PAYING ATTENTION TO THE DETAILS: RARE GENETIC VARIATION IN THE DOPAMINE TRANSPORTER AND ADHD

By

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confidence is my abilities have helped me push forward and finish this process with confidence.

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LIST OF ABBREVIATIONS

5-HT-5-Hydroxytryptamine

AADC-Aromatic acid decarboxylase

AChE- Acetylcholineesterase

ADE-Anomalous dopamine efflux

ADHD-Attention-deficit hyperactivity disorder

Akt-Protein kinase B

AMPH-Amphetamine

BPD-Bipolar disorder

CaMKII-Calcium/calmodulin-dependent protein kinase II

CK2-Casein kinase 2

COMT Catechol-*O*-methyl transferase

CTxB- Cholera toxin B subunit

DA- Dopamine

DAergic- Dopaminergic

DAT- Dopamine transporter/human dopamine transporter

DIP- Dopamine transporter interacting protein

EL- Extracellular loop

ERK- Extracellular signal-regulated kinase

fMRI- Functional magnetic resonance imaging

GABA- gamma-aminobutyric acid

GAT1- GABA transporter 1

GLUT- Glucose transporter

GLYT1- Glycine transporter 1

GPCR- G protein-coupled receptor

HPLC- High-pressure liquid chromatography

ICQ- Intensity correlation quotient

IPD- Infantile Parkinsonian Dystonia

KO- knock-out

$LeuT_{Aa}$- *Aquifex aeolicus* Leucine Transporter

MAO- Monoamine oxidase

MAPK- Mitogen-activated protein kinase

METH -Methamphetamine

mGluR- metabotropic glutamate receptor

mPFC- medial prefrontal cortex

nAChR- Nicotinic acetylcholine receptor

NE- Norepinephrine

NET- Norepinephrine transporter

NO- Nitric oxide

NOS- Nitric oxide synthase

PET- Positron emission tomography

PI3K- Phosphatidylinositol 3-kinase

PICK1- Protein interacting with C kinase I

PKA- Protein Kinase A

PKC- Protein Kinase C

PMA- Phorbol 12-myristate 13-acetate

RACK1- Receptor for activated C kinase

SERT- Serotonin transporter

SN- Substantia Nigra

SNAP-25- Synaptosomal-associated protein 25

SNP- Single nucleotide polymorphism

TAAR- Trace amine-associated receptor

TGCE- Temperature gradient capillary electrophoresis

TH- Tyrosine hydroxylase

TMD- Transmembrane domain

VMAT- Vesicular monoamine transporter

VNTR- Variable number tandem repteats

VTA- Ventral tagmental area

WT- Wild type

Y2H- Yeast 2-hybrid

CHAPTER I

INTRODUCTION

Overview of Dopamine and the Dopaminergic System

The neurotransmitter dopamine (3,4-dihydroxyphenethylamine, DA) makes a significant contribution to brain function through its modulatory role in pathways controlling reward, locomotor activity, and attention (Carlsson, 1987, Robbins, 2003). DA is synthesized in a two-step process involving first, the hydroxylation of tyrosine by tyrosine hydroxylase (TH) to yield L-dihydroxyphenylalanine (L-DOPA) that is subsequently decarboxylated by L-aromatic amino acid decarboxylase (AADC) to yield DA. The action of TH is rate-limiting in this reaction due to the higher turnover rate and lack of substrate saturation of AADC. In pathways that utilize DA as a neurotransmitter, DA is packaged for release or processed further intracellularly to synaptically inactive metabolites. DA is packaged for release into synaptic vesicles using vesicular monoamine transporter-2 (VMAT-2). DA is transported into the synaptic vesicles using proton gradient present across the synaptic vesicle membrane. VMAT-2 function and expression is often altered by use of psychostimulants (Little et al., 2003). Another VMAT isoform, VMAT-1, is mostly associated with large secretary granule vesicles, whereas VMAT-2 is predominantly present on small synaptic vesicles (Henry et al., 1994). In most brain regions, including striatum, free

intracellular DA is enzymatically degraded by isoforms of monoamine oxidase (MAO) into 3,4-dihydroxyphenylacetic acid (Jonason, 1969). In the prefrontal cortex, however, enzymatic breakdown of DA occurs by catechol-*O*-methyl transferase (COMT) into 3-methoxytyramine (Yavich et al., 2007). Other brain and peripheral pathways use DA as a precursor to produce norepinephrine (NE) and epinephrine. Synthesis and degradation of DA is summarized in Figure 1.

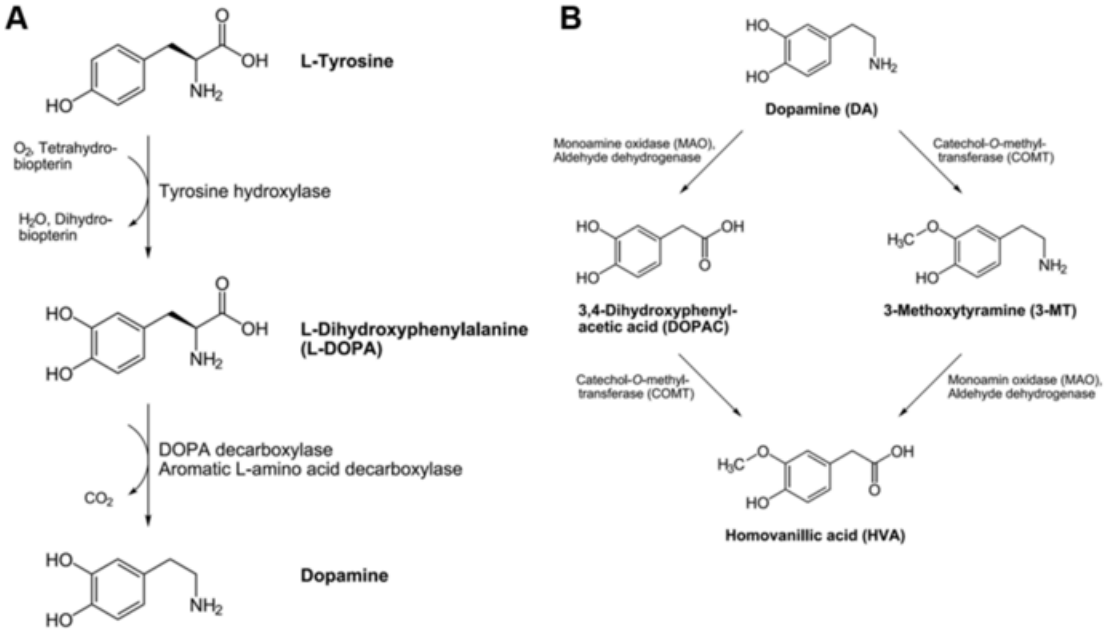


Figure 1 Biosynthesis and Degradation of Dopamine: (A) Biosynthesis of DA from the actions of enzymes, tyrosine hydroxylase, and dihydroxyphenylanine (DOPA) decarboxylase (also known as AADC due to its lack of specificity for DA). **(B)** Degradation of DA by monoamine oxidase and catechol-*O*-methyl transferase.

Four major brain pathways utilize DA as a neurotransmitter: the tuberoinfundibular pathway, the nigrostriatal pathway, the mesocortical pathway, and the mesolimbic pathway (Figure 2). The tuberoinfundibular pathway originates from of neurons in the hypothalamic arcuate nucleus and projects to

the median eminence, where DA secretion produces prolactin secretion from the anterior pituitary gland. The nigrostriatal pathway, which modulates movement, and that is lost in Parkinson's disease, originates in neurons of the substantia nigra (SN), so named for their pigmented appearance in unstained sections.

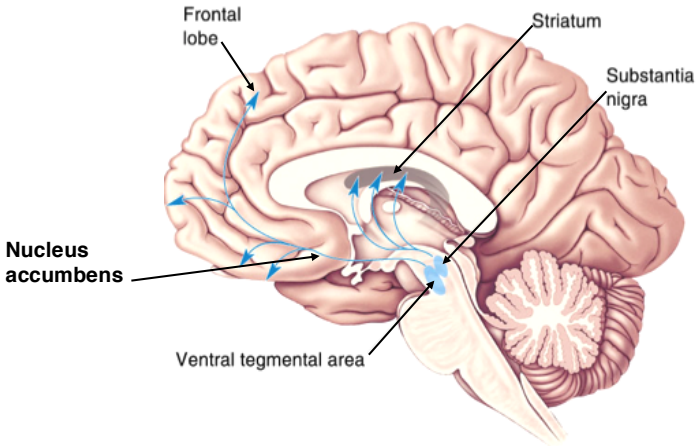


Figure 2 Schematic Illustration of Dopaminergic Projections: DA neurons in the VTA project to the nucleus accumbens and prefrontal cortex through the mesolimbic and mesocortical pathways, respectively. DA neurons in the SN project to the striatum via the nigrostriatal pathway. The tuberoinfundibular pathway is not depicted. Adapted from Neuroanatomy, An Atlas of Structures, Sections, and Systems

Dopaminergic (DAergic) axons from the SN terminate in nuclei of the basal ganglia, termed the striatum in rodents, but divided into the caudate nuclei and putamen in primates. Both the mesocortical and the mesolimbic pathways originate in the ventral tagmental area (VTA), projecting to the prefrontal cortex and nucleus accumbens, respectively. The mesocortical pathway is important in the modulation of motivation and emotion, whereas the mesolimbic pathway is implicated in reward and pleasure.

Due to the actions of DA in the regulation of motor and cognitive function, as well as the recognition of incentive salience and reward, alterations in DAergic tone can lead to multiple neurological and psychiatric disorders. These include Parkinson's disease (Chase et al., 1998), dystonia (Kurian et al., 2009), attention-deficit hyperactivity disorder (ADHD) (Mazei-Robison et al., 2005), addiction (Ritz et al., 1987), and schizophrenia (Horn and Snyder, 1971).

The first evidence for altered DA neurotransmission in a brain disorder involved the demonstration that rats given reserpine displayed features of Parkinson's disease (Carlsson et al., 1966), findings that would ultimately bring Arvid Carlsson the Nobel Prize in 2000. Subsequently, Hornykiewicz established a loss of DA in the brains of Parkinson disease subjects (Ehringer and Hornykiewicz, 1960). The reversal of Parkinsonian symptoms by the DA precursor, L-DOPA, provided clear evidence of the role of DA in the disorder (Birkmayer and Hornykiewicz, 1962). Hornykiewicz would go on to show that Parkinson's disease arises from degeneration of nigrostriatal DA neurons, resulting in denervation of basal ganglia that form the extrapyramidal motor system (Hornykiewicz, 1972).

Although Parkinson's disease patients, particularly in the latter stages, often exhibit cognitive and emotional problems, the idea that DAergic dysfunction is involved in psychiatric disorders derives in large measure from research demonstrating altered DA metabolism and signaling in patients with schizophrenia (Hokfelt et al., 1974, Lindvall et al., 1974). Indeed, classical

antipsychotic drugs, including chlorpromazine and haloperidol, block D2 subtype DA receptors, thus suggesting a role for hyperdopaminergic signaling (Horn and Snyder, 1971). Another strong evidence of altered DAergic neurotransmission contributing to a neuropsychiatric disorder came from ADHD patients. ADHD is the most commonly diagnosed childhood disorder affecting 3-5% of school age children. Multiple studies point to a contribution of variation in genes expressed in DAergic neurons as influencing risk for ADHD (Gill et al., 1997, Qian et al., 2003, Bobb et al., 2005, Mazei-Robison et al., 2005). I will discuss relationship between ADHD and DAergic transmission later in this chapter. Whereas L-DOPA and chlorpromazine have therapeutic efficacy, other drugs targeting DA signaling can be highly addictive, including the psychostimulants cocaine and amphetamine (AMPH), Long-term use of these agents produces molecular, cellular and circuit-level plasticities that drive a desire and search for drug, despite the recognition of negative consequences (Kauer and Malenka, 2007, Dietz et al., 2009). Taken together, alterations in DA signaling can be seen to underlie multiple, devastating disorders that affect tens of millions of individuals worldwide. My thesis research derives from the belief that a better understanding of DA signaling mechanisms, and how these mechanisms are perturbed by disease states, can lead to advances in the prevention, diagnosis and therapy.

Discovery of Presynaptic DA Transport and Demonstration of Specific DA Transporter (DAT) Binding Sites

Acetylcholine, the first identified neurotransmitter, is inactivated by degradation via the extracellular enzyme acetylcholinesterase (AChE). Although metabolism of the catecholamine NE by MAO and COMT to inactive products has been established, acute inhibition of these enzymes does not impact synaptic DA inactivation. Axelrod and colleagues discovered that radiolabeled NE could be sequestered into the terminals of sympathetic neurons (Hertting and Axelrod, 1961), and subsequently demonstrated that NE accumulation could be blocked by both antidepressants and psychostimulants (Axelrod et al., 1961, Hertting et al., 1961). These studies lead to the hypothesis that synaptic NE inactivation was mediated by rapid clearance through a presynaptic uptake process (Axelrod, 1971). Later, investigators would demonstrate that brain slices could accumulate both NE and DA and that psychostimulants could inhibit this process (Carlsson et al., 1966, Glowinski and Axelrod, 1966, Ross and Renyi, 1967). Subsequently, Ross and Renyi demonstrated that the pharmacological profiles of DA uptake inhibition in striatum and cortex were distinct from that of NE uptake inhibition (Ross and Renyi, 1967). They demonstrated that whereas AMPH and cocaine were potent inhibitors of DA and NE uptake in both brain regions, desipramine was a weak inhibitor of DA uptake in striatum, but a potent inhibitor of NE in cortex (Ross and Renyi, 1967). These findings suggested that the DA uptake mechanism was distinct from that supporting NE clearance,

contributing further to the idea that DA was not simply a precursor for NE but also a neurotransmitter in its own right. Snyder and Coyle would go on to demonstrate that the specificities of the DA and NE uptake processes for isomers of AMPH were distinct (DA uptake equivalently inhibited by D- and L-AMPH, NE uptake preferentially inhibited by D-AMPH) (Snyder and Coyle, 1969). Ritz and coworkers provided further dissociation of the actions of cocaine on DA and NE uptake sites by demonstrating cocaine's potency to inhibit DA uptake in rat brain extracts was significantly higher than cocaine's potency for inhibiting NE uptake (Ritz et al., 1987). Ritz and colleagues also reported important *in vitro* findings that the antagonist potency in blocking brain DA uptake correlated well with the potency of cocaine determined from self-administration studies with non-human primates (Ritz et al., 1987), further strengthening the hypothesis that the abuse liability of the psychostimulant derived from an inhibition of DA transport and inactivation.

The studies described above prompted efforts to identify the DA transporter, first by demonstrating the presence of specific cocaine binding sites in brain and their relationship to the DA reuptake process. Autoradiographic studies using the cocaine analog [^{3}H] 2 beta-carbomethoxy-3 beta-(4-fluorophenyl)tropane ([^{3}H]CFT) demonstrated specific binding presence in monkey brain, specifically in the caudate and putamen, a dopamine-rich area, and one that had been previously implicated in the behavioral effects and abuse potential of cocaine (Canfield et al., 1990). Kennedy and Hanbauer, using rat

striatal synaptosomes, demonstrated the presence of presynaptic, Na^+-dependent cocaine binding sites (Kennedy and Hanbauer, 1983). Today, these cocaine binding sites are known to reflect the presence of DAT protein. Such binding approaches remain valuable as they are used with *in vitro* studies of DAT regulation as well as for *in vivo* analyses, as with positron emission tomography (PET) imaging techniques to monitor DAergic nerve terminals in Parkinson's disease (Antonini et al., 2001, Song et al., 2012).

