroxytamoxifen asymmetrically modulate transporter activity, but it is a question worthy of further investigation.

In our investigation of the effects of tamoxifen and its metabolites on the dopamine transporter, it was necessary that we consider the potential effects of the drugs' SERM activity on dopamine transporter function. We found that antagonists of the estrogen receptors had no effect on amphetamine-stimulated dopamine efflux or dopamine uptake on their own, and did not alter the ability of tamoxifen or its metabolites to inhibit forward or reverse transport. By failing to antagonize the effects of tamoxifen and its metabolites on the dopamine transporter, these experiments demonstrated that tamoxifen is not exerting its effects on the dopamine transporter through an estrogenic mechanism. Furthermore, because the antagonists themselves had no effect on uptake or efflux, an antiestrogenic effect of tamoxifen is insufficient to explain its inhibition of dopamine transporter function. These conclusions are further supported by previous observations that fulvestrant but not tamoxifen is capable of antagonizing the effect of estrogen on amphetamine-stimulated efflux (Xiao et al., 2003). Notably, endoxifen and 4-hydroxytamoxifen are 100-fold more

potent in their actions at the estrogen receptors, relative to tamoxifen (Lien et al., 1991), but all of the compounds tested display IC_{50}s in the micromolar range for reduction of dopamine transporter function.

In conclusion, we demonstrated that two of tamoxifen's metabolites, endoxifen and 4-hydroxytamoxifen, asymmetrically inhibit dopamine transporter function. We also demonstrated the validity and identified some of the caveats of a cell based assay for further probing of this phenomenon. Finally, we conclusively demonstrated that the effects of tamoxifen and its metabolites on dopamine uptake and amphetamine-stimulated dopamine efflux occur independently of the compounds actions at the estrogen receptor.

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CHAPTER 4. DISCUSSION

This thesis demonstrates for the first time that the breast cancer therapeutic tamoxifen inhibits dopamine transporter function independently of its actions as a selective estrogen receptor modulator. Furthermore, I provide evidence supporting the characterization of tamoxifen as a new atypical dopamine uptake inhibitor.

In Chapter 2 I demonstrated that tamoxifen blocks both uptake and amphetamine-stimulated efflux of dopamine through the dopamine transporter. I demonstrated that tamoxifen alters the conformational equilibrium of the dopamine transporter, and that it appears to interact directly with the transporter at the putative S2 site of the transporter. *In vivo,* tamoxifen inhibited amphetamine-stimulated locomotor activity, yet exhibited no effects on basal locomotor activity. In Chapter 3, I demonstrated that the blockade of dopamine transporter function by tamoxifen occurs independently of its actions as a selective estrogen receptor modulator. I also characterized the effects of tamoxifen's chief metabolites on dopamine transporter function and noted discrepancies and similarities in their effects on uptake and efflux in cells and synaptosomes.

A direct interaction with the transporter?

Though the data presented here do not conclusively indicate a direct interaction between tamoxifen and the dopamine transporter, such an interaction seems the most likely explanation. Inhibition of dopamine uptake and efflux could occur through intracellular modulation of the regulatory pathways known to target the dopamine

transporter; however, we demonstrated that tamoxifen stabilizes an outward-facing conformation of the dopamine transporter. Previous work demonstrated that stabilization of the outward facing conformation of the dopamine transporter enhances [^{3}H]WIN 35,428 binding, yet we see a competitive decrease in [^{3}H]WIN 35,428 binding. I believe this strongly suggests that tamoxifen is directly interacting with the transporter to block [^{3}H]WIN 35,428 binding. Indeed, our collaboration with the laboratory of Dr. Maarten Reith, NYU, supports the hypothesis that tamoxifen is interacting with the so-called S2 allosteric binding site of the dopamine transporter. Mutation to alanine of aspartic acid 476 in the transporter, an important residue in the coordination of the S2 binding site, eliminates the ability of tamoxifen to inhibit dopamine uptake (Zhen and Reith, 2016). This suggests a direct interaction with the S2 binding site. However, previous work has indicated that compounds binding this allosteric site in the serotonin transporter (Chen et al., 2005) and in the dopamine transporter (Pariser et al., 2008) will slow dissociation of [^{3}H]*S*-citalopram or [^{3}H]WIN 35,428 from each respective transporter. This observation in the dopamine transporter is consistent with the position of the S2 site extracellular to the substrate S1 and [^{3}H]WIN 35,428 binding sites of the transporter. However, in a pilot study I conducted, I found that tamoxifen does not slow dissociation of [^{3}H]WIN binding (see Figure 4-1 for a more in depth explanation). Instead, tamoxifen appears to have a similar effect on [^{3}H]WIN 35,428 binding as the addition of a large concentration of unlabeled WIN 35,428. This would suggest that tamoxifen is interacting with the WIN 35,428 binding site of the dopamine transporter. This is consistent with my observation, reported in chapter 2, that tamoxifen competitively inhibits [^{3}H]WIN 35,428 binding. Though WIN 35,428 has long been thought to bind near the S1 site, newer computer modeling studies have

demonstrated a potential interaction between cocaine analogues (such as WIN 35,428) and the S2 site (Huang et al., 2009). The D476A and I159A dopamine transporter mutants exhibit a significantly different B_{max}, but not K_d, for [^{3}H]WIN 35,428 binding compared to wild type (Zhen and Reith, 2016). These observations, in addition to the results reported here, suggest that tamoxifen may be inhibiting [^{3}H]WIN 35,428 binding by competitive inhibition of the S2 site

Atypical dopamine uptake inhibitors

In this dissertation I present evidence that tamoxifen falls within the class of atypical dopamine uptake inhibitors. These compounds are capable of blocking the dopamine transporter, yet unlike a typical inhibitor such as cocaine, they fail to exhibit psychostimulant properties of their own. I found that tamoxifen fulfilled both of these characteristics. Similar to some atypical dopamine uptake inhibitors, tamoxifen also antagonized amphetamine-stimulated hyperactivity, and has been previously demonstrated to antagonize hyperactivity stimulated by treatment with the typical uptake blocker, methylphenidate (Pereira et al., 2011a).

In recent years, atypical uptake inhibitors have gained traction as potential therapeutics for the treatment of psychostimulant abuse (Tanda et al., 2009). Previous attempts to antagonize the effect of psychostimulants of the dopamine transporter have yielded compounds with similar abuse liability to cocaine and amphetamine. The atypical uptake inhibitors may prove an opportunity to antagonize psychostimulants without the risk of their own abuse potential.

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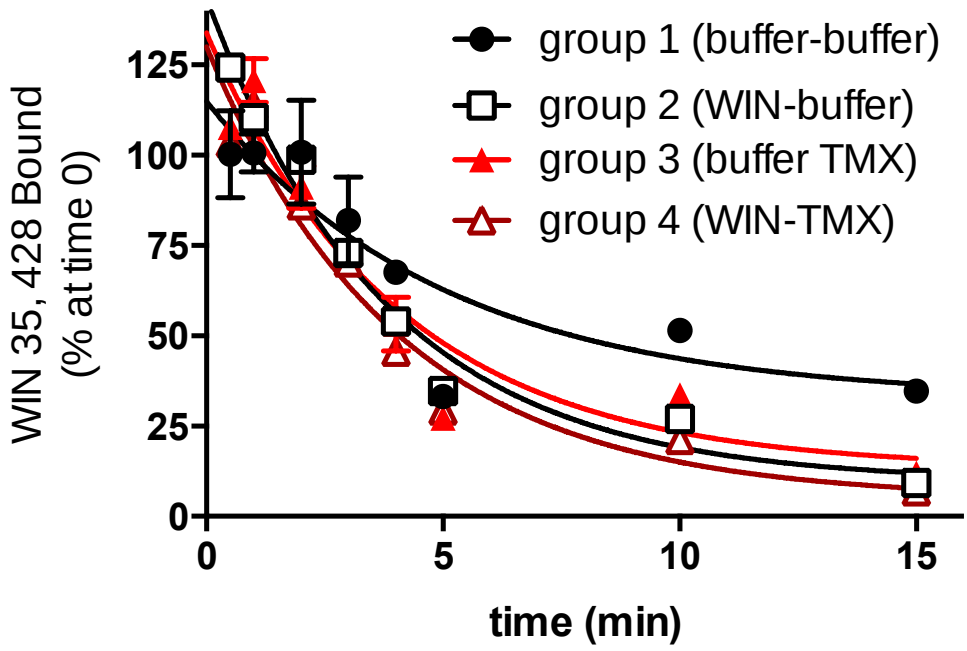

Figure 4-1. Preliminary Data. Effects of tamoxifen on dissociation of [^{3}H]WIN 35,428 from the dopamine transporter.
Rat striatal membranes were prepared as described in Chapter 2 and incubated with 10 nM [^{3}H]WIN 35,428 in binding assay buffer on ice for one hour, in order to allow binding to the dopamine transporter to reach equilibrium. To initiate dissociation, 20 µl of "complex solution" ([^{3}H]WIN 35,428 bound to striatal membranes) was added to 500 µl of dissociation treatment. The samples were divided up into four groups which differed by their dissociation treatment. Group 1: binding assay buffer with vehicle, Group 2: 10 µM unlabeled WIN 35,428, Group 3: 10 µM tamoxifen, Group 4: 10 µM unlabeled WIN 35,428 and 10 µM tamoxifen. This assay format has been used previously to probe the effects of substrate bound to the S2 site on dissociation of a centrally binding ligand (Pariser et al., 2008). The dissociation in group 1 is actually a combination of disassociation and reassociation, whereas in group 2, excess unlabeled WIN 35,428 competes with [^{3}H]WIN 35,428 for reassociation. In group 3, we see that tamoxifen affects dissociation similarly to group 2, indicating that tamoxifen is similarly preventing reassociation. Previously, drugs that interact with the S2 or allosteric site have been proposed to slow dissociation of a centrally binding ligand, but we do not see such an effect with tamoxifen. Group 4 looks no different from group 2 or 3, indicating that tamoxifen and WIN 35,428 are likely exerting their effects on [^{3}H]WIN 35,428 dissociation at the same site.

The unifying characteristics of the atypical uptake inhibitors are that they block the dopamine transporter, yet fail to exhibit one or more of the expected characteristics of a typical dopamine transporter blocker. A typical dopamine transporter blocker is expected to, at a high enough concentration:

1. fully inhibit DA uptake
2. fully inhibit the binding of another blocker
3. fully block the reverse transport of substrate.

Behaviorally, typical dopamine transporter blockers are expected to

4. stimulate locomotor behavior
5. exhibit reinforcing characteristics, indicating a potential for abuse in human users (Reith et al., 2015).