Cloning of DAT cDNAs

In 1988, Blakely and colleagues demonstrated the transport of radiolabeled L-glutamate, gamma-aminobutyric acid (GABA), glycine, DA, serotonin (5-HT), and choline in Xenopus laevis oocytes microinjected with brain mRNAs. mRNAs competent for expression of neurotransmitter transporter demonstrated a regional enrichment consistent with the anatomical distribution of neurotransmitter synthesizing soma and thereby demonstrated the opportunity for expression cloning of neurotransmitter transporter proteins (Blakely et al., 1988). Following the cloning of the norepinephrine (NET) (Pacholczyk et al., 1991) and serotonin transporters (SERT) (Blakely et al., 1991), four groups reported the cloning of cDNAs encoding rat and bovine DATs (Giros et al., 1991, Kilty et al., 1991, Shimada et al., 1991, Usdin et al., 1991). Using *in situ* hybridization, several of these groups demonstrated that DAT mRNA was expressed in the SN and VTA, consistent with the localization of DA neuron cell

bodies (Giros et al., 1991, Kilty et al., 1991, Shimada et al., 1991). Heterologous expression of rat DAT cDNA revealed the induction of a Na^+- and Cl- dependent, DA uptake process that was absent in non-transfected cells. The transport of DA displayed a high-affinity for DA, with a K_M estimated at between 300 and 900 nM. Finally, the *in vivo* DA uptake blockers, GBR12909 (IC_{50}=12 nM), mazindol (IC_{50}=27 nM), cocaine (IC_{50}=336 nM), and D-AMPH (IC_{50}=881 nM) inhibited DA uptake (Giros et al., 1991) with the same potencies previously reported with rat brain synaptosomes (Ritz et al., 1987). Together, these studies validated the contention that the DAT transcript produced a protein with the properties expected for the DA uptake process, including its antagonist-binding site, in the CNS. They also revealed that no additional, brain-specific subunits were required to produce functional DAT protein.

Oligonucleotides based on rat DAT cDNA sequence were used to design probes to clone human dopamine transporter (DAT) cDNA from SN libraries (Giros et al., 1992, Vandenbergh et al., 1992). DAT is now understood to be a 620 amino acid protein that displays ~92% amino acid identity to rat DAT. DAT displays reduced, but significant identity to other members of the Na^+/Cl^--dependent neurotransmitter transporter family (SLC6) members, with highest amino acid sequence identify observed with hNET (66%) and the human serotonin transporter (hSERT, 50%). Hydropathy analysis of DAT, like that of other SLC6 transporters, predicts 12 transmembrane domains (TMDs) with N- and C- termini located intracellularly (Figure 3). Three sites for N-linked

glycosylation are located in extracellular loop 2 (EL2) that connects TMDs 3 and 4. Mutating all three canonical glycosylation sites as well as enzymatic degradation and blockade of glycosylation resulted in reduced DAT surface expression; significantly diminished catalytic activity and inhibitor sensitivity compared to wildtype (WT) DAT (Li et al., 2004). Recently, a novel isoform of DAT produced by alternative splicing was discovered from human blood cells (Sogawa et al., 2010). The spliced isoform lacked exon 6, resulting in the deletion of TMD5 and an extracellular C-terminus. Further characterization showed expression differences between brain and peripheral tissues, indicating tissue-specific, alternative splicing. Expression of the spliced isoform resulted in loss of DAT activity and when expressed with the WT DAT, the spliced isoform acted

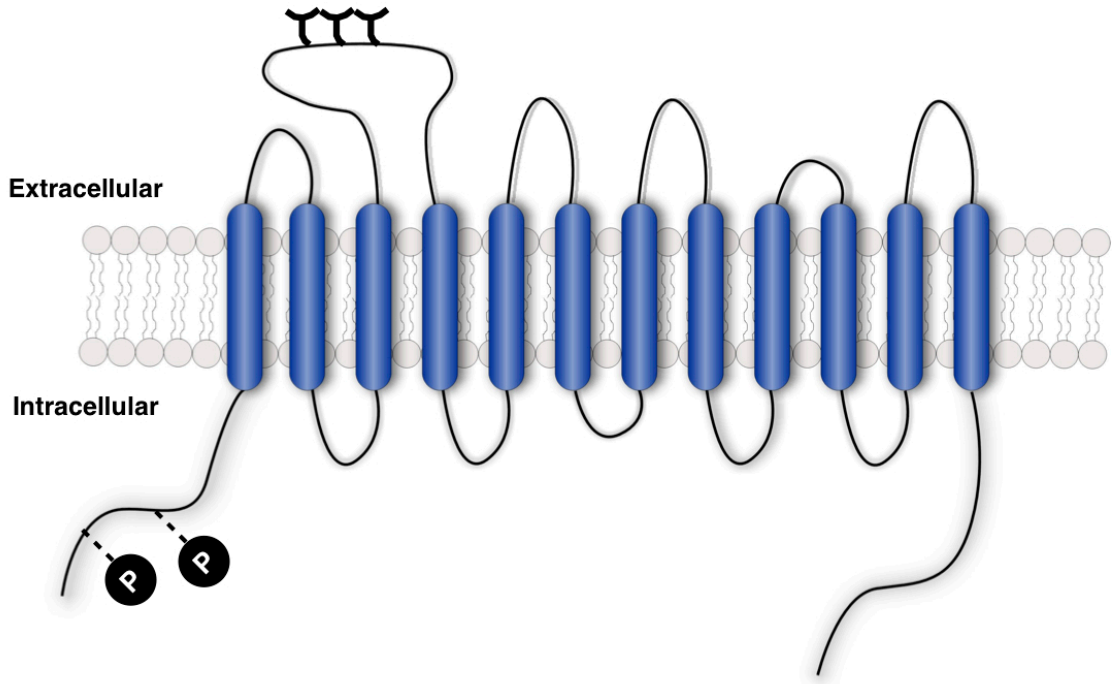


Figure 3 Schematic Illustration of DAT Topology: The DAT protein contains 12 TMDs with N- and C-termini located intracellularly. EL2 contains 3 N-linked glycosylation sites. Numerous consensus phosphorylation sites, depicted by P in a black circle, are found on the N- and C-termini as well as intracellular loops.

dominantly, reducing overall DA uptake (Sogawa et al., 2010). The functional significance of these observations *in vivo*, however, is unknown. Additionally, in schizophrenia patients, Talkowaski and colleagues identified a novel cassette exon within intron 3 of DAT gene that is conserved among primates (Talkowski et al., 2010). The presence of this cassette exon introduces multiple in-frame stop codons resulting in a truncated product, and that may undergo nonsense-mediated decay. The presence of this spliced variant was confirmed using RNA from postmortem human SN. Factors that increase use of this cassette exon splice variant could reduce the amount of unspliced RNA encoding WT DAT, with attendant functional consequences (Talkowski et al., 2010).

Dopamine Transporter Structure and Function

The characterization of DAT structure/function relationships has largely relied on heterologous expression studies performed *in vitro*. Data from studies expressing DAT and NET chimeras provided initial evidence that discrete domains within the transporter were involved in distinct functions such as substrate recognition, translocation, and affinity (Buck and Amara, 1994, Giros et al., 1994, Syringas et al., 2000). In these studies, TMDs 1-3 and 10-11 were identified as important for determining substrate affinity, whereas TMDs 5-8 were identified as critical for substrate translocation and the selectivity of inhibitors (Buck and Amara, 1994, Giros et al., 1994). Using similar approaches, TMDs 1-3

and 9-12 were identified as important for the Na^+ and Cl^- dependence exhibited by DAT, as well as other SLC6 transporters (Syringas et al., 2000).

Whereas chimera studies implicated broad regions of the transporter in key DAT properties, site-directed mutagenesis studies provided insights into more defined structure-function relationships, identifying in some cases single residues important for transport function and regulation. These studies indicated that, aside from support for N-glycosylation, EL2 appears to play a role, or be influenced by, inhibitor interactions, as inhibitor binding could be shown to protect DAT from trypsin digestion in this region (Gaffaney and Vaughan, 2004). In contrast, DAT substrates do not protect against proteolytic digestion (Gaffaney and Vaughan, 2004). Additionally, zinc immobilization of EL2 was shown to block DA transport through coordination of residues of EL2 and EL4, suggesting that movement of EL2 might be crucial for substrate translocation, possibly by structural movement of TMDs 3 and 4 (Norregaard et al., 1998). Interestingly, zinc immobilization of EL2 and EL4 increases ion conductance, which negatively impacts transport and stimulates efflux (Meinild et al., 2004). The later studies highlight the importance of electrophysiological approaches in providing mechanistic insights of mutation-induced changes in DAT function.

To further our understanding as to how both substrates like DA and antagonists like cocaine interact with DAT, mutagenesis studies have been conducted to identify residues critical for ligand coordination. In these studies, alanine substitution was used to modify residues in or near TMDs, including all

tryptophan (Lin et al., 2000b), proline (Lin et al., 2000a), and phenylalanine (Lin et al., 1999) residues. The importance of Asp79 (Kitayama et al., 1992) and Phe105 (Wu and Gu, 2003) residues in cocaine recognition has been studied in detail. DAT immunogold labeling has shown that in the nigrostriatal neurons, these transporters are strategically localized to extrasynaptic plasma membrane to re-uptake diffused DA back into the presynaptic terminal (Nirenberg et al., 1997). Using chemical cross-linkers, Fluorescent Resonance Energy Transfer (FRET) analysis, and expression studies with differentially tagged DAT constructs, DAT have been shown to exist in oligomeric form at the plasma membrane (Hastrup et al., 2001, Sorkina et al., 2003b).

Although the three dimensional structure of DAT is unknown, the generation of a high-resolution crystal structure for a bacterial SLC6 family member from *Aquifex aeolicus*, the leucine transporter ($LeuT_{Aa}$) supports the prediction of a 12 TM topology for DAT and homologs (Yamashita et al., 2005). The orientation and arrangement of TMDs of $LeuT_{Aa}$ is depicted in Figure 4. Unfortunately the leucine transporter has short cytoplasmic N- and C-termini that are unstructured in the crystal structure. Molecular modeling efforts based on the information obtained from the crystal structure of $LeuT_{Aa}$ helped Beuming and colleagues investigate the relationship between DA and cocaine binding sites on DAT (Beuming et al., 2008). Modeling and mutagenesis approaches provided evidence that the binding sites for cocaine and cocaine analogs are buried deeply between TMDs 1, 3, 6, and 8 and overlap with the binding site for DA and

AMPH (Beuming et al., 2008). Recently, using steered molecular dynamics simulation and homology modeling, a second DA binding site has been proposed to lie in the DAT extracellular vestibule and that this second substrate-binding site allosterically modulates transport mechanism (Shan et al., 2011). This proposal remains a significant point of debate among investigators studying the structural determinants of the function of SLC6 family members (Piscitelli et al., 2010).

The cytosolic tails and intracellular loops of DAT contain numerous serine,

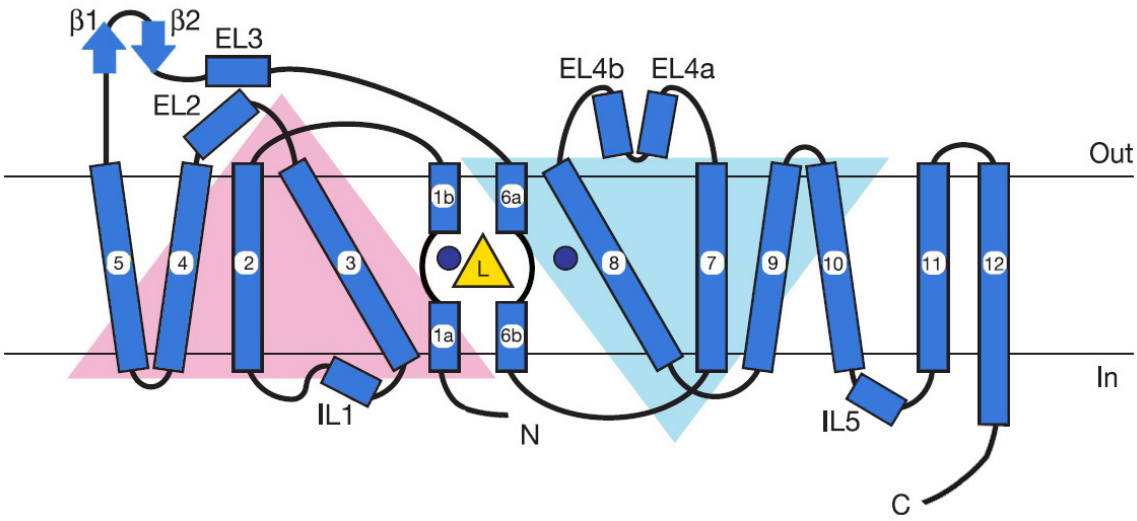


Figure 4 Schematic Topology of DAT Based on LeuTaA crystal structure: TMDs1-5 and 6-10 form a pseudo two-fold axis in the membrane plane (inverted triangle configuration) and fold over forming substrate translocation pathway. Yellow triangle with L and blue circles depict leucine and sodium ions respectively. TMDs 11 and 12 flank the outer surface of TMDs 9 and 10, and possibly contributing to the oligomerization. Adapted from Yamashita et al., 2005.

and threonine residues, some of which are located in consensus sites for protein kinase C (PKC), protein kinase A (PKA), and calcium calmodulin-dependent kinase II (CaMKII). However, the exact residues phosphorylated by these kinases remains an active area of investigation (see below).

The transport of DA is Na^+ and Cl^- dependent, with a predicted stoichiometry of $2Na^+$, $1Cl^-$ and one DA^+ (DA is positively charged at physiological pH) (Amara and Kuhar, 1993). In keeping with the proposed net charge transfer per transport cycle, DA transport through DAT is electrogenic and a voltage dependent process, where hyperpolarization increases and depolarization decreases DA transport (Sonders et al., 1997). Electrophysiological studies reveal that DA transport through DAT produces a larger current than is expected for stoichiometric charge transfer determined by the ion-dependence of DA transport. This observation appears to reflect the presence of channel-like states that operate to permit ion permeation during the DA transport cycle (Carvelli et al., 2004, Kahlig et al., 2005).

As a DAT substrate, AMPH competitively inhibits DA reuptake, causing an increase in synaptic DA that otherwise would have been cleared by re-uptake. Additionally, AMPH stimulates the release of DA through DAT in the brain (Fischer and Cho, 1979, Jones et al., 1998) and in transfected cells expressing DAT (Khoshbouei et al., 2003). AMPH-induced DA efflux has been considered to be mediated by a facilitated-exchange diffusion process, in which the inward transport of AMPH increases the availability of inward-facing binding sites of the transporter (Fischer and Cho, 1979), thereby leading to increased efflux of cytosolic DA. As noted below, this perspective is altogether too simple as multiple kinase-mediated signaling mechanisms can shift DAT into an “efflux willing” state. Finally, AMPH is a weak base and as a VMAT substrate, can collapse the

synaptic vesicle proton gradient needed for VMAT to package DA (Sulzer et al., 1995). Via this process, AMPH can elevate cytoplasmic DA levels and enhance the probability of DAT-mediated DA efflux.