The atypical dopamine transporter blockers are united only in that they each fail to exhibit at least one, but usually more, of the above effects of typical blockers. As such, it is easy to imagine the huge level of diversity among the actions studied in these compounds, making them particularly difficult to discuss as a group. The most intriguing of the atypical uptake inhibitors have exhibited the additional characteristic of being able to antagonize the behavioral effects of psychostimulants, without exhibiting psychostimulant effects of their own. In order to simplify I will focus on one of the more successful atypical inhibitors tested thus far and draw comparisons to my results with tamoxifen.

The earliest atypical inhibitors to be subjects of extensive study and development were the benztropine analogs. These compounds possess the distinctive tropane group of cocaine with the addition of a diphenyl moiety (Reith et al., 2015). The compound with, perhaps, the most therapeutic potential that has been investigated to date is JHW007

(Figure 4-2, note the congruency between the diphenyl groups of JHW007 and tamoxifen). When first synthesized, JHW007 was found to have a K_i of ~25 nM for the dopamine transporter, with roughly 100-fold selectivity for the dopamine transporter over the norepinephrine and serotonin transporter. Its IC_{50} for inhibition of uptake (roughly 25 nM) was similar to its K_i (Agoston et al., 1997). JHW007 was demonstrated to associate slowly with the dopamine transporter. At doses of 1-10 mg/kg it failed to produce locomotor stimulation (Hiranita et al., 2009) or conditioned place preference in mice (Velazquez-Sanchez et al., 2010). Tamoxifen similarly failed to produce locomotor stimulation in rats. Unlike cocaine, there was no correlation between behavioral stimulation and dopamine transporter occupancy, as measured by *ex vivo* displacement of the dopamine transporter ligand [^{125}I]RTI-121. JHW007 also failed to maintain self-administration, indicating that it lacks the reinforcing characteristics of typical uptake inhibitors like cocaine (Hiranita et al., 2009). Pretreatment with 10 mg/kg JHW007 completely antagonized the effects of cocaine on locomotor activity. In cocaine discrimination, JHW007 shifted the dose effect curve of cocaine to the right, suggesting that JHW007 was competitively antagonizing cocaine (Desai et al., 2005). This observation was consistent with molecular modeling studies that revealed that the benztropine analogs bind to a site on the dopamine transporter that overlaps with those of cocaine and dopamine (Bisgaard et al., 2011). However, whereas pretreatment with a typical dopamine uptake inhibitor such as methylphenidate shifted the cocaine self-administration dose response curve to the left, pretreatment with JHW007 completely antagonized self-administration of cocaine, instead leading to a flattening of the dose response curve (Hiranita et al., 2009). JHW007 also dose dependently blocked self-administration of methamphetamine (Hiranita et al., 2014) and the development of

behavioral sensitization when administered concurrently with amphetamine (Velazquez-Sanchez et al., 2013). Comparatively, tamoxifen blocked amphetamine-stimulated hyperactivity.

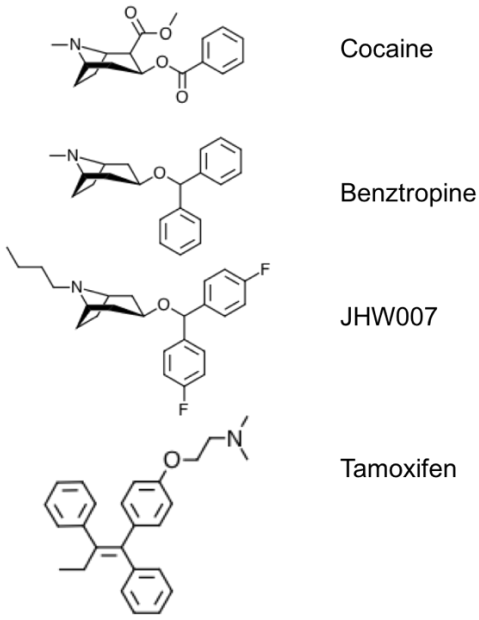

Figure 4-2. Inhibitors of dopamine uptake

Mechanism of action of atypical uptake inhibitors

Numerous mechanisms have been proposed as explanations for the differential nature of the atypical uptake inhibitors, yet for every explanation suggested, an exception is discovered. It was proposed that the lack of psychostimulant and reinforcing properties may be due to a slower onset of action. Indeed, slow infusion of cocaine is significantly less reinforcing in monkeys compared to a rapid infusion of the same dose (Woolverton and Wang, 2004) and several of the benztropine analogs exhibit a slow onset of action (Schmitt et al., 2013). However, fast-acting benztropine analogs, which rapidly block dopamine uptake *in vivo,* have been found that still lack the reinforcing properties of cocaine (Li et al.,

2011). One particularly intriguing hypothesis posited that the reinforcing effects of atypical inhibitors of uptake were directly related to the effect the inhibitor had on the dopamine transporter conformation. Loland et al. (2008) demonstrated a positive correlation between the degree to which an uptake inhibitor would substitute for cocaine in drug discrimination trials and that drug's preference to bind the outward facing conformation of the dopamine transporter. The atypical inhibitors, which exhibited minimal, if any, substitution for cocaine in drug discriminating rats, exhibited either no preference for the outward facing conformation of the transporter, or a preference for the inward-facing or occluded conformation. This led to an examination of the intriguing "transceptor" hypothesis (Schmitt et al., 2013) first introduced in nutrient transporters (Kriel et al., 2011), in which the conformation of the transporter may be capable of translocating a signal across the membrane in the manner of a membrane receptor and independently of its function as a transporter. Though this hypothesis still has merit with some atypical uptake inhibitors, the recent discovery of atypical uptake inhibitors which stabilize the outward facing conformation of the dopamine transporter have precluded the possibility of this hypothesis as one which encompasses all atypical uptake blockers. As we demonstrated, tamoxifen stabilizes the outward facing conformation of the dopamine transporter, and thus disagrees with the conformation hypothesis of atypicality. Additional explanations have been posited regarding off-target effects observed in many of the atypical inhibitors, including antagonism of M1 muscarinic receptors, antagonism of H1 histamine receptors, agonism of dopamine D2 receptors, and allosteric modulation of cannabinoid 1 (CB1) receptors. The muscarinic hypothesis was dismissed with the observation that pure anticholinergics, both general and selective for the M1 receptor

actually potentiated the effects of cocaine, in direct disagreement with the hypothesis that antagonism at M1 may be masking the reinforcing effects of the atypical dopamine uptake inhibitors (Tanda et al., 2007). Antagonism of H1 was similarly dismissed with the observation that H1 antagonists failed to modify the subjective effects of cocaine (Campbell et al., 2005). Modulation of D2 and CB1 receptors were dismissed as viable hypotheses for the atypicality of the benztropine analogs because JHW007 could antagonize the effect of cocaine-stimulated locomotor activity in CB1 and D2 knockout mice (Desai et al., 2014).

The most promising of the hypotheses set forward have concerned antagonism of the sigma receptors. The sigma receptors are small intracellular chaperone like proteins, and a great deal remains unknown about them (Katz et al., 2016). Antagonists of the sigma receptors have demonstrated efficacy in blocking some of the behavioral effects of cocaine, and cocaine is known to bind the sigma receptors (Sharkey et al., 1988; Matsumoto et al., 2003). Several atypical dopamine transporter inhibitors that successfully antagonize the effects of cocaine also antagonize the sigma receptor (Hiranita et al., 2011). Combinations of dopamine transporter inhibitors with sigma antagonists have proven effective against the self-administration of both cocaine (Hiranita et al., 2011) and methamphetamine (Hiranita et al., 2014). It is worth noting that a screen performed by the NIMH-funded Psychoactive Drug Screen Program at the University of North Carolina showed that tamoxifen binds the sigma 1 and sigma 2 receptors with affinities of ~200 nM and ~30 nM, respectively (data not shown). It is unknown whether tamoxifen is acting as a sigma antagonist or agonist. However, not all of the atypical inhibitors bind the sigma receptor, so, once again, this hypothesis is incapable of explaining atypical inhibition of uptake as a whole (Hiranita et al., 2014). At this point, it seems unlikely that there will be one over-

arching mechanism which unites all atypical dopamine uptake inhibitors, but rather several different hypotheses, including slow-onset, the transceptor hypothesis, and antagonism of the sigma receptor, which eventually explain the diverse actions of the atypical uptake inhibitors.

Tamoxifen as a protein kinase C inhibitor

The data presented in this dissertation focus on an interaction between tamoxifen (or its metabolites) and the dopamine transporter. However, our original hypothesis when we began working with tamoxifen pertained to its ability to inhibit protein kinase C. Tamoxifen is the only known brain permeable protein kinase C inhibitor. We have found that tamoxifen inhibits protein kinase C with micromolar potency in N2A cells.

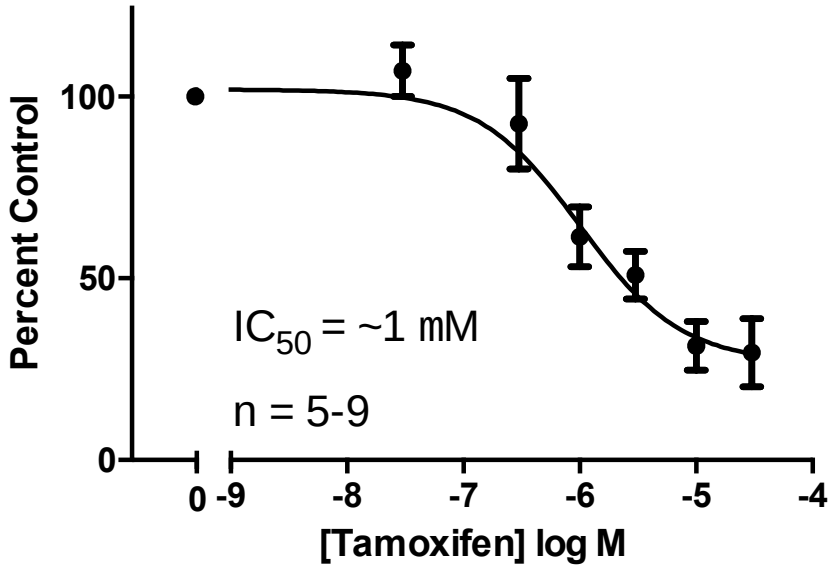

Figure 4-3. Tamoxifen inhibition PMA-stimulated phosphorylation of the protein kinase C substrate GAP43.
N2A cells were incubated with tamoxifen for 1 hour prior to stimulation of protein kinase C activity with 20 nM of the phorbol ester and protein kinase C activator, PMA for 5 min at 37 °C. Phospho-ser40-GAP43 was detected by western blotting and quantified. Data are represented as mean ± S.E.M.