Regulation of the Dopamine Transporter

Although DAT was recognized early in its documentation as a target for psychoactive drugs, the transporter was discussed more as a static component of presynaptic homeostasis, inexorably clearing DA upon synaptic release. Mechanisms that could influence DAT activity and clearance were unknown. An early hint that DAT might be locally regulated arose from studies of Kuhar's group, where irreversible inhibitor manipulation of striatal DAT revealed that the time for newly synthesized DAT to replace inactivated transporters in terminals is very long, on a synaptic transmission scale, with only ~50% of DAT replaced in 6 days (Fleckenstein et al., 1996). Although later studies reduced this figure to 2-3 days (Kimmel et al., 2000), the point remains that somatic biosynthetic mechanisms are too slow to effect significant changes in DAT-mediated DA clearance to match the more rapid changes in the firing rates of DA neurons (Goto et al., 2007). Today, evidence exits that rapid (sec-minutes) DAT regulation is imposed by presynaptic receptors, associated proteins and intracellular signaling networks, with evidence that both functional changes and/or membrane environment dictate the capacity for DA clearance (Zahniser and Doolen, 2001, Torres, 2006, Chen et al., 2010).

Presynaptic receptors

DA acts on both post- and pre-synaptic receptors, with presynaptic D2 receptors belonging to the Gi/Go family of G-protein coupled receptors (GPCR) that decrease adenylate cyclase activity (among other actions). D2 receptors have two isoforms as a result of alternative splicing with different spliced forms expressed pre or post-synaptically. D2 long (D2L) receptors, which have additional 29 amino acids in third intracellular loop and are exclusively expressed on postsynaptic neurons (Giros et al., 1989). On the other hand, D2 short (D2S) receptors that lack 29 amino acid insert are expressed presynaptically and act as DA autoreceptors. In response to elevated extracellular DA, D2S receptor provide inhibitory feedback in altering DA synthesis, release, and re-uptake (Meiergerd et al., 1993), with DAT regulation mediated by initiation of a second messenger cascade (Meiergerd et al., 1993). D2S receptors have been reported to increase DAT activity through activation of extracellular signal-regulated kinases (ERK1 and 2) (Bolan et al., 2007). The same group also provided evidence that D2S receptor modulation of DAT involves a phosphoinositide-3 kinase (PI3K)-independent mechanism (Bolan et al., 2007).

Type 1 metabotropic glutamate receptors (mGluRI) have been reported to regulate DAT expressed by dendrites of substantia nigra DA neurons (Falkenburger et al., 2001). These investigators demonstrated that stimulation of subthalamic nucleus afferents resulted in DAT-mediated DA efflux. Pharmacological inhibition of DAT, as well as antagonism of mGluR1 receptors,

attenuated DA release from DA neurons. In another study, mGluR5 was reported to regulate DAT *in vitro*. Activation of mGluR5 by its selective agonist (S)-3,5-dihydrophenylglycine (DHPG) decreased DA transport by DAT in striatal synaptosomes (Page et al., 2001). In these studies, mGluR5-dependent inhibition of DAT activity was dependent on signaling by CaMKII and PKC pathways.

Nicotinic acetylcholine receptors (nAChRs) have been reported to regulate DAT function. *In vivo* voltammetry studies provide evidence that systemic nicotine administration increases DA clearance in striatum, medial prefrontal cortex (mPFC) (Middleton et al., 2004), and nucleus accumbens (Hart and Ksir, 1996). The dose-response profile of nicotine in modulating DA clearance in striatum and mPFC is different, possibly owing to differential expression of nAChR subunits. AMPH-stimulated DA release from prefrontal cortex brain slices was reported to increased following 5 μM nicotine application (Drew et al., 2000). AMPH-mediated DAT downregulation following nicotine administration were found to be PKC-dependent but CaMKII-independent (Drew and Werling, 2001). Thus, activation of PKC via mGluRI and nAChR may drive opposite effects on DAT activity. One possible explanation for this could be that processes supporting DA uptake and DAT-mediated DA efflux may be governed by different mechanisms.

Regulation of DAT by σ2 receptors (formally classified as opioid receptors) has also been reported. Activation of σ2 receptor by (+)-pentazocine enhances AMPH-stimulated DAT-dependent DA release in striatal slices (Izenwasser et al.,

1998), as well as in PC12 cells (Weatherspoon and Werling, 1999). σ2 receptors have been suggested to regulate intracellular Ca^{2+} levels through their action on endoplasmic reticulum Ca^{2+} stores (Vilner and Bowen, 2000). In rat striatal slices, activation of σ2 receptors has been reporter to act through Ca^{2+} and PKC-dependent mechanisms to stimulate DAT activity (Derbez et al., 2002). Conversely, in PC12 cells, σ2 receptors activation was found to increase AMPH-stimulated DA efflux, stimulation dependent on CaMKII activation (Weatherspoon and Werling, 1999). Choice of model system and experimental designs likely dictate these regulatory differences. These studies with σ2 receptors reinforce the complexity in DAT regulation and demonstrate a need for more in-depth investigation using more precise pharmacological and genetic tools.

A recent addition to the list of receptors reported to regulate DAT is the trace amine-associated receptor 1 (TAAR1). TAAR1 is a G_s-coupled GPCR that signals through increasing cAMP production (Wainscott et al., 2007). TAAR1 is activated by trace amines like beta-phenylethylamine, as well as classic biogenic amines, and amphetamine-related psychostimulants. TAAR1 is expressed by substantia nigra DA neurons in primates and rodents (Xie and Miller, 2007, Xie et al., 2007) and its activation by DA or methamphetamine (METH) appears to lead to a PKA- and PKC-mediated reduction in DAT-mediated DA uptake. TAAR1 activation also induced an increase in DA efflux via a PKC-dependent mechanism (Xie and Miller, 2007). Further studies of DAT regulation by TAAR1 may be of clinical relevance in the treatment of METH addiction.

DAT-interacting proteins

Multiple DAT-interacting proteins (DIPs) have been identified using yeast two-hybrid (Y2H) screens with specific DAT sequences as bait, with confirmation of interactions pursued in cells or brain preparations by co-immunoprecipitation (Torres, 2006). DIPs are theorized to regulate DAT somatic export, synaptic localization, and surface trafficking, as well as DAT functional properties. None of these interactions has as yet been shown to influence DAT *in vivo*.

Torres and colleagues, using the DAT C-terminus as bait in a Y2H screen, described the first DIP, Protein Interacting with C kinase-1 (PICK1) (Torres et al., 2001). PICK1 is a PDZ (postsynaptic density 95/Discs large/zona occludens 1) domain containing protein that has been argued to be important for synaptic localization of DAT. The PDZ domain of PICK1 binds to a large number of membrane proteins especially proteins containing C-terminus PDZ type II binding motif. PICK1 also has a BAR (Bin/amphiphysin/Rvs) domain. The BAR domain of PICK1 binds to lipids, mainly phosphoinositides (Xu and Xia, 2006). PICK1 was originally identified as a PKC binding protein (Staudinger et al., 1995) and therefore, the PICK1-DAT interaction may be important in PKC-mediated regulation and trafficking of DAT. The Torres studies, the interactions between PICK1 and DAT were found to be mediated by the PDZ domain of PICK1 and the class II PDZ binding motif (LKV) that ends the C-terminus of DAT. PICK1/DAT interactions were reported to increase the number of surface DAT molecules, leading to an increase in DA uptake (Torres et al., 2001). However, more recent

studies from Gether's lab argue that surface targeting of transporter does not depend on PICK1 binding to the transporter's PDZ binding motif, but rather derives from residues (RHW) upstream of these sequences (Bjerggaard et al., 2004). Moreover, these authors suggest that the ability of PICK1 to modulate DAT surface expression may derive from an effect on the export of DAT from ER/Golgi biosynthetic stores. Recent studies by Madsen and colleagues show that deletion of PDZ domain from PICK1 results in clustering of PICK1 and BAR domain-dependent redistribution of PICK1 to endosomal compartments (Madsen et al., 2008). These authors further show that under basal conditions, the PDZ domain and the linker region connecting PDZ domain and BAR domain fold over leading to inhibit interactions of the BAR domain with its targets. Disinhibition of the BAR domain leads to membrane interactions, membrane curvature, and altered protein trafficking (Madsen et al., 2008). These findings raise the possibility that the action of PICK1 with respect to DAT may be constrained by BAR domain interactions with the PICK1 PDZ domain, and thus be one target of the regulatory cascades impacting DAT trafficking out of the ER/Golgi or to and from the cell surface.

In the Y2H screen where PICK1 was identified, Caron's group identified a second DIP, the multiple Lin-11, Isl-1, and Mec-3 (LIM) domain-containing adaptor protein, Hic-5 (Carneiro et al., 2002). Hic-5 is a member of a focal adhesion-associated adaptor protein family and is closely related to paxillin. In addition to its membrane localization, Hic-5 has also been found localized to

nuclear compartments (Shibanuma et al., 2004), with suggestion that the protein may cycle between these compartments, though a functional significance of this idea has not been determined with respect to DAT. Hic-5/DAT interactions arise through Hic-5 LIM domains and membrane-proximal sequences of the DAT C-terminus. Carneiro and colleagues reported that expression of DAT and Hic-5 in HEK 293 cells results in reduced DA uptake activity that arose from decreased DAT surface expression (Carneiro et al., 2002). Even though the physiological importance of DAT/Hic-5 interaction *in vivo* is unknown, Hic-5 is targeted by numerous signaling molecules (Shibanuma et al., 2012), and thus DAT interactions could connect the transporter to multiple intracellular signaling pathways. Recently, Carneiro and Blakely extended the generality of interactions of Hic-5 to other SLC6 family transporters, as well as their dynamic nature and occurrence in native preparations, with demonstration of PKC-dependent, SERT/Hic-5 interactions in human and mouse platelets (Carneiro and Blakely, 2006). Co-immunoprecipitation (co-IP) and 5-HT transport studies revealed that Hic-5 produces SERT inactivation and targets SERT to a membrane compartment that is permissive for PKC-dependent relocation of transporters from the cell surface. Interestingly, SERT/Hic-5 associations were reduced by PKC activation over the same time course as SERT internalization. Since association of intracellular SERT with Hic-5 was not detected, these authors concluded that Hic-5 participates in chaperoning SERT to membrane domains competent for PKC-dependent endocytosis (Carneiro and Blakely, 2006, Steiner

et al., 2008) and then dissociates thereafter or shortly following SERT endocytosis. Studies examining DAT/Hic-5 interactions in DA terminals are needed to determine whether the SERT/Hic-5 model extends to DAT.

The synaptic vesicle associated protein α-synuclein is yet another protein found to interact with DAT, once again mediated by sequences in the transporter's C-terminus (Lee et al., 2001). Mutations in α-synuclein have been implicated in familial forms of Parkinson's disease (Higuchi et al., 1998, Kruger et al., 1998), a disorder characterized by the degeneration of DA neurons. The physiological role of α-synuclein *in vivo* remains an active area of investigation (Chandra et al., 2005, Jin et al., 2011). DAT/α-synuclein interactions were first identified via a Y2H screen, and thereafter confirmed in neurons and co-transfected cells (Lee et al., 2001, Lee et al., 2007). DAT/α-synuclein interactions have been found to cluster the transporter at the plasma membrane, to increase uptake activity and to enhance DA-induced cellular apoptosis (Lee et al., 2001), possibly via enhanced oxidative stress produced by oxidation of the neurotransmitter. The functional outcome of this interaction is somewhat controversial as one group has reported that DAT/α-synuclein interactions following heterologous expression of these molecules leads to decreased DAT activity (Wersinger and Sidhu, 2003). The same group also reported that disruption of microtubule networks results in increased DAT activity as a consequence of DAT-α-synuclein interactions (Wersinger and Sidhu, 2005). As these studies have largely been conducted via heterologous expression, the

importance of DAT/α-synuclein interactions for presynaptic DA homeostasis and signaling remains unclear. Indeed, α-synuclein knockout mice do not show significant changes in DAT activity or protein expression (Abeliovich et al., 2000), though regulatory control of DAT has yet to be examined in this model.

In another Y2H screen using the DAT C-terminus as bait, Fog and colleagues identified CaMKIIα as a DIP (Fog et al., 2006). DAT/CaMKIIα interactions were confirmed using GST pulldown experiments and co-IP studies of both transfected cells and brain lysates, as well as through immunocytochemical colocalization studies. These researchers demonstrated that CaMKIIα binds to the DAT C-terminus and leads to phosphorylation of the transporter, likely through sites in the N-terminus of DAT. Additionally, CaMKII binding and DAT phosphorylation were shown to be necessary for AMPH-mediated DA efflux through DAT (Fog et al., 2006), providing evidence that conformational changes in the transporter that mediate DA efflux do not simply arise from substrate-mediated conformational changes, but involve a stabilized transition of DAT to an "efflux-willing" state. Whether DAT/CaMKIIα associations are constitutive, regulated or sensitive to disease-associated DAT mutations is unknown.

Recently, Melikian's group used a Y2H screen with a bait that encoded DAT C-terminal sequences 587-596 to identify a Ras-like GTPase, Rin, as a DIP. This interaction was confirmed through GST pull-downs, co-IP studies, and co-localization studies using heterologous expression system. DAT/Rin interactions

are suggested to be essential for PKC-induced DAT downregulation and internalization (Navaroli et al., 2011). The actual mechanism by which DAT-Rin interaction leads to PKC-mediated DAT downregulation is still under investigation. Rin binds to, and activates calmodulin (Lee et al., 1996). Since CaMKIIα also interacts with the DAT C-terminus, it is possible that Rin may serve to activate DAT-bound CaMKII via its activation of calmodulin. Rin is also required for nerve growth factor (NGF)-stimulated neurite extension (Spencer et al., 2002) and p38 mitogen-activated protein kinase (MAPK) activation in response to NGF signaling (Shi et al., 2005). Since DAT trafficking has also been linked to MAPK signaling (Moron et al., 2003, Bolan et al., 2007), studies are needed to determine if Rin participates in this process.

In summary, the DAT C-terminus is a site of interaction for multiple cytosolic proteins that have been argued to regulate DAT activity and expression. Understanding whether all of these associations occur together, are constitutive, or arise at different phases in the life cycle of the transporter needs further study. Since artificial systems provide the bulk of the data supporting the physiological significance of these findings, additional studies with DA neurons in vitro and in vivo are needed.

Using the N-terminus of DAT as Y2H bait, two additional DIPs have been identified; Receptor for Activated C Kinase (RACK1), and syntaxin 1A. RACK1 was originally identified as protein that binds activated PKC and recruits it to the plasma membrane, in proximity of its substrates (Ron et al., 1994). Co-

immunoprecipitation from rat brain extracts has shown the presence of a ternary complex involving DAT-RACK1-syntaxin 1A (Lee et al., 2004). It is tempting to speculate that this interaction between DAT and RACK1 may modulate function of the transporter via recruiting PKC to the membrane. Syntaxin 1A is a major component of the SNARE-mediated mechanism of synaptic vesicle fusion and neurotransmitter release (Sudhof and Rothman, 2009). GABA transporters (GAT1) (Beckman et al., 1998), glycine transporters (GLYT1) (Geerlings et al., 2000), NET (Sung et al., 2003b) and SERT (Quick, 2002), have also been shown to interact with syntaxin 1A. Cervinski and colleagues have shown that syntaxin 1A/DAT complexes are present in rat striatal synaptosomes, that syntaxin 1A cleavage enhances DAT activity, supporting a role for these complexes in DA neurons, though other mechanisms downstream of syntaxin 1A cleavage are possible (Cervinski et al., 2010).

Syntaxin 1A transfection into NET expressing cells leads to increased transporter surface expression, likely due to the SNARE-mediated fusion role of syntaxin 1A. In contrast, this expression also leads to a decrease in NE uptake capacity, accompanied by a reduction in NE-activated NET currents (Sung et al., 2003a). These authors showed that activation of PKC leads to a dissociation of NET and syntaxin 1A paralleled by NET internalization. Cervinski and colleagues have found that in rat striatal synaptosomes, cleavage of syntaxin 1A does not preclude PKC-dependent downregulation of DAT (Cervinski et al., 2010). Together, these findings suggest that NET and DAT interactions with syntaxin 1A

are likely to be part of a dynamic cycle by which transporters are brought to the presynaptic membrane, possibly to specialized microdomains (see below), and then regulated to provide for neural-activity dependent chances in transport function.

The ability of syntaxin 1A to modulate currents has also been reported with SERT, where syntaxin 1A can dictate whether the transporter exhibits an electroneutral or electrogenic 5-HT transport cycle (Quick, 2003). Recent studies have shown that DAT/syntaxin 1A associations promote AMPH-induced DA efflux (Binda et al., 2008) and can regulate DAT channel activity (Carvelli et al., 2008). In the *C. elegans* model, DAT/syntaxin 1A interactions have been reported to impact DA neurotransmission and behavior (Carvelli et al., 2008), to date the only evidence that these complexes are important *in vivo* As to the dynamic nature of transporter/syntaxin 1A interactions, Quick demonstrated that PKC activation with phorbol esters destabilized GAT1/syntaxin 1A interactions, possibly through phosphorylation of the syntaxin 1A-associated protein munc-18 (Beckman et al., 1998). Recent studies by the Galli lab suggest that phosphorylation of syntaxin 1A by casein kinase 2 (CK2) destabilize DAT/syntaxin 1A interactions, leading to enhanced AMPH-mediated DA efflux (Cartier 2011). How these findings reflect changes in second messenger-based signaling pathways, such as those mediated by PKC and CaMKII requires further analysis.

With regard to coordinating DA release and reuptake, Lee and colleagues have reported that a direct interaction between DAT and D2S receptors, mediated by the DAT N-terminus, can modulate the DAT function (Lee et al., 2007). This protein-protein interaction has great significance as both proteins are present at DA terminals and the associations may be part of a mechanism to synchronize DA release and clearance.

DAT-membrane microdomain associations

The term “Lipid raft” refers to the dynamic clustering of sphingolipids and cholesterol to form membrane microdomains that within the lipid bilayer. Rafts are proposed to function as platforms for the attachment of proteins when membranes are moved around inside the cell and during signal transduction (Simons and Ikonen, 1997, Lingwood et al., 2009). For a long time, membrane microdomain associations have been speculated to impact DAT expression, function, trafficking, and lateral mobility on the cell surface. Recently, Adkins and colleagues demonstrated the localization of DAT in specialized membrane microdomains, limits the lateral mobility of DAT and regulates DA transport capacity (Adkins et al., 2007). Since then a number of groups have confirmed this finding (Foster et al., 2008, Hong and Amara, 2010, Cremona et al., 2011). Foster and colleagues have suggested that PKC activation by β-PMA leads to DAT internalization through a non-raft fraction and that raft-associated DAT might undergo cholesterol-dependent, trafficking-independent regulation (Foster et al., 2008).

With respect to DAT trafficking, the redistribution of DAT from the plasma membrane to intracellular compartments in response to PKC activation has been reported to be a clathrin-dependent and dynamin-dependent endocytic mechanism (Daniels and Amara, 1999, Sorkina et al., 2005, Foster et al., 2008). AMPH-induced DAT trafficking has also been reported to be clathrin-dependent. These studies suggest that membrane domains supporting PKC and psychostimulant-induced endocytosis may originate from a common, or similar, membrane compartment, discussed further below.

Hong and Amara recently demonstrated the effects of cholesterol depletion on DAT conformation (Hong and Amara, 2010). After a mild-detergent extraction, DAT was found in cholesterol-rich lipid rafts. Increasing membrane cholesterol content using water-soluble cholesterol, these investigators observed an increase in DAT binding to cocaine analogs without a parallel increase in DAT surface expression. Using accessibility of DAT cysteine residues to a membrane-impermeable maleimide-biotin conjugate, DAT was found to favor an outward-facing conformation in lipid rafts, which may indicate a modulatory role of surrounding lipid content on DAT ligand interactions (Hong and Amara, 2010). The authors suggest that harsher treatments to remove cholesterol in earlier studies may have led to changes in DAT activity through non-specific mechanisms. The methods described in this study should be very useful in future studies that probe the functional significance and dynamic status of DAT in membrane microdomains.

Consistent with the findings of Hong and Amara, Cremona and colleagues demonstrated an association of DAT with the lipid-raft associated protein flotillin-1, (Cremona et al., 2011). DAT/flotillin-1 interactions were shown to be required for both the localization of DAT to lipid rafts and AMPH-induced DA efflux, as well as for PKC-mediated DAT internalization. Treatment of cells with phorbol esters was found to lead to phosphorylation of flotillin-1 at Ser 315, a modification that was found to be essential for DAT endocytosis. These studies may explain the finding that PKC activation leads to DAT internalization in a DAT phosphorylation-independent manner (Granas et al., 2003). Although DAT is phosphorylated in cells and synaptosomes by activators of PKC (Huff et al., 1997, Vaughan et al., 1997), this phosphorylation may play a role in DAT function versus DAT trafficking, with phosphorylation on raft-associated proteins of greater significance in endocytosis. Since AMPH-induced DA efflux, as noted above, is dependent on DAT N-terminal phosphorylation and CaMKII activation(Khoshbouei et al., 2004, Fog et al., 2006), a critical role for flottilin-1 reinforces the idea that DAT membrane compartmentation, trafficking, and catalytic function are coordinated via membrane microdomains. Cremona and colleagues also provide evidence that flotillin-1 participates in PKC-mediated DAT endocytosis. Both SERT and DAT demonstrate endocytic and functional regulation, though the signaling pathways supporting their regulation appear somewhat distinct (Steiner et al., 2008), and SERT-mediated 5-HT efflux has not received the same degree of attention as DAT-mediated DA efflux.

DAT regulation by intracellular signaling pathways

In addition to (but likely in concordance with) regulation of DAT by DIPs, DAT is believed to be regulated through covalent modifications, including phosphorylation, ubiquitination and lipid modification. The importance of these modifications for DAT trafficking, function and turnover are, to date, based on *in vitro* studies, most conducted following heterologous expression though several studies have been conducted ex vivo with brain synaptosomes (Vaughan et al., 1997). The development of transporter knock-in mouse models (Herrstedt Hansen, 2011, Mergy, 2011), as well as studies of DAT in powerful invertebrate models, including *C. elegans* (Jayanthi et al., 1998, Nass et al., 2001, Carvelli et al., 2004, McDonald et al., 2006) and *Drosophila melanogaster* (Porzgen et al., 2001), portend an increased opportunity to tackle questions related to *in vivo* relevance of DAT post-translational modifications in the coming years.

Canonical Ser/Thr phosphorylation sites, and many potential noncanonical Ser/Thr sites, are found on the DAT N- and C-termini, as well as on intracellular loops. Numerous studies demonstrate that DAT activity is associated with activation or inhibition of Ser/Thr protein kinases, including PKC, PKA, MAPKs, such as ERK1 and 2, CaMKII, and PI3K (Zapata and Shippenberg, 2002, Bolan et al., 2007, Blakely and Edwards, 2012). No studies to date have reported evidence of basal or regulated DAT phosphorylation following activation of tyrosine kinases. Although evidence supporting insulin receptor modulation of DA clearance and DAT trafficking has been advanced (Carvelli et al., 2002, Daws et

al., 2011), it is unclear whether this modulation is constitutive or dynamic since the paradigms examined so far either exploit treatments with PI-3K inhibitors or monitor DAT following insulin deprivation, followed by rescue of normal DAT trafficking/function with insulin restoration (see below).

Activation of PKC, either by exogenous application of phorbol esters such as β-PMA (Huff et al., 1997, Vaughan et al., 1997, Zhang et al., 1997, Zhu et al., 1997, Daniels and Amara, 1999, Loder and Melikian, 2003, Foster et al., 2008), or indirect stimulation through the G protein-coupled substance P receptor (Granas et al., 2003), has been reported to produce significant DAT downregulation along with decreased cell surface expression. PKC-dependent downregulation of DAT activity is thought to be primarily due to the intracellular accumulation of the transporter (Zhang et al., 1997, Zhu et al., 1997, Melikian and Buckley, 1999, Granas et al., 2003, Loder and Melikian, 2003), and was initially believed to involve PKC-dependent DAT phosphorylation. Although PKC activation using β-PMA treatment on striatal synaptosomes does increase DAT phosphorylation (Huff et al., 1997, Vaughan et al., 1997, Foster et al., 2002), more recent evidence suggests that DAT phosphorylation is not necessary for DAT downregulation (Foster et al., 2002, Granas et al., 2003). Possibly, N-terminal phosphorylation part of a redundant mechanism that insures appropriate control of transporter trafficking. We must also admit that our tools are likely quite limited with respect to following more subtle properties of “DAT activity”, as well as in detecting nuances in DAT trafficking pathways that may be altered by loss

of phosphorylation sites. We also have to recognize that no studies investigating a role of DAT phosphorylation involve an *in vivo* manipulation of DAT phosphorylation sites. Thus, current models, even ones reported in the present thesis, should be cautiously interpreted and will need extending through more physiologically relevant preparations.

The lack of a requirement for DAT phosphorylation in PKC-mediated DAT downregulation led to a search for other post-translational modifications that might be critical for regulated DAT trafficking. In this regard, PKC activation has been found to increase DAT N-terminal ubiquitination. In MDCK cells, DAT ubiqutination has been associated with lysosomal degradation (Daniels and Amara, 1999), though in other cells, the modification has been associated with endocytosis of the transporter, without a discernible reduction in protein levels (Miranda et al., 2005, Miranda et al., 2007).

Despite a lack of a direct role in DAT trafficking, PKC-induced phosphorylation of DAT may be critical for other aspects of DAT function, such as DA efflux (Kantor and Gnegy, 1998, Johnson et al., 2005b). Indeed, Cowell and colleagues showed in striatal slices that DAT phosphorylation following PKC activation is paralleled by DAT-dependent DA efflux (Cowell et al., 2000). These data are consistent with both trafficking-dependent and independent roles for PKC in regulating DAT function. Ramamoorthy's (Jayanthi et al., 2004) and Blakely's groups (Zhu et al., 2005, Steiner et al., 2008) have provided evidence that NET and SERT undergoes both trafficking-dependent and trafficking-

independent modes of regulation, suggesting that the control of DAT described here may be further evidence of a more general phenomenon.

The finding by Carvelli and co-workers that a 5 hour treatment of serum-starved, DAT-transfected HEK-293 cells with 1 μM insulin stimulates DAT activity, lead to an association of PI-3 kinase-linked pathways to DAT regulation (Carvelli et al., 2002). Garcia and colleagues demonstrated that a dominant negative form of Akt, an effector downstream of PI-3K, was able to block the effects of insulin of DAT expressed by these cells (Garcia et al., 2005). Consistent with these findings, studies by Galli's lab have demonstrated that treatment of rat brain synaptosomes with a PI-3K inhibitor reduces DAT function (Carvelli et al., 2002). The findings that insulin application to cells expressing DAT displayed increased DA uptake (Carvelli et al., 2002), and that rats made hypoinsulinemic by fasting exhibited decreased rate of DA uptake (Patterson et al., 1998) is consistent with a supportive role of insulin signaling and PI-3K linked pathways in sustaining DAT function.

Interestingly, AMPH self-administration is reduced in rats that are made hypoinsulinemic by injection of streptozotocin (Owens et al., 2005). Collectively, *In vitro* studies suggest that hypoinsulinemia may regulate the actions of AMPH by inhibiting the insulin downstream effectors PI3K and Akt, which we have previously shown are able to fine-tune DAT cell-surface expression (Carvelli et al., 2002, Garcia et al., 2005, Wei et al., 2007b) Finally, *in vivo*, using functional magnetic resonance imaging (fMRI) the ability of AMPH to elicit positive blood

oxygen level-dependent signal changes in the striatum was dependent on intact insulin signaling (Williams et al., 2007) Recently, Galli's group has extended their investigations of a role of PI3K/Akt pathways in DAT regulation to implicate a downstream target of Akt, mTOR, in NET trafficking and NE transport (Robertson et al., 2010, Siuta et al., 2010). These latter findings, derived from studies of conditional mTOR-knockout mice, included behavioral changes, such as disrupted pre-pulse inhibition (PPI), that are found in schizophrenic subjects. PPI deficits could reversed by the NET antagonist desipramine (Siuta et al., 2010), a surprising finding given that the brains of mTOR knockout mice exhibit gross changes linked to the role of mTOR in protein translation. Regardless, these studies warrant further consideration of PI3K-Akt-mTOR-DAT/NET pathways in modulation of catecholamine transporter capacity as well as in neuropsychiatric disorders.

Multiple MAPKs have been implicated in DAT regulation. Initially, inhibitors of p42 and p44 MAPK were found to induce a reduction in DAT activity and to increase internalization in transfected cells and striatal synaptosomes (Lin et al., 2003, Moron et al., 2003). Unlike PI-3K and MAPK, inhibition of PKA has been shown to increase DAT activity and surface expression in heterologous expression systems (Pristupa et al., 1998). However, striatal synaptosomes display opposite effects upon PKA activation (Page et al., 2004) leaving the role of PKA signaling in DAT regulation unclear

Together, the studies describe above paint a complex and incomplete picture regarding control of DAT activity and DA clearance. Regulation of DAT likely involves multiple intracellular signaling pathways, acting across different time domains, through multiple DIPs. A further characterization of DAT regulatory events could inform our understanding and treatment of DA-linked neuropsychiatric disorders, including schizophrenia, ADHD and addiction.

Impact of Psychostimulants on DAT

Given the dynamic role of DAT in controlling DA signaling, it is no surprise that drugs targeting DAT have profound biochemical and physiological effects. These effects can be beneficial, such as the use of methylphenidate (Ritalin®) and mixed amphetamine formulations (e.g. Adderall®) for the treatment of ADHD. Other drugs, however, are highly addictive and can lead to devastating consequences for the individual, his or her family, and their community.

Cocaine is a conventional, and rapidly acting, competitive DAT antagonist whereas the actions of AMPH are more complex, as noted above. In addition to their rapid effects in blocking DA uptake and/or producing DAT-mediated DA efflux, these agents also influence the subcellular localization of DAT proteins (Saunders et al., 2000, Daws et al., 2002, Gnegy et al., 2004). Although effects of cocaine on DAT cell surface expression have been reported, the findings are somewhat inconsistent. Daws and colleagues reported that 10 min application of 10 μM cocaine increases DA uptake and DAT cell surface expression in DAT

transfected HEK 293 cells (Daws et al., 2002). In contrast, Vaughan's group have suggested that cocaine produces no effects on DAT expression and regulation in heterologous cell expression in system (Gorentla and Vaughan, 2005). Chronic cocaine use has been reported to increase DAT binding in brains of abusers (Little et al., 1993, Mash et al., 2002), though others have reported that striatal DAT protein levels are decreased in cocaine abusers (Wilson et al., 1996). Possibly, these observations relate to the changes in conformation for ligand recognition that were seen with membrane cholesterol depletion (Hong and Amara, 2010). Alterations in DA neurotransmission and presence of excess DA could also lead to downregulation of DAT as a compensatory mechanism due to psychostimulant addiction. Further investigation of these phenomena is warranted; as such changes may underlie the psychiatric component associated with addiction and relapse.