We have demonstrated previously, and extensively, that inhibition of protein kinase C (particularly the β isoform) attenuates amphetamine-stimulated dopamine efflux, without altering normal dopamine uptake (Kantor and Gnegy, 1998; Johnson et al., 2005; Zestos et al., 2016).

Ample evidence exists supporting the therapeutic potential of tamoxifen as a protein kinase C inhibitor. Post-mortem brain tissue from patients with bipolar mania have elevated protein kinase C activity (Wang and Friedman, 1996) and patients experiencing a manic episode exhibit elevated protein kinase C activity in their platelets (Friedman et al., 1993; Wang et al., 1999). Preclinically, activation of protein kinase C with the phorbol ester PMA enhances risk taking behavior in rats (a mania-like behavioral marker)(Abrial et al., 2013). Tamoxifen treatment has proven effective in multiple preclinical models of bipolar mania (Armani et al., 2014). The data presented in this dissertation may render questionable those results performed with models which are dependent on pharmacological modulation of the dopamine transporter. However, tamoxifen successfully inhibited the paradoxical sleep-deprivation model of mania as well as apomorphine-induced stereotypy (Pereira et al., 2011b; Armani et al., 2012; Abrial et al., 2015), both models that should be independent of the dopamine transporter. Furthermore, *in vivo* treatment with tamoxifen effectively blocked amphetamine-stimulated phosphorylation of GAP-43 in rat striata, demonstrating that tamoxifen was inhibiting protein kinase C in these animals (Einat et al., 2007). Of course, based on our results, it is possible that the inhibition of protein kinase C activity *in vivo* is the result of a blockade of amphetamine action. However, we know from our work in cells that tamoxifen inhibits protein kinase C independently of any action on the dopamine transporter (Figure 4-3). Beyond the

preclinical models, tamoxifen has been used successfully in several clinical trials for the treatment of bipolar mania (Bebchuk et al., 2000; Zarate et al., 2007; Yildiz et al., 2008; Amrollahi et al., 2011; Yildiz et al., 2016). Preclinical studies have also compared tamoxifen with other antiestrogens to demonstrate that tamoxifen's success in treating mania is independent of its SERM activity (Pereira et al., 2011a).

Therapeutic potential of a protein kinase C inhibitor – Beyond mania

Inhibition of protein kinase C has therapeutic potential beyond the treatment of mania. As discussed, protein kinase C plays an important role in amphetamine-stimulated dopamine efflux. Protein kinase C inhibition decreases amphetamine-stimulated dopamine efflux *in vivo* (Zestos et al., 2016) and *in vitro* (Kantor and Gnegy, 1998; Johnson et al., 2005), attenuates amphetamine-stimulated locomotor activity *in vivo* (Browman et al., 1998; Zestos et al., 2016), and prevents the development of conditioned place preference to amphetamine (Aujla and Beninger, 2003).

An additional potential mechanism for protein kinase C inhibition in the treatment of psychostimulant abuse lies in protein kinase C regulation of the dopamine autoreceptor. The dopamine autoreceptor, which is part of the D2 family of dopamine receptors, resides extrasynaptically on the presynaptic neuron and provides a negative feedback mechanism for the reduction of exocytotic dopamine release. Though relatively few studies have investigated the effects of psychostimulant abuse on dopamine autoreceptors, novelty seeking and impulsivity, both predictors for susceptibility to drug abuse (Bardo et al., 1996; Weafer et al., 2014), are associated with lower levels of dopamine autoreceptor (Zald et al., 2008; Buckholtz et al., 2010). Amphetamine self-administration in rats causes a decrease in dopamine D2 receptor (autoreceptors) function in the midbrain dopamine cells (Calipari et

al., 2014). Preclinical data in rat models of psychostimulant abuse and relapse suggest that some D2/D3 partial agonists may translate to the clinic as aids in maintenance of abstinence in recovering addicts, potentially through activation of dopamine autoreceptors (Neisewander et al., 2014). Alternatively, a therapeutic which enhanced autoreceptor signaling or prevented desensitization might be able to serve a similar role. The Gnegy lab has found that protein kinase C beta inhibition enhances the function of the dopamine autoreceptor by increasing the surface levels of the D2 dopamine receptor (Luderman et al., 2015). With this in mind, a protein kinase C inhibitor might be able to serve the same role as a D2 partial agonist.

Though the focus of the Gnegy lab has been on presynaptic regulation of extracellular dopamine levels, others have identified important roles for protein kinase C in the post synaptic response to the dopamine signal, particularly with regards to cocaine abuse. Protein kinase C zeta/lambda phosphorylation (a marker of activation) is increased in rats exhibiting cocaine-induced behavioral sensitization (Chen et al., 2007). Intracerebral injection of protein kinase C inhibitors blocks cocaine seeking (Schmidt et al., 2013; Ortinski et al., 2015; Schmidt et al., 2015), the development of conditioned place preference for cocaine, (Cervo et al., 1997), and the expression of behavioral sensitization to cocaine (Pierce et al., 1998). With the above data in hand, it indeed appears that inhibition of protein kinase C may be a viable treatment for psychostimulant abuse.