With prolonged treatments, amphetamines (AMPH and METH) produce a reduction in DA transport, accompanied by a loss in cell surface expression (Saunders et al., 2000, Gulley et al., 2002). METH, a highly addictive psychostimulant, produces a significant reduction in brain DA content and a loss of DAT protein and/or DA axon degeneration with chronic use (McCann et al., 1998, Sekine et al., 2001). The endocytic effects of AMPH are dependent on AMPH transport and PKC activity, as they can be blocked by cocaine and PKC inhibitors (Kantor and Gnegy, 1998, Saunders et al., 2000).

AMPH-induced DA efflux is regulated by both PKC (Kantor and Gnegy, 1998) and CaMKII (Fog et al., 2006). Consistent with these observations, AMPH, likely through its weak base properties, induces a rise in intracellular Ca^{2+} (Gnegy et al., 2004). Activation of either kinase leads to DAT phosphorylation, with evidence consistent in both cases with modification of N-terminal serine residues as 1) elimination of these sites leads to a loss of phorbol ester-activated DAT phosphorylation (Foster et al., 2002, Granas et al., 2003) and 2) alanine substitution at N-terminal serines abolishes AMPH-induced efflux (Khoshbouei et al., 2004). As noted above, CaMKII interacts directly with distal sequences of the DAT C-terminus (Fog et al., 2006). No such interaction has been suggested for PKC, although PKCβ has been shown to both regulate DAT and colocalize with the transporter in mesencephalic neurons (Chen et al., 2009, O'Malley et al., 2010a). Together, these studies suggest a role of DAT phosphorylation in modulating DAT reversal and DA efflux. Along with findings of a PKC-modulated interaction of DAT with the lipid raft-associated protein flotillin-1 (Cremona et al., 2011), these studies suggest a model whereby AMPH acts initially to prevent DA clearance, and then after AMPH transport, the psychostimulant elevates cytosolic DA and Ca^{2+}, with a subsequent activation of PKC and CaMKII. Kinase activation leads to phosphorylation of DAT on the transporter's N-terminus to stabilize transporters, located in lipid rafts, in an efflux-competent state. The latter effect is likely produced by alterations in the association of the DAT N-terminus with DIPs, including syntaxin 1A and flotillin-1, but likely several other proteins

Dopamine Transporter and Human Disorders

Disruption of DA neurotransmission has been linked to multiple neurodegenerative and psychiatric disorders. Here, I focus on two of these disorders, juvenile dystonia, and ADHD, as they relate to genetic alterations in DAT that form the basis of this thesis.

ADHD is the most commonly diagnosed disorder of childhood, affecting 3-5% of US school-age children (AmericanPsychologicalAssociation, 1994). The disorder is characterized by deficits in attention/cognitive function and evidence of hyperactivity/impulsivity. ADHD is highly heritable, with males four times more likely to be affected than females (AmericanPsychologicalAssociation, 1994). ADHD has been found to exhibit significant comorbidity with bipolar disorder (BPD). The American Psychiatric Association has developed criteria to clinically diagnose the disorder that involves behavioral assessment of the child as well as parent and teacher observations. There are no known biological markers that can serve to help diagnose ADHD, which makes it difficult to distinguish the disorder from other mental disorders with cognitive deficits.

Brain imaging and genetic studies are consistent with a contribution of altered brain DA signaling to ADHD risk. Using iodine-123 labeled N-(3-iodopropen-2-yl)-2beta-carbomethoxy-3beta-(4-chlorophenyl) tropane ([^{123}I]IPT) single-photon emission tomography (SPECT), DAT binding in the basal ganglia has been found to be increased in Asian children with ADHD compared to normal children (Cheon et al., 2005). Multiple studies have also found an association

between DAT binding and adults with ADHD (Dresel et al., 2000, Krause et al., 2000) with one exception (van Dyck et al., 2002). ADHD traits have significant heritability and multiple genetic studies demonstrate association between ADHD and DAT polymorphisms or haplotypes, with greatest focus being a variable number tandem repeat (VNTR) in the 3'-untranslated region of the human DAT (DAT) gene, a variant whose functional relevance remains debated (Cook et al., 1995). Association studies have also implicated the DA D4 receptor in ADHD (Holmes et al., 2002, Kirley et al., 2004). In addition to these genes, significant association has been found with genes that encode DA D5 receptors, DA beta-hydroxylase, SERT, 5-HT1B receptors, and synaptosomal associated protein-25 (SNAP-25) (Brookes et al., 2006). Although multiple genes appear to contribute to the heritability of, and risk for, ADHD, it seems reasonable to speculate that groups of these genes ultimately will be linkable into coherent molecular networks that control the development, function and plasticity of brain synapses, in general, and of DA synapses in particular.

DAT knock-out (KO) and knockdown mice display hyperactivity (Giros et al., 1996, Zhuang et al., 2001) as well as cognitive alterations (Giros et al., 1996, Pogorelov et al., 2005). These findings suggested that loss of DAT expression or function could contribute to ADHD, with the mice described above as models that might be of valuable to further dissect the disorder in ways impossible with human subjects. However, there is no evidence for DAT loss of function mutations in ADHD subjects, raising concern as to the legitimacy of these mouse

models for the study of ADHD. Reinforcing these concerns, Kurian and colleagues recently identified subjects with infantile Parkinsonism-dystonia (IPD) that possess homozygous, loss-of-function DAT mutations (Kurian et al., 2009). Although these findings do not support a role for severe reductions in DAT expression in ADHD, they do encourage further investigation of DAT alterations as a risk for movement disorders, particularly in syndromes associated with dystonia. An important, though not obligate reason for considering DAT with respect to ADHD risk factors is the fact that multiple medications used to successfully treat ADHD also target DAT. As noted earlier, methylphenidate (Ritalin®) blocks DAT whereas AMPHs (typified by the mixed AMPH, Adderall®) acts as a DAT substrate and induces DA efflux. The efficacy of DAT-directed agents in the treatment of ADHD, along with their rapid action, is consistent with compromised DA signaling in the disorder. However, these findings are just as consistent with the utility of these agents in enhancing signaling at perfectly normal DA synapses that can overcome deficits originating elsewhere.

Detailed characterization of previously identified, but not clinically associated DAT coding variants failed to demonstrate specific deficits in either DAT function or regulation (Mazei-Robison and Blakely, 2005), except for the V382A variant, which displayed an intriguing regulatory deficit. PMA treatment (1 μM, 30 minutes) of V382A transfected cells was found to reduce DAT activity without accompanying endocytosis, revealing a capacity of transporters to display catalytic inactivation, as well as transporter endocytosis, as a mechanism

of DAT downregulation (Mazei-Robison and Blakely, 2005). Although these findings hint that natural variation in DAT may provide insights into components of DAT regulation more difficult to observe in the WT transporter, the lack of a clinical correlation with the V382A variant prevents further extrapolation to this variant's potential physiological significance.

Owing to a lack of evidence of functionally perturbed, DAT coding variants associated with DA-linked disorders, the Blakely lab pursued a search for functional polymorphisms in subjects with ADHD (Mazei-Robison et al., 2005, Mazei-Robison et al., 2008). These studies resulted in the identification of the A559V coding variant, carried by two brothers diagnosed with hyperactive predominant ADHD or combined-type ADHD. Both brothers were also 10/10 for the DAT 3' VNTR, and thus homozygous for the VNTR allele most often associated with ADHD, suggesting the possibility of a "double-hit" model. Although the kindred containing these subjects is small, Mazei-Robison and coworkers were able to demonstrate transmission across two generations, passed from grandmother to mother to the two boys.

The DAT A559V variant was the first coding variant to be identified in independent studies, having been separately identified in a female subject with BPD (Grunhage et al., 2000, Mazei-Robison et al., 2005). Despite the screening of many hundreds of affected and unaffected subjects since, the A559V variant has not been uncovered, indicating that it is indeed a rare variant as opposed to a common polymorphism. Interestingly, BPD and ADHD are comorbid (Faraone

and Tsuang, 2003) and the manic phases of BPD often display hyperactivity and impulsivity. Children of BPD subjects are three times more likely to have ADHD, and children of ADHD patients are twice as likely to develop bipolar disorder in their adulthood (Faraone and Tsuang, 2003). In both cohorts in which DAT A559V was identified, subjects are heterozygous for the substitution, suggesting that the variant, if it contributes to disease risk, likely acts dominantly.

Mazei-Robison and colleagues evaluated the functional impact of the DAT A559V in transfected HEK-293 cells. Under basal conditions, A559V exhibits normal DAT activity, and surface expression (Mazei-Robison et al., 2005). Remarkably, DAT was found to display anomalous DA efflux (ADE) under basal conditions that could be blocked by both AMPH and methylphenidate (Mazei-Robison et al., 2008). These investigators also observed that A559V ADE could be greatly enhanced by membrane depolarization, and DA efflux was supported by D2 receptor stimulation and increased DAT channel activity (Mazei-Robison et al., 2008, Bowton et al., 2010). The properties of the DAT A559V variant led these investigators to propose that idiopathic ADHD may feature disrupted DAT function or regulation, thereby disrupting the fidelity of DA signaling that can be restored through the use of psychostimulants. Possibly, ADHD-associated ADE may have parallels in other biogenic amine transporter-linked disorders, such as anxiety and depression (associated with altered SERT and/or 5-HT signaling) and cardiovascular disorders (associated with altered NET and/or NE signaling).

Specific Aims

This dissertation specifically seeks to characterize functional alterations possessed by a novel, ADHD-associated coding variant DAT-R615C (DAT 615C) and determine whether there are shared or distinct properties with the previously identified DAT variant A559V, To achieve these goals, I have pursued the following aims:

1) Determine impact of DAT 615C variant on basal total and surface expression as well as DAT activity including DA uptake and DA efflux.
2) Monitor regulatory changes associated with DAT 615C and A559V after AMPH and β-PMA treatment (Surface and total expression, DA uptake)
3) Examine protein-protein interactions that might alter due to DAT 615C variant
4) Determine whether DAT 615C can act dominantly
5) Monitor DAT phosphorylation levels for DAT 615C and A559V
6) Verify the DAT 615C and A559V mutations do not alter membrane microdomain (lipid raft) associations

CHAPTER II

MATERIALS AND METHODS

Materials

DMEM and FBS were from Gibco. Mouse anti CaMKII antibody (MA1-048), TCEP, Sulpho-NHS-SS-Biotin, and Streptavidin agarose resin were from Thermo Fisher. [^{3}H]DA (30-50 Ci/mmol) and [^{32}P]orthophosphoric acid (8500-9120 Ci/mmol) were from PerkinElmer. Alexa Fluor 647 CTxB was from Invitrogen. KN-93, KN-92 and β-PMA were from EMD biosciences. Rat and rabbit anti-DAT antibodies were from Millipore (MAB369 and AB5803 respectively). Mouse anti-flotillin-1 antibody was from BD Biosciences (Catalog # 610820). Secondary antibodies were obtained from Jackson Laboratories. Other reagents were obtained from Sigma.

ADHD subject collection and ascertainment

Subject recruitment and ascertainment for U.S. ADHD probands has been previously described (Mazei-Robison et al., 2005, Mazei-Robison et al., 2008). Additional subjects from Ireland were also enrolled from child guidance clinics and ADHD support groups around Ireland. The age range of the probands was between 5 and 14 years, with males accounting for 88% of the cases. To

establish DSM-IV diagnoses, one or both parents were interviewed using the Child and Adolescent Psychiatric Assessment (CAPA). To fulfill DSM-IV ADHD criteria for symptom pervasiveness, information regarding ADHD symptoms at school was obtained from teachers using a semistructured teacher telephone interview. ADHD symptom dimensions, as well as comorbid disorders such as Oppositional Defiant Disorder (ODD) and Conduct Disorder (CD) were obtained from the Child and Adolescent Psychiatric Assessment (CAPA). A positive family history of ADHD was defined by at least one parent scoring 36 or greater on the 25 item subscale of the Wender Utah Rating Scale (WURS). A 25-item sub scale was used in with a cutoff score of 36 or greater being 96% sensitive and specific for a retrospective diagnosis of childhood ADHD in the parent.

PCR amplification of DAT exons and polymorphisms screening via temperature gradient capillary electrophoresis (TGCE)

PCR amplification, screening and sequence confirmation was performed as described previously (Mazei-Robison et al., 2005).

Cell culture, transfections, and stable cell line generation

Flp-In™ 293 cells were used to generate stable lines expressing either DAT 615R or DAT 615C following the manufacturer's protocol (Invitrogen™). Selection media contained 100 μg/ml Hygroycin B. HEK 293T cells used for

transient transfections were cultured, maintained and transfected as described previously (Mazei-Robison and Blakely, 2005).

DA transport assays

[^{3}H] DA transport assays were performed in triplicate or quadruplet in 24 well plates as described previously (Apparsundaram et al., 1998). Briefly, cells were washed with Krebs-Ringers-HEPES (KRH) (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, pH 7.4) buffer and were incubated in the uptake assay buffer (KRH, 10 mM glucose, 100 μM pargyline, ascorbic acid, and 10 μM tropolone) before incubation with [^{3}H] DA for 10 min followed by three 4^{0}C washes. To determine non-specific uptake, parallel wells were incubated with 1 μM GBR 12909 5 min prior addition of [^{3}H] DA. For single point uptake assays, 50 nM [^{3}H] DA was used. For saturation kinetic analyses, six different DA concentrations were used (0.05, 0.1, 0.5, 1, 2, and 3 μM or 0.05, 0.1, 0.5, 1, 3, and 6 μM). Serial dilution from a stock made with 5% [^{3}H] DA was used for saturation analyses, except for the 50 nM concentration where 100% tritiated substrate was used. For all inhibitors, six concentrations (0.001, 0.01, 0.1, 0.5, 1, 10 μM) were tested to determine IC_{50} values. With AMPH treatments, cells were rapidly washed with ice-cold KRH buffer and warmed back up to 37^{0}C using uptake assay buffer for 5 min before addition of [^{3}H] DA for a further 3 min incubation. The shorter uptake time was used to reduce confounds from DA efflux due to the presence of intracellular AMPH.

MicroScint scintillation cocktail was added to the wells at the end of the assay and DA uptake was measured using a TopCount Scintillation Counter. Mean +/- SEM values reported derive from 3-6 independent experiments.

IDT 307 Uptake Assays

Flp-In HEK cells were plated onto PDL coated 96 well plates and cultured for 24-36 hrs. Cells were washed twice with warm KRH and incubated with KRH for 10 min with or without GBR 12909 before addition of IDT 307. For saturation kinetic analyses, five different IDT 307 concentrations were used (0.5, 1, 5, 10, and 20 μM). Plate was placed in the FlexStation and fluorescence was measured at 400 nM for 10 min. and endpoint fluorescence was used to generate the saturation curve. Data was analyzed using Soft-Max Pro, Microsoft Excel, and Prism softwares.

To determine AMPH-induced IDT 307 efflux, cells were preloaded with IDT 307 for 10 min prior to washing the cells with KRH to remove excess extracellular IDT 307 and addition of AMPH. Change in fluorescence was measured for 15 min. as described above. Data was normalized to 100% at a time point 100 sec. prior to AMPH addition. This was necessary to ensure IDT 307 fluorescence was stable before addition of AMPH.

Cell surface biotinylation, biotinylation internalization, and biotinylation recycling assays

Cells were seeded in 6 well plates and incubated for 36-48hrs before experiments. Cells were washed twice with warm KRH buffer before drug treatments. At the end of the treatment time, cells were placed on ice and washed rapidly twice with ice-cold PBS (136 mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2PO_4, 2.8 mM glucose) containing 0.1mM $CaCl_2$ and 1mM $MgS0_4$ Cells were incubated with sulphosuccinimidyl1-2-(biotinamido)ethyl-1,3-dithioproprionate-biotin (Sulpho-NHS-SS-Biotin) for 30 min at 4^0C. Excess biotin was quenched by 2 washes with 0.1 M glycine in PBS and cells were solubilized using radioimmunopreciptation assay (RIPA) (100 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1 % SDS, 1 % TritonX-100, 1 % sodium deoxycholate) buffer for 30 min at 4^0C.

To determine the internalization rate, we used a modified version of a previously published protocol (Holton et al., 2005). Briefly, cells seeded on 2 different plates were labeled with Sulpho-NHS-SS-Biotin for 30 min at 4^0C and quenched with 0.1 M glycine in PBS. One plate was mainlined at 4^0C to determine total surface expression and stripping efficiency. The other plate was warmed by 3 rapid washed with warm PBS and cells were incubated with warm PBS at 37^0C for 30 min either with or without AMPH to allow trafficking of the surface proteins. At the end of incubation, plates were transferred onto ice and cells and washed 3 times with ice-cold PBS followed by 3 washes with NT buffer

(150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM Tris pH 8.6). The biotin signal remaining on the surface from the 37^0C plates and stripping control wells from 4^0C plates were stripped using 50 mM tris(2-carboxyethyl)phosphine (TCEP) in NT buffer for 60 min at 4^0C. Fresh stripping solution was added after the first 30 min to ensure optimal stripping efficiency. Cells were washed rapid three times with ice-cold NT buffer followed by ice-cold PBS and solubilized using RIPA buffer for 30 min at 4^0C. Protein concentration was determined using the BCA protein assay and equal protein amounts were incubated with pre-washed streptavidin beads for 2hr or overnight at 4^0C. Streptavidin beads were washed three times with RIPA buffer and bound proteins were eluted using Laemmli sample buffer (LSB) and analyzed using SDS-PAGE, followed by western blotting. A rat anti-DAT antibody (Millipore-MAB 369, 1:5000) was used to visualize DAT and mouse anti β-actin (Sigma-a5316, 1:5000) antibody was used to detect β-actin as a loading control. HRP-conjugated goat anti rat and mouse secondary antibodies (1:10000) were used.

Recycling rates were estimated using a biotinylation-recycling assay. Cells were labeled with Sulpho-NHS-SS-Biotin for 60 min at 37^0C to achieve labeling of both surface and recycling intracellular proteins. Cells were placed on ice and cooled by washing with ice-cold PBS, followed by NT buffer three times each. Surface biotin labeling was stripped using 50 mM TCEP in NT buffer for 90 min at 4^0C. After an initial 60 min of stripping, fresh TCEP solution was added for the remaining 30 min to ensure efficient stripping, assessed via western blots.

Following stripping, cells were washed rapidly three times with ice-cold NT buffer followed by three times with ice-cold PBS buffer. One plate was maintained at 4^0C, and the other plate was warmed by washing three times with 37^0C PBS. Cells were then incubated for 30 min either with or without AMPH at 37^0C. At the end of the incubation, cells were again placed on ice and washed three times with ice-cold PBS followed by NT buffer. Biotin signal from intracellular DAT that recycled to the surface was stripped by incubating cells in 50 mM TCEP in NT buffer on ice for 60 min, with fresh stripping buffer added after 30 min. Cells were then washed three times with ice-cold NT buffer followed by three washes with ice-cold PBS buffer and solubilized using RIPA buffer for 30 min at 4^0C. Streptavidin pulldown and western blots were performed as described above.

Co-immunoprecipitation (co-IP) assays

DAT-CaMKII co-IP

HEK 293T cells were seeded on 10 cm dishes and co-transfected with pEYFP-HA-DAT-WT (615R) (gift from Dr. Alexander Sorkin) or the pEYFP-HA-DAT 615C variant and CaMKIIα (gift from Dr. Roger Colbran) 24hrs later. The pEYFP-HA-DAT-WT construct has an N-terminal YFP tag and the HA tag derives from modifications of extracellular loop 2, as described elsewhere (Sorkina et al., 2003a, Sorkina et al., 2006). The presence of YFP- or HA- tag does not interfere with expression, function and trafficking of DAT (Sorkina et al., 2006). Cells were

lysed using co-IP buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% TritonX-100) containing protease inhibitors 48hr post-transfection. Immunoprecipitation was performed overnight using goat anti CaMKII antibody (gift from Dr. Roger Colbran) and co-immunoprecipitated DAT was visualized using rat anti-DAT antibody as for western blots noted above. Mouse anti-CaMKII antibody (1:5000) was used to visualize immunoprecipitated CaMKII. Goat IgG was used in parallel co-IP experiments to insure specificity of immunoprecipitation.

DAT-flotillin-1 co-IP

Flp-in™ HEK stable cells were seeded on 10 cm dishes. Cells were lysed using co-IP buffer 48hr after plating. Immunoprecipitation was done using rat anti-DAT antibody and co-precipitated flotillin-1 was visualized using a mouse anti flotillin-1 antibody (1:1000). Rabbit anti-DAT antibody (1:5000) was used to visualize immunoprecipitated DAT. Extracts from Flp-In HEK parent line were used as a negative control.

Metabolic labeling to assess DAT phosphorylation

[^{32}P]orthophosphate metabolic labeling was performed as described previously (Bowton et al., 2010). Briefly, Flp-In HEK stable cells transfected with either WT DAT or DAT 615C were seeded on 6 well plates and cultured for 48hrs. Cells were washed once with phosphate free DMEM and incubated in the same medium for 1hr pool at 37^{0}C to deplete the intracellular ATP. Cells were

then supplemented with 1 mCi/ml of [^{32}P]orthophosphoric acid and incubated for 4hrs at 37^{0}C. Cells were then washed 3 times with ice-cold PBS and lysed with RIPA buffer containing protease inhibitors and 1 μM okadaic acid and microcystin each for 30 min at 4^{0}C. Lysates were immunoprecipitated with rat anti-DAT antibody overnight at 4^{0}C. Immunoprecipitated DATs were visualized using SDS-PAGE, followed by autoradiography and densitometry.

Cholera toxin B (CTxB) labeling and confocal microscopy

HEK 293T cells were plated on glass bottom MatTek plates. Cells were transfected with pEYFP-HA-DAT-WT (615R) or pEYFP-HA-DAT 615C 24hrs later and cultured for 48 hrs. Cells were washed 3 times with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgSO_4$ and incubated with 1 μg/mL Alexa 647 conjugated CTxB for 30 min at 4^{0}C. Cells were washed 3 times with ice-cold PBS and fixed using 4% paraformaldehyde for 10 min at room temperature. Fixed cells were washed with PBS and stored in PBS until imaging at 4^{0}C. Confocal imaging was performed in the VUMC Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, HD15052, DK59637 and EY08126). Laser output was adjusted to assure that all fluorescence monitored was non-saturating with respect to fluorophore emission. Intensity correlation quotient (ICQ) to assess colocalization of DAT with CTxB labeled membranes was obtained as previously described (Steiner et al., 2009, Baucum et al., 2010) using the Integration Colocalization Analysis section of WCIF Image

J http://www.uhnresearch.ca/facilities/wcif/imagej/. This analysis computes a correlation in the intensity of two probes across pixels in regions of interest, with ICQ values distributed between -0.5 and +0.5. ICQ values that report evidence for colocalization fall between 0 and + 0.5 and are evaluated for significance using a One sample t-test comparing against a zero ICQ value. Genotype differences in ICQ values were determined by a two tailed, Student's t-test.

Detection of palmitoylated DAT using click chemistry

Flp-in™ HEK stable cells were seeded on 10 cm dishes. 48 hrs. post plating cells were serum starved for 1 hr. and metabolically labeled with 17-octadecynoic acid (17-ODYA) for 6 hrs. at 37^0C. Cells were then chilled on ice and lysed using RIPA buffer. Immunoprecipitation was performed using rat anti-DAT antibody followed by on-bead, copper-catalyzed click chemistry reaction as described elsewhere (Zhang et al., 2010). Briefly, beads were resuspended in 20µL of PBS and 2.25 µL freshly premixed click-chemistry reaction mixture biotin-azide (100 µM, 5 mM stock solution in DMSO), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50 mM freshly prepared stock solution in deionized water), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (100 µM, 2mMstock solution in 1:4 DMSO:t-butanol) and CuSO4·5H2O (1 mM, 50 mM freshly prepared stock solution in deionized water)] for a total approximate reaction volume of 25 µL for 1 h at room temperature. The beads were washed three times with 1 mL of ice-cold RIPA buffer and bound

proteins were eluted using LSB followed by SDS-PAGE and transfer onto PVDF membrane. Attachment of 17-ODYA (palmitoylation mimic) was confirmed using western blot with streptavidin-HRP(1:1000). The same membrane was stripped and reprobed for DAT using rabbit anti-DAT antibody.

Detection of AMPH using High-Pressure Liquid Chromatography (HPLC)

Flp-In HEK cells were seeded in 6 well plates and incubated for 36-48hrs before experiments. Cells were washed twice with warm KRH buffer before incubation with 10 μM AMPH for 5 minutes at 37^0C. Cells were rapidly washed 3 times using ice-cold KRH buffer and lysed using 10 mM TRIS/1 mM EDTA, pH 8.0 buffer (TE) containing protease inhibitors. Total protein concentration was determined using BCA protein assay and used to normalize the AMPH uptake. AMPH uptake was determined using HPLC analysis and data are expressed as nmol AMPH transported per μg protein.

Amperometry

Unpatched amperometric currents were recorded as previously described(Bowton et al., 2010) using Flp-In™ HEK stable cells. Briefly, cells were plated at a density of 10^3 per 35-mm culture dish. To preload cells with DA, attached cells were washed with KRH assay buffer containing 10 mM D-glucose, 100 μM pargyline, 1 mM tropolone, and 100 μM ascorbic acid, and then

incubated with 1 μM DA in assay buffer for 20 min at 37°C. Dishes containing DA loaded cells were then washed three times with external solution (130 mM NaCl, 10 mM HEPES, 34 mM D-glucose, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1.3 mM KH_2PO_4, adjusted pH to 7.35, and 300 mOsm). A carbon fiber electrode (ProCFE; fiber diameter of 5 μm; obtained from Dagan Corporation), juxtaposed to the plasma membrane and held at +700 mV (a potential greater than the oxidation potential of DA), was used to monitor basal and AMPH-evoked DA efflux through DAT as a consequence of DA oxidation. To determine basal DAT-dependent efflux, cells were treated with 10 μM cocaine following establishment of a stable recording baseline. Cells were not voltage-clamped in basal or AMPH (10 μM)-evoked DA efflux experiments. Amperometric currents in response to an addition of AMPH were recorded using an Axopatch 200B amplifier (Molecular Devices, Union City, CA) with a low-pass Bessel filter set at 1 kHz. Traces were digitally filtered offline at 1 Hz using Clampex9 software (Molecular Devices). DA efflux was quantified as the mean peak amperometric current (in picoamperes) +/- SEM.

Quantification and statistics

Western blots were quantified using NIH Image J software, with multiple exposures taken to insure linearity of signal detection. Student's t-test or one-way ANOVA with Bonferroni's post hoc test was used wherever appropriate. GraphPad Prism was used to determine V_{max} and K_m for saturation kinetic

analyses and IC_{50} for inhibition analyses. Significant differences between DAT 615R and 615C values of V_{max}, K_m, and IC_{50} were determined using Student's t-test and a *P* value of 0.05 was considered as evidence of significance.

Chapter III

Altered Regulation and Trafficking Associated with the ADHD-Associated Human DAT Variant R615C

Introduction

The neurotransmitter DA provides critical modulatory influences over circuits subserving reward, locomotor activity, and attention (Carlsson, 1987, Robbins, 2003). As such, alterations in DA signaling contribute to multiple neurological and psychiatric disorders including Parkinson's disease (Chase et al., 1998), attention-deficit hyperactivity disorder (ADHD) (Mazei-Robison et al., 2005), and addiction (Ritz et al., 1987). The re-uptake of DA through presynaptic DATs is a primary mechanism for terminating DA action at presynaptic and postsynaptic receptors and is a major target for psychostimulants, such as cocaine and AMPH.

Multiple studies point to a contribution of variation in the genes encoding DAT, catechol-O-methyl transferase (COMT) and/or DA receptors as influencing risk for ADHD (Gill et al., 1997, Qian et al., 2003, Bobb et al., 2005, Mazei-Robison et al., 2005), the most commonly diagnosed disorder of school age children in the U.S. A further link between DAT function and ADHD is suggested from the therapeutic utility of DAT-interacting psychostimulants, including methylphenidate (Ritalin®) and AMPH preparations (e.g. Adderall®). Brain

imaging studies also point to deficits in DA signaling as a key feature of ADHD (Swanson et al., 2007). Genetic elimination of DAT expression in mice reduces presynaptic DA stores, elevates extracellular DA, and produces hyperactivity in a novel environment (Giros et al., 1996). However, humans that are homozygous for loss of function DAT (*SLC6A4*) alleles exhibit infantile neonatal dystonia rather than ADHD (Kurian et al., 2009), raising questions as to the relevance of compromised DAT function and DA signaling as a key feature in ADHD.

Reasoning that genetic alterations that perturb DAT regulation, as opposed to DAT elimination, could reconcile data from rodent and human studies, we screened ADHD subjects for rare variation producing coding variation in DAT. Previously, we described the anomalous, non-vesicular release of DA by the DAT A559V coding variant (Mazei-Robison et al., 2008), leading to the hypothesis that dysregulated DA availability may be a determinant of risk for ADHD. Here we provide additional evidence for this idea through study of the properties of a second DAT variant, R615C.

Before presenting the results obtained through a characterization of the DAT-R615C variant (DAT 615C), I wish to highlight the evidence from the literature that indicate common regulatory pathways linked to the WT (DAT 615R) transporter. Earlier I described in detail the effects of alterations in intracellular signaling on DAT 615R. Here, I summarize findings pertinent to studies described in this chapter. Acute treatment with β-PMA (200nM, 30 minutes), leads to a reduction in DA uptake activity and a loss of DAT from the plasma

membrane (Huff et al., 1997, Loder and Melikian, 2003, Sorkina et al., 2005). Recently, Cremona and colleagues presented evidence that PKC-induced loss of surface DAT occurs through flotillin-1 enriched membrane rafts (Cremona et al., 2011), a finding previously reported to occur via clathrin-mediated mechanism (Sorkina et al., 2005). The data presented in this thesis are consistent with a membrane raft-mediated internalization mechanism subserving regulated DAT trafficking. Acute AMPH treatment has been shown to produce flotillin-1- and CaMKII-dependent DA efflux (Fog et al., 2006, Cremona et al., 2011), to reduce DA transport activity, and to increase DAT endocytosis (Wei et al., 2007b, Chen et al., 2010). In this chapter, I describe the results I obtained from studies that compare the alterations between DAT 615R and DAT 615C with β-PMA and AMPH, with an emphasis on changes in AMPH-mediated regulation of DAT 615C.