Future Directions

Tamoxifen, protein kinase C, and the dopamine transporter

Within this body of work, I was unable to further investigate the potential role for protein kinase C inhibition in tamoxifen's effects on the dopaminergic system, but several

pilot studies I conducted suggest a potential dual mechanism for tamoxifen, with regards to the dopamine transporter. As revealed in Chapter 3, we find that suprafusion-based efflux assays are more capable of demonstrating the effects of protein kinase C inhibition on amphetamine-stimulated dopamine efflux than are plate-based efflux assays. Whether this discrepancy is the result of a difference in model (rat tissue vs cultured cells) or assay (suprafusion vs plate) remains unclear.

My pilot studies indicate that tamoxifen is capable of inhibiting protein kinase C-mediated phosphorylation of GAP-43 with only 10 minutes' pretreatment (data not shown). In this vein, pretreatment with 10 µM tamoxifen for 6 minutes prior to initiation of efflux in a suprafusion assay was sufficient to create a robust reduction in amphetamine-stimulated dopamine efflux (treatment: $F(1, 4) = 38.23$; time: $F(19, 76) = 174.2$; interaction: $F(19, 76) = =25.62$).

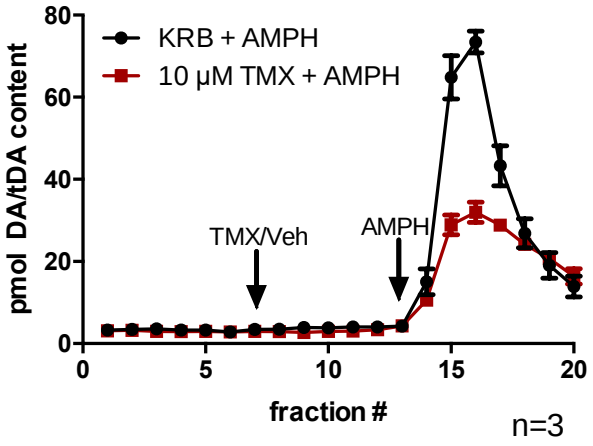

Figure 4-4. Tamoxifen rapidly inhibits amphetamine-stimulated dopamine efflux in rat striatal synaptosomes.
The suprafusion assay was conducted as described in chapters 2 and 3 with the exception that 10 µM tamoxifen/vehicle was added to the wash buffer at fraction 7 and 10 µM amphetamine was added at fractions 13 and 14, as indicated by the arrows. Results are expressed mean ± S.E.M. RM two-way ANOVA indicates $p<0.01$ for treatment, $p<0.0001$ for time, $p<0.0001$ for interaction. N=3.

If we quantify baseline dopamine release from synaptosomes over the hour following the addition of tamoxifen to the suprafusion buffer, we see a slow but steady increase in baseline dopamine levels that is calcium dependent, consistent with a slow-onset blockade of dopamine transport (Figure 4-5). Within 1-hour of initiating tamoxifen treatment, this increase in basal dopamine release returns to those of untreated synaptosomes, possibly due to negative feedback inhibition of dopamine release by the D2-like autoreceptor.

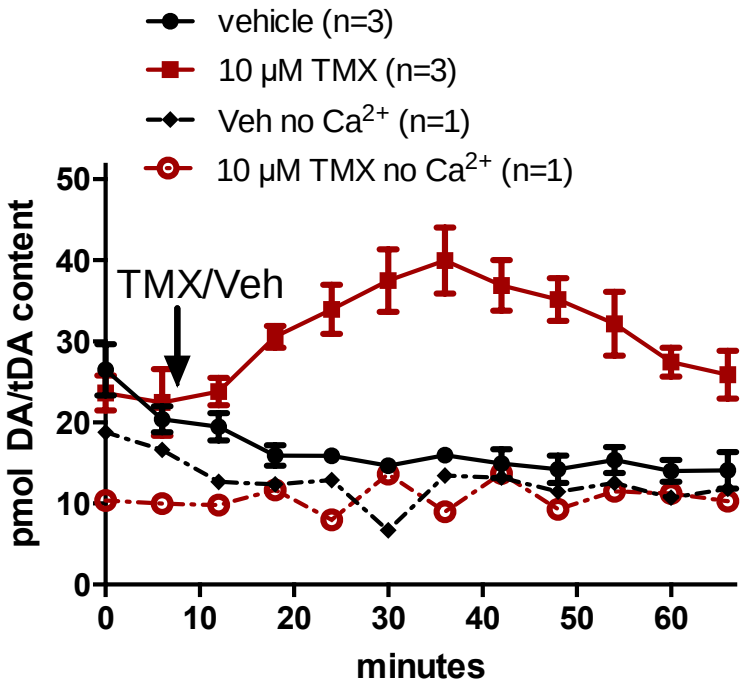

Figure 4-5. Basal dopamine release during treatment with 10 μM tamoxifen.
Synaptosomes were perfused on a Brandel suprafusion apparatus with normal Kreb's Ringer Buffer or with buffer lacking Ca^{2+} at 800 μl/min. 10 μM tamoxifen or vehicle was added at 5 minutes and the synaptosomes were perfused for an hour. Every 6 minutes, a 2-minute fraction was collected for quantification of dopamine in the perfusate via HPLC couple to electrochemical detection. Tamoxifen significantly increased dopamine in the perfusate. This increase was blocked by the removal of calcium from the buffer, suggesting that the increase in extracellular dopamine was due to exocytotic dopamine release rather than reverse transport through the dopamine transporter. These results are consistent with a blockade of dopamine uptake.