Results

Identification of a functional DAT coding variant in an ADHD subject

Using polymorphism discovery methods (Li et al., 2002), we screened coding exons and intron-exon junctions of the SLC6A4 gene in 417 ADHD subjects. Although a number of variants have been identified that alter the coding of DAT, all known variants are rare (allele frequency <1%) (Mazei-Robison et al., 2005). In an Irish cohort of 100 subjects receiving a DSM-IV diagnosis of ADHD

(Bellgrove et al., 2009), we identified a nonsynonymous, single nucleotide polymorphism (SNP, 2026 T/C) that converts a highly conserved Arg residue at position 615 to Cys (R615C). R615 is completely conserved in mammalian DATs and resides in a generally well-conserved region of the DAT C-terminus (Figure 5), a region implicated in transporter somatic export, surface trafficking and protein-protein interactions (Torres et al., 2001, Bjerggaard et al., 2004, Fog et

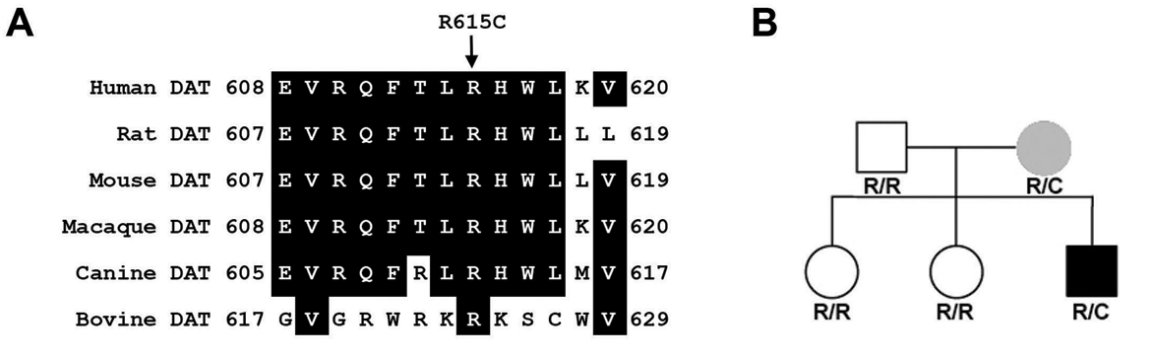


Figure 5 Sequence Alignment and Pedigree Analysis of DAT 615C: (A) Sequence alignment showing conservation of Arg 615 across mammalian DATs. **(B)** Pedigree of the ADHD proband carrying the R615C variant. The subject (black box) is heterozygous for the R615C-DAT variant. Transmission of the mutant allele occurred from mother (grey circle). Other family members (father and two older sisters) are unaffected.

al., 2006). The subject, a Caucasian male of European origin and heterozygous for the R615C variant was 13 years old at the time of assessment and possessed a strong clinical and research diagnosis of combined-type ADHD (Connor's parent rating scale-inattentive symptoms, T=70; hyperactive symptoms, T=75, total score=74, WISK-III UK IQ=141). Pedigree genotyping revealed that the mutation was transmitted from the proband's mother who is retrospectively suspected to have suffered from ADHD as a child (Wender-Utah=59), although

no clinical diagnosis is currently available. The subject's father and two older sisters, who do not carry the variant, are unaffected (Figure 5). The R615C proband has been successfully treated with methylphenidate.

To search for functional evidence that the R615C variant may be causal in the proband's ADHD, we compared the activities of DAT 615R and DAT 615C in

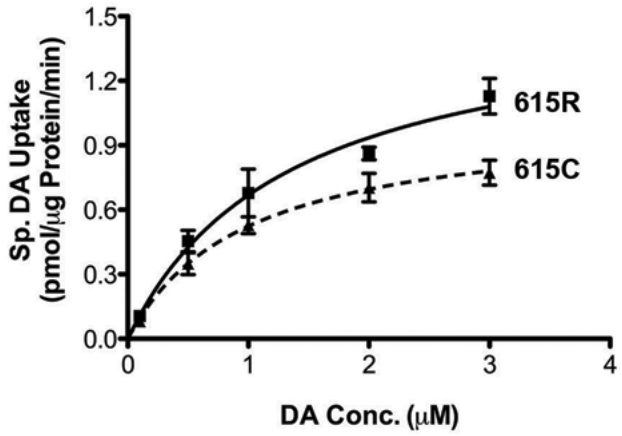


Figure 6 DA Transport Saturation Kinetic Analysis: DA transport V_{max} and K_m are expressed as pmol/µg protein/min and µM respectively. Data derive from a nonlinear, single-site curve fit to n=3 saturation kinetic assays with error bars indicating the SEM at each concentration.

stably transfected HEK cells. To limit confounds associated with different sites of integration, we created stable Flp-In™ HEK lines that express either of the two transporters from a common locus, under the control of the same promoter (Sauer, 1994). Saturation kinetic analysis revealed a significant reduction in DA transport V_{max} for the R615C variant (615R, 1.6±0.2 vs. 615C, 1.0±0.1 pmol/µg protein/min, $P<0.005$, two-tailed Student's t-test) without a significant change in DA K_M (1.3±0.4 vs. 1.0±0.3 µM) (Figure 6).

The reduced DA transport capacity was associated with a reduction in steady-state DAT protein levels (86±3% of DAT 615R; n=6, P<0.005, two-tailed

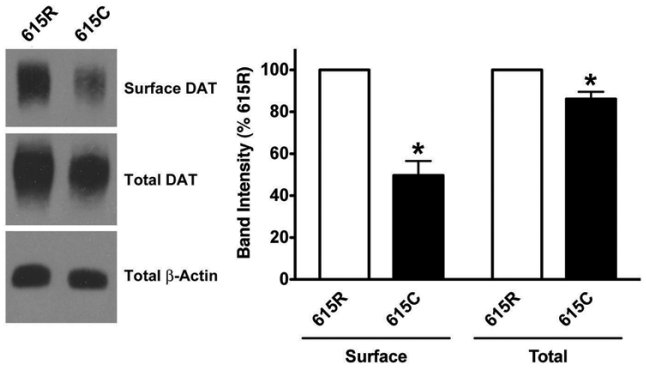


Figure 7 Analysis of Total and Surface DAT Protein: Left: Representative western blot showing reduction in the total and surface DAT for the R615C variant compared to 615R. Total protein levels of β-actin, an intracellular loading control, were similar for both 615R and 615C. **Right:** Quantification from six independent experiments +/- SEM, indicating a significant reduction in the R615C variant compared to 615R for both total and surface expression. In the blot shown, total protein samples were diluted 5 fold compared to the surface protein fraction to retain signal linearity

Student's t test) and an even greater loss of transporter surface transporter expression (50±7% of DAT 615R; n=6, P<0.0001, two-tailed Student's t test) (Figure 7), the latter determined by whole-cell biotinylation. However, we observed no significant differences in the IC_{50} values for cocaine (615R, 7.7±2.0 vs. 615C, 10.0±2.1 nM), GBR 12909 (615R, 9.7±3.0 vs. 615C, 10.9±2.0 nM), or AMPH (615R, 329±135 vs. 615C, 226±101 nM). We did find a small, but statistically significant loss of potency for methylphenidate (615R 585±290 vs. 615C, 821±160 nM, P<0.05, two-tailed Student's t-test). Thus, although surface levels and DA transport capacity are reduced, DAT 615C appears to exhibit comparable interactions with DA and DAT antagonists as seen with DAT 615R.

Anomalous modulation of DAT 615C by AMPH

AMPH produces two alterations in DAT-expressing cells (Sulzer et al., 2005). First, AMPH induces (sec-min) non-vesicular DA release (efflux), mediated by

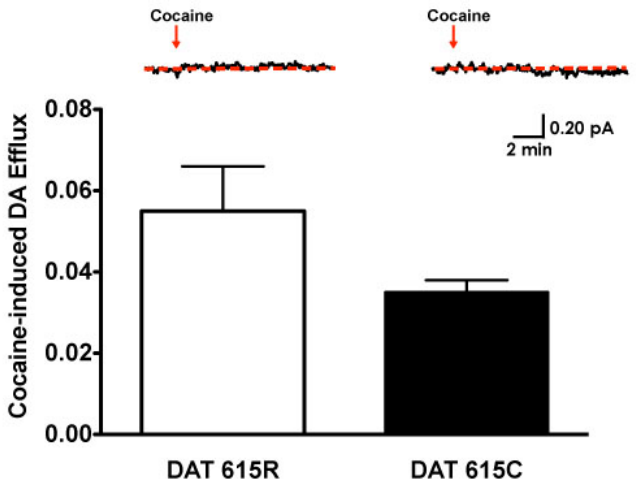


Figure 8 Amperometric Detection of Basal DA Efflux: DA preloaded Flp-In HEK stable cells display no significant differences in basal DA efflux between DAT 615R and DAT 615C (n=5, *P*>0.05, Student's t-test). Data collected in collaboration with Dr. Aurelio Galli by Dr. Erica Bowton.

reverse transport of cytoplasmic DA that is supported by CaMKII-mediated phosphorylation of the DAT N-terminus (Khoshbouei et al., 2003, Fog et al., 2006). Second, AMPH treatment of DAT-expressing cells has been reported to produce both a rapid translocation of the transporter to the cell surface (Furman et al., 2009a), with more prolonged treatments (10-30 min) producing net transporter internalization (Chen et al., 2010). Using carbon fiber amperometry (Bowton et al., 2010), we monitored basal efflux from DAT 615R and DAT 615C

cells that were preloaded with DA (Figure 8). We found no significant difference in DA efflux comparing WT and mutant transporters.

We also monitored AMPH-triggered DA efflux from DA-preloaded cells and, as shown in Figure 9A, 615R and 615C-expressing cells exhibited comparable AMPH-evoked DA efflux, measured using the peak amplitude of amperometric responses. This finding was surprising due to the reduced steady-

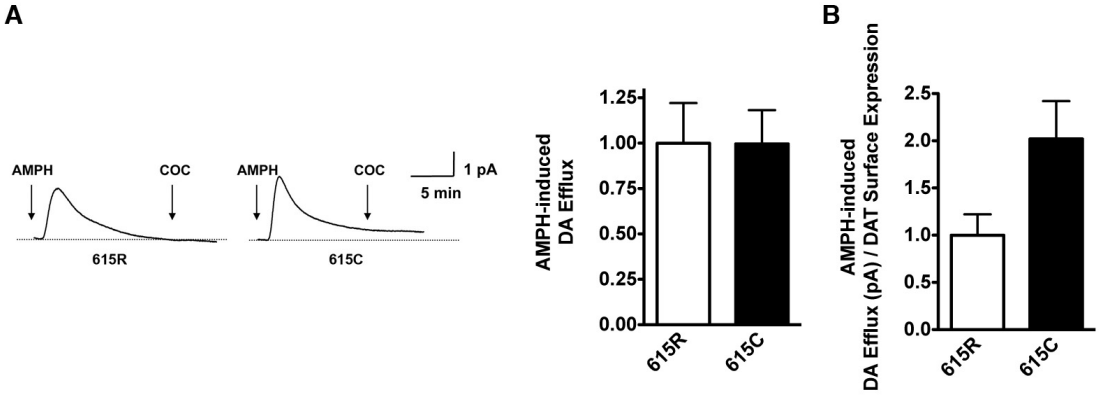


Figure 9 AMPH-induced DA Efflux: (A) Flp-In 293 stable cells were preloaded with 1 µM DA for 20 min and AMPH-induced DA efflux was measured using carbon fiber amperometry. AMPH-induced DA efflux from DAT 615C was not significantly different than 615R (1.0±0.22 pA for 615R vs. 0.996±0.18 pA for 615C). (n=5, P>0.05, Student's t-test) Data was collected by Peter Hamilton. **(B)** AMPH-induced DA efflux from DAT 615C was ~ 2 times more when the efflux was normalized to the respective surface expression.

state DAT surface expression of the R615C variant noted above. Indeed when we normalized the AMPH-induced DA efflux to respective DAT surface expression, a significantly greater DA efflux was detected from DAT 615C expressing cells (Figure 9B). Since it was not possible to obtain DA efflux and surface expression from the same experimental setup no further statistical analysis was performed on the data. We found a possible answer to this conundrum in examining the impact of AMPH on transporter surface expression.

Whereas treatment of 615R expressing cells with 10 µM AMPH for 30 min produced the expected reduction in transporter surface expression, without any change total DAT protein levels (Figure 10), the same treatment failed to reduce surface expression of DAT 615C.

Consistent with these findings, AMPH reduced DA transport from DAT 615R cells, but not from DAT 615C cells (Figure 11). Equivalent findings were also obtained following transient transfection of DAT 615R or DAT 615C into HEK-293T cells (data not shown) or CAD cells, a catecholamine-producing neuronal cell line (Qi et al., 1997) (Figure 12).

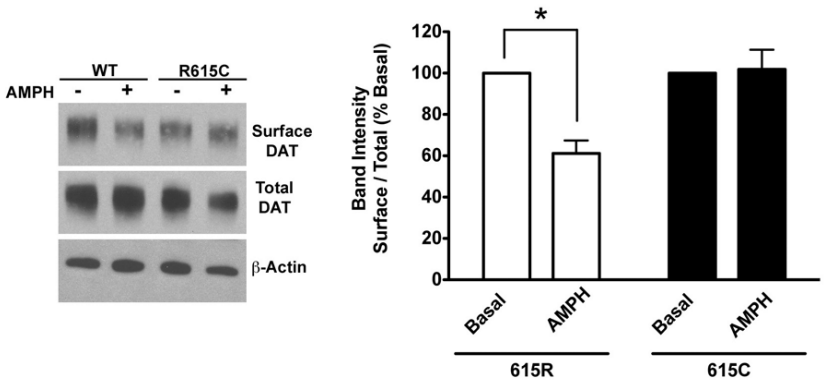


Figure 10 Effect of AMPH DAT Surface Expression: Left: Representative western blot showing total and surface DAT protein levels with or without AMPH treatment (10 µM, 30 minutes) **Right:** Quantification of AMPH-induced decrease in DAT surface protein levels expressed as surface/total. Data were normalized to basal DAT surface/total expressions as 100%. AMPH treatment caused a significant (62±7%) decrease in DAT 615R surface expression whereas no change was detected in 615C expression (95±8%). Significant differences were calculated using Sstudent's t-test. (n=7, $P<0.0001$)

DAT internalization is known to occur following activation of PKC, by phorbol esters such as β-PMA (Loder and Melikian, 2003). As shown in Figure 13, we found that unlike DAT 615R, DAT 615C displayed no net endocytosis

(109±9% of vehicle treated cells) and, consistent with this finding, also displayed no reduction in DA transport activity (98±2% of control), following β-PMA treatment (200 nM, 30 min). On the other hand, similar β-PMA treatments caused internalization of DAT 615R (72±4% of vehicle treated cells, P<0.005, two-tailed Student's t-test) and a significant reduction in DA transport activity (61±6% of control, P<0.001, two-tailed Student's t-test)

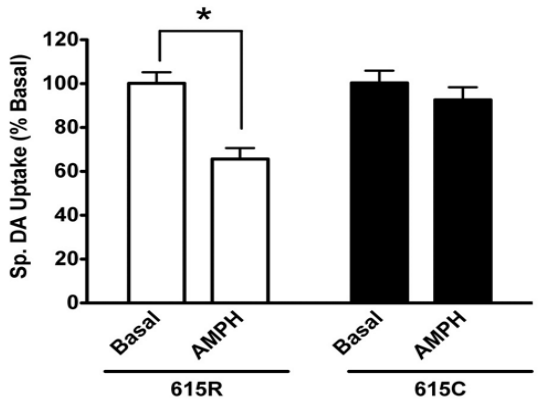


Figure 11 Effect of AMPH DA Uptake Activity: AMPH-mediated (10 μM, 30 minutes) reduction in the DA transport seen in DAT 615R (white bar; AMPH) versus 615C (black bar; AMPH). Basal DA transport levels of each cell lines were used to normalize AMPH-mediated reductions. AMPH caused a significant decrease (66±5 % of basal) in the DA transport of DAT 615R whereas no significant changes were detected for 615C (93±6 % of basal). Significant differences were calculated using Student's t-test. (n=3, P<0.001) .