Though we were unable to further investigate the phenomena shown here, I hypothesize that we are seeing a two-fold modulation of the dopamine transporter by tamoxifen. Tamoxifen rapidly inhibits protein kinase C, allowing for the attenuation of amphetamine-stimulated dopamine efflux that we see after only 10 minutes' pre-treatment, then as tamoxifen slowly associates with the dopamine transporter, we see a steady increase in dopamine in the perfusate as uptake is blocked. When efflux is stimulated with amphetamine following an hour treatment with tamoxifen we are likely seeing the results of a combination of protein kinase C inhibition and direct blockade of the dopamine transporter.

To a certain degree this hypothesis is even supported by our results in the cell based assay: we found that a one-hour incubation was absolutely necessary in order to see a reduction in dopamine uptake and amphetamine-stimulated efflux. A ten-minute incubation with tamoxifen yielded only a weak inhibition of uptake or amphetamine-stimulated efflux.

These results support the hypothesis that the direct effects of tamoxifen on the dopamine transporter have a slow onset and are consistent with the slow increase in baseline I saw in the suprafusion assay. Additionally, according to the above hypothesis, at this point in time protein kinase C inhibition would be the primary mechanism by which tamoxifen was affecting the dopamine transporter, and as I have discussed, efflux in the plate-based assay is resistant to effects from protein kinase C inhibition. Further studies and replication of the above preliminary data will be necessary in order to pull apart tamoxifen's actions on protein kinase C and its effects on the dopamine transporter, but

such studies will need to be done in a suprafusion based assay of amphetamine-stimulated dopamine efflux.

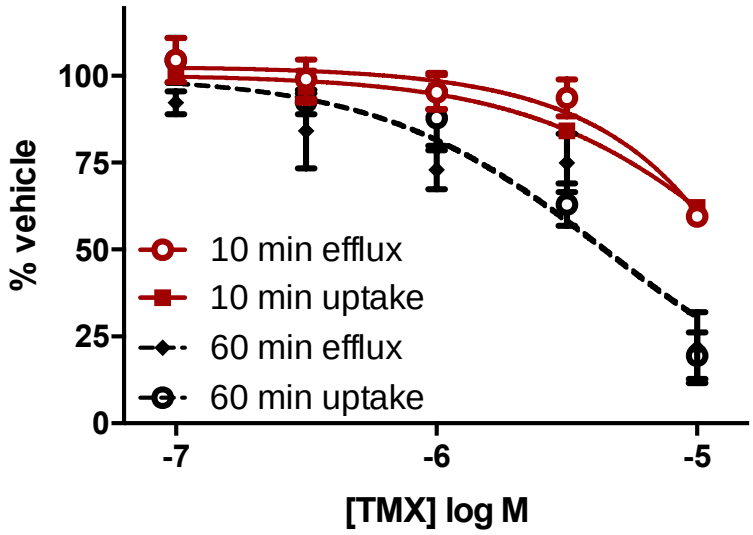

Figure 4-6. Tamoxifen inhibition of uptake and amphetamine-stimulated efflux in hDAT-N2A cells after only 10 minutes pretreatment.
The experiment was carried out identically to those described in chapter 3 with the following exceptions: 1) in the uptake assay, tamoxifen was added only 10 minutes prior to the initiation of uptake; 2) in the efflux assay, following five 10 minute washes, tamoxifen was added in the last wash prior to stimulation of efflux with 20 μM amphetamine. Shown for comparison, uptake and efflux following 60 min pretreatment, from figure 3-3a (dashed line).

Time dependence of tamoxifen action in vivo

One intriguing phenomena that we were unable to sufficiently explain is the observation that an extended pretreatment time with tamoxifen was necessary in order for us to observe an attenuations of amphetamine-stimulated hyperactivity (data not shown). It is, as yet, unclear whether this is the result of a pharmacodynamic or a pharmacokinetic characteristic of the drug. One hypothesis was that the extended pretreatment was necessary in order for sufficient levels of tamoxifen or one of its metabolites to reach appreciable concentration in the brain. However, one microdialysis study demonstrated

that a systemic injection of tamoxifen induced a slight but significant increase in extracellular dopamine levels in the striatum within an hour of administration, indicating that sufficient brain concentrations were being reached within that time frame for tamoxifen to be affecting the dopamine transporter (Chaurasia et al., 1998). If such concentrations are being reached in the brain so soon after administration, it seems that brain penetrance is an insufficient explanation for the delayed *in vivo* effect.

Though we have found that tamoxifen inhibits the dopamine transporter independently of its action as a selective estrogen receptor modulator, we have not confirmed that this effect on dopamine transport is also the reason for the blockade of amphetamine action that we see in vivo. It is possible that tamoxifen is affecting amphetamine-mediated behaviors through its actions as a selective estrogen receptor modulator. Indeed, the delayed onset of the genomic effects of estrogen receptor modulation would seem to fit with this timeline. This problem may be able to be probed more in depth with estrogen receptor knockout mice. A similar but alternative hypothesis is that one of tamoxifen's other mechanisms of action, such as protein kinase C inhibition, is acting in a manner that requires complex downstream changes before an effect can be seen.

Effects on psychostimulant-mediated behaviors

We have provided evidence that tamoxifen will block the dopamine transporter and, indeed, tamoxifen appears to be behaving as an atypical inhibitor of dopamine uptake. I have already demonstrated that tamoxifen antagonizes amphetamine-stimulated hyperactivity, but we do not know how it will affect the reinforcing characteristic of amphetamine, or whether it will impact the behavioral effects of cocaine. Future studies

with tamoxifen will aim to determine whether tamoxifen, like other atypical uptake inhibitors, is capable of antagonizing the effects of psychostimulants.

4-hydroxytamoxifen and endoxifen

We demonstrated that 4-hydroxytamoxifen and endoxifen differentially modulate dopamine uptake and efflux, depending on the system being observed. 4-hydroxytamoxifen was significantly more efficacious as an inhibitor of dopamine uptake than as an inhibitor of amphetamine-stimulated dopamine efflux in synaptosomes. The reasons for this asymmetry in the modulation of the transporter remain unclear. It is possible that 4-hydroxytamoxifen is interacting with the transporter in a way that asymmetrically regulates transporter function, or that it is acting intracellularly to affect one of the many regulatory pathways known to act on the dopamine transporter. No such difference in the effects of 4-hydroxytamoxifen on the dopamine transporter were seen in hDAT-N2A cells, but due to the above discussed caveats of the plated cell assay, it is difficult to draw any hypotheses concerning a difference between cells and synaptosomes.

Endoxifen, on the other hand, was a much more potent inhibitor of amphetamine-stimulated dopamine efflux than of dopamine uptake in cells. Future work will aim to determine whether endoxifen is similarly effective at inhibiting amphetamine-stimulated dopamine efflux in synaptosomes. Inhibition of protein kinase C presents an attractive hypothesis for the effect of endoxifen on amphetamine-stimulated dopamine efflux, however, this asymmetry was seen in the plated cell assay, which, as I have discussed, fails to fully demonstrate an effect of protein kinase C on amphetamine-stimulated dopamine efflux. Thus it seems unlikely that the effects of endoxifen on amphetamine-stimulated dopamine efflux in this assay are the result of inhibition of protein kinase C. With this in

mind, it may be that endoxifen is the opposite side of the same coin as 4-hydroxytamoxifen, and is interacting with the transporter to asymmetrically affect transporter function, but in a manner converse to what is seen with tamoxifen. More in depth studies of the effects of these two compounds on dopamine transporter function, conformation, and WIN binding, as were already carried out with tamoxifen here, may provide additional insight into these compounds mechanisms of action.

The tamoxifen scaffold in drug design

In its capacity as both a protein kinase C inhibitor and an atypical dopamine transporter blocker, the tamoxifen structure exhibits enormous therapeutic potential for the treatment of diseases of dopaminergic dysfunction. The development of a similar drug that lacks the selective estrogen receptor modulating activity of tamoxifen promises to provide the advantages of a protein kinase C inhibitor and atypical uptake inhibitor, without the negative estrogenic effects attributed to tamoxifen. Interestingly, tamoxifen appears to inhibit protein kinase C through a different mechanism than the typical bisindoylmaleimide protein kinase C inhibitors. Tamoxifen inhibits protein kinase C through the regulatory domain of the enzyme, competing with binding for phosphatidylserine (O'Brian et al., 1990). The bisindoylmaleimide inhibitors (staurosporine-based compounds) act competitively at the ATP binding site of the enzyme. As the regulatory site is less tightly conserved across isoforms, the design of a protein kinase C inhibitor based on the tamoxifen structure may allow for increased isoform selectivity, a goal elusive thus far in the development of protein kinase C inhibitors (with the exception of the □ isoform). Though we have treated tamoxifen as a general protein kinase C inhibitor in our use of it so far, there is some indication that it may selectively

inhibit those isoforms which are dependent phospholipids and calcium for activation (Su et al., 1985).

Thanks in part to a thorough characterization of the structure activity relationships between tamoxifen's various moieties and their effects on estrogen receptor binding, protein kinase C inhibition and calmodulin binding (de Medina et al., 2004), we have already had some success in the development of such a compound. CCG215103 is a tamoxifen analogue currently under investigation in our lab which exhibits no binding to the estrogen receptor at concentrations up to 10 μM, has an improved potency for inhibition of protein kinase C relative to tamoxifen, and has been seen to successfully attenuate amphetamine-stimulated locomotor activity and self-administration (Carpenter et al. in preparation).

Conclusions

I demonstrated that tamoxifen inhibits dopamine uptake and amphetamine-stimulated dopamine efflux through the dopamine receptor in a non-competitive manner, and competitively inhibits binding of the selective dopamine transporter ligand WIN 35,428. Combined with my observation that tamoxifen antagonizes amphetamine-stimulated hyperactivity, yet exhibits no stimulating properties of our own, I make the argument that tamoxifen falls within the class of atypical dopamine uptake inhibitors. Additionally, I demonstrated that two of tamoxifen's active metabolites exhibit similar actions at the dopamine transporter, though these two compounds appear to asymmetrically regulate dopamine uptake vs. amphetamine-stimulated dopamine efflux. I also found that while a plated cell-based model of dopamine transporter function is useful in the characterization of these compounds' effect directly on dopamine transporter

function, it is insufficient to identify potential indirect methods of regulation of the dopamine transporter such as protein kinase C inhibition.

My results provide insight into some of the previous observations made concerning tamoxifen and the dopaminergic system. More significantly, my results provide valuable ground work for a targeted drug design program that has already had some success in preclinical models of drug abuse. Additionally, I demonstrate a potential opportunity for the therapeutic repurposing of a drug with a well-known safety profile for the treatment of psychostimulant abuse, a widespread condition with a paucity of therapeutic options.

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