AMPH transport is required to modulate DAT trafficking as demonstrated by studies of a transport incompetent DAT mutant. (Kahlig et al., 2006). Intracellular injection of AMPH, however, produces internalization of this mutant, suggesting that intracellular AMPH activates signaling pathways sufficient to produce DAT internalization. Thus, the R615C variant could transport AMPH more efficiently than wildtype DA, leading to the appearance of equivalent DA

efflux despite reduced surface expression. However, HPLC assays of DAT 615C cells treated with AMPH under the same conditions used for efflux assays revealed similar levels of intracellular AMPH for DAT 615R and DAT 615C cells (615R, 4.9±1.5 nM vs. 615Cm 5.1±1.5 nM, n=4, P>0.05, two-tailed Student's t-test).

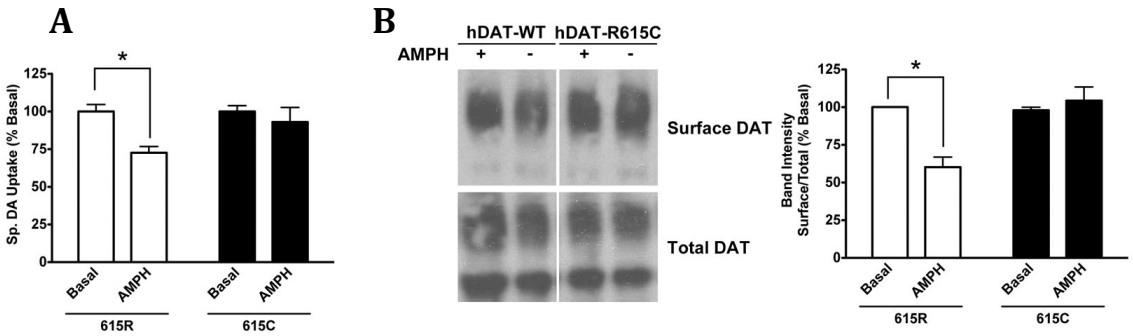


Figure 12 Effect of AMPH on DA uptake and DAT surface expression in transiently transfected CAD cells: (A) Basal DA transport levels were normalized to respective cell lines and AMPH-mediated reductions were calculated. AMPH caused a significant decrease (72±4 % of basal) in the DA transport of DAT 615R whereas no significant changes were detected for 615C (93±9 % of basal). Significant differences were calculated using one-way ANOVA followed by Bonferroni's post hoc test. (n=4, P<0.01) **(B) Left:** Representative western blot showing surface DAT protein levels after AMPH treatment (10 µM, 30 minutes). **Right:** Quantification of AMPH-induced decrease in DAT surface levels, expressed as surface/total. Data is normalized to basal DAT surface/total expressions as 100%. AMPH treatment caused a significant (63±6%) decrease in the DAT 615R expression while no change was detected in 615C expression (104±9%). Significant differences were calculated using Student's t-test. (n=4, P<0.0001).

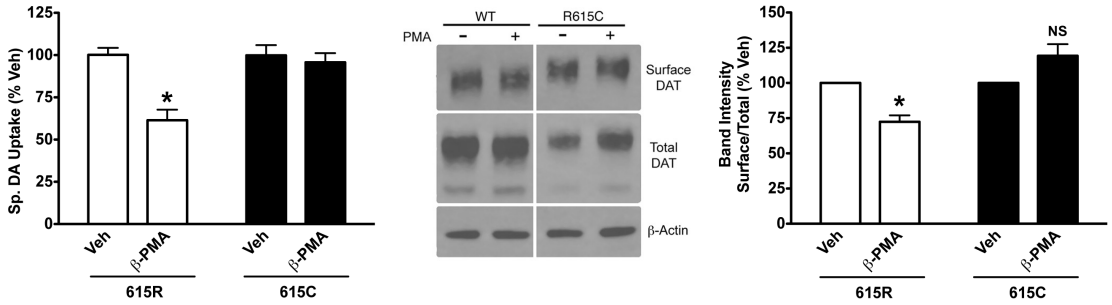


Figure 13 Effect of β-PMA on DA Uptake and DAT Surface Expression in Flp-In HEK Cells: (A) Data are normalized to vehicle treatments for individual cell lines. β-PMA caused a significant decrease (61±6 % of veh.) in the DA transport of DAT 615R while no significant changes were detected for 615C (95±5 % of veh.). Significant differences were calculated using one-way ANOVA followed by Bonferroni's post hoc test. (*p< 0.01; n=3) **(B) Left:** Surface DAT protein level after β-PMA treatment (200 nM) for R615C variant is significantly greater than DAT 615R protein level. **Right:** Data is normalized to vehicle treated DAT 615R surface/total expressions as 100%. β-PMA treatment caused a significant (72±4%) decrease in the DAT 615R expression while no change was detected in 615C expression (119±9%). Significant differences were calculated using Student's t-test. (*p<0.05; n=3)

DAT R615C exhibits accelerated rates of constitutive endocytosis and recycling.

To define the mechanisms supporting loss of AMPH-induced surface trafficking of the DAT R615C variant, we implemented kinetic surface biotinylation assays that report rates of membrane protein endocytosis and recycling (Deken et al., 2003, Holton et al., 2005). By monitoring the amount of surface biotinylated DAT that displays resistance to a biotin-stripping reagent, we can quantify the extent of transporter internalization under basal and AMPH-treated conditions. Here, we discovered that the DAT R615C variant displays an accelerated, constitutive internalization rate relative to the wildtype transporter (Figure 14). Moreover, whereas AMPH significantly increased the rate of

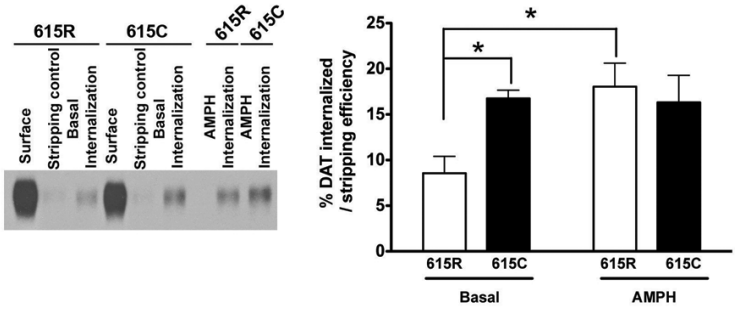


Figure 14 Accelerated constitutive Endocytosis Associated with DAT 615C: Left: Representative western blot showing increased basal endocytosis associated with the R615C variant. AMPH treatment (10 µM, 30 minutes). **Right:** Quantification of internalization rates normalized to stripping efficiency. Basal internalization rate of DAT 615C was significantly increased compared to 615R (16.7±0.9% for 615C vs. 8.5±1.8% for 615R; n=3, $P<0.05$, Student's t test). AMPH treatment significantly increased internalization rate of DAT 615R (8.5±1.8% for basal vs. 18±2.6% for AMPH; n=3, $P<0.05$, Student's t test) without affecting the R615C variant (16.7±0.9% for basal vs. 16.3±2.9% for AMPH; n=3, $P>0.05$, Student's t test).

internalization for DAT 615R, we observed no change in DAT 615C internalization rate over that seen under basal conditions.

Possibly, accelerated internalization of DAT 615C under basal conditions explains this transporter's significantly diminished basal surface expression. Alternatively, a change in surface recycling could also explain this difference, and also account for insensitivity to AMPH. We therefore determined the extent of DAT 615R and 615C recycling by biotinylating the full pool of recycling transporter molecules at 37^0C, followed by stripping away any surface resident transporters at 4^0C. Upon warming the cells back to 37^0C, we determined the rate of reappearance of either wildtype or DAT 615C. Thirty minutes after stripping and shifting back to 37^0C, approximately 40% of wildtype DAT remained intracellular under basal conditions, and the extent of recycling was not further decreased by AMPH. In contrast, approximately 90% of the DAT R615C variant

recycled under basal conditions. This capacity for recycling was also not diminished by AMPH (Figure 15). Thus, AMPH treatment produced a net internalization of wildtype DAT protein by enhancing transporter endocytosis rates without a compensatory increase in recycling rates. DAT 615C constitutively endocytosis and recycles at a much faster rate than wildtype transporters, and this difference is not impacted by AMPH treatment. Thus, the failure of DAT 615C to exhibit AMPH-triggered internalization and a commensurate reduction in DA uptake is most consistent with the transporter's

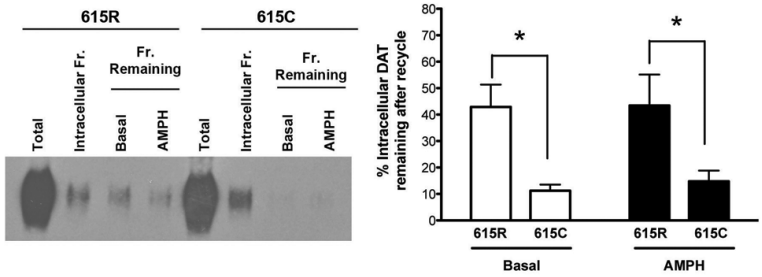


Figure 15 DAT 615C Exhibits Accelerated Recycling: Left: Representative western blot showing accelerated recycling of R615C variant. AMPH treatment (10 µM, 30 minutes) did not impact the recycling rate for both DAT 615R and 615C. **Right:** Quantification of recycling rate normalized to intracellular DAT fraction. A bigger fraction of DAT still remains intracellularly after constitutive recycling for DAT 615R compared to 615C (42.9±8% for 615R vs. 11.2±2.3% for 615C; n=6, $P<0.005$, Student's t test). AMPH treatment did not alter fractional DAT remaining inside after recycling (basal-42.9±8% vs. AMPH-43.4±11.7% for DAT 615R and basal-11.2±2.3% vs. AMPH-14.8±4.1% for DAT 651C; n=3-6, $P>0.05$, Student's t test).

efficient recycling through a pathway that cannot support acceleration of trafficking rates, which are already elevated.

Discussion

ADHD is the most commonly diagnosed disorder of childhood (Smith et al., 2009). Multiple lines of evidence, noted in Chapter I, suggest that alterations in DA signaling, including changes in DAT expression or function (Mazei-Robison et al., 2005), can increase risk for the cognitive and hyperactive perturbations found in ADHD. In a meta-analysis of association studies examining *SLC6A4* polymorphisms in ADHD, Gizer and colleagues concluded that the locus is likely to harbor multiple functional variants whose variable influence across families can account for differences in effect sizes detected (Gizer et al., 2009). To date, however, the majority of genetic studies implicating DAT gene variation with ADHD, or the efficacy of ADHD medications, derive from analysis of a variable number tandem repeat (VNTR) in the 3'-untranslated region. As the functional impact of this VNTR remains ill defined (Winsberg and Comings, 1999, Kirley et al., 2002, Kirley et al., 2003), aligning these studies with specific alterations in DA signaling is difficult. Rather than focus on common DAT variation of uncertain impact, we sought insights into the DA contributions to ADHD via a search for highly penetrant, DAT coding variants. Studies of rare, heritable forms of Alzheimer's disease (Lemere et al., 1996) and Parkinson's disease (Groen et al., 2004) provide cogent examples of how the elucidation of rare gene variation can lead to novel pathophysiological concepts. Moreover, intensive study of rare variants is justified as such studies can help define broader networks that may

elucidate a common underlying pathophysiology (Abrahams and Geschwind, 2010).

Recently, we described properties of the DAT coding variant, A559V, identified in two male siblings with ADHD (Mazei-Robison et al., 2005). We observed that A559V displays increased DAT channel activity and spontaneous, nonvesicular DA release that can be greatly enhanced by membrane depolarization (Mazei-Robison et al., 2008, Bowton et al., 2010). Moreover, spontaneous DA efflux can be attenuated by the ADHD therapeutic AMPH. Here, we describe a second, rare, ADHD-associated DAT coding variant, R615C, where substitution establishes profound basal and regulatory alterations. Although the pedigree harboring the R615C variant is small, with only a single affected carrier, the regulatory disruption we report provides further evidence that changes in DAT-dependent DA signaling contributes to risk for ADHD. Studies with knock-in mice that harbor the R615C variant, similar to those underway in our lab involving the A559V variant (Mergy M.A. and Blakely R.D., unpublished observations) should allow us to assess which of the multiple *in vitro* alterations in trafficking and regulation we have identified occurs *in vivo*, and how these or other changes impact synaptic DA inactivation.

Multiple elements of DAT's cytoplasmic N- and C-termini have been implicated in basal and regulated control of DAT surface expression, stability and activity. The DAT N-terminus has been the focus of much investigation that examines AMPH-induced DA efflux (Khoshbouei et al., 2004, Fog et al., 2006,

Binda et al., 2008). In contrast, Holton and colleagues have proposed that sequences in the DAT C-terminus (residues 587-596) influence basal and PKC-modulated transporter trafficking (Holton et al., 2005). The distal C-terminus (residues 618-620) bears a type II PDZ domain interaction motif that has been shown to dictate interactions of the transporter with the PDZ domain protein, PICK1, and possibly enhance DAT surface expression (Torres et al., 2001). Interestingly, Bjerggaard and colleagues found that substitution of AAA for the RHW sequence immediately upstream of the PDZ recognition motif leads to ER retention, but preserves the ability of mutant DAT to bind PICK1 (Bjerggaard et al., 2004). The R615C mutation lies in the RHW sequence, and thus an effect on ER/Golgi export may contribute to the transporter's reduced surface expression and DA transport V_{max}. As previously reported, we found AMPH treatments produce net transporter endocytosis (Saunders et al., 2000, Kahlig et al., 2004, Boudanova et al., 2008a). Activation of PKC isoforms with β-PMA also induces net transporter internalization (Miranda et al., 2007, Boudanova et al., 2008b, Cremona et al., 2011), an effect proposed to be mediated by DAT C-terminal sequences (Holton et al., 2005). Remarkably, we found that the R615C variant demonstrates complete resistance to the trafficking effects of both AMPH and PKC activation. These findings led us to monitor the kinetics of DAT 615C basal and regulated surface trafficking. We found that DAT 615C displays a significantly accelerated rate of both endocytosis and recycling as compared to wildtype DAT. In our cells, treatment with AMPH accelerates the endocytic rates

of wildtype DATs, with no detectible change in transporter recycling. Thus, it seems likely that DAT 615C lacks the dynamic range needed to display regulated endo/exocytosis due its high rate of constitutive trafficking.

Alterations in basal, as well as AMPH- and β-PMA-induced trafficking suggest that the response of DAT 615C to intracellular signaling mechanisms are perturbed, either due to an inaccessibility of transporters to critical proteins controlling endocytosis through regulated pathways or a mislocalization of transporters to compartments that do not support regulated endocytosis, or both. In the next chapter, I describe studies that establish a novel role of CaMKII to support DAT 615C activity, along with changes in the mutant transporter's membrane microdomain association.

Chapter IV

Role of Signaling Pathways, Microdomains, and the C-Terminus in the Altered Behavior of DAT 615C

Introduction

Our initial characterization of the DAT R615C variant indicated a reduced basal DA transport V_{max}, a decreased basal total and surface protein expression, a lack of β-PMA and AMPH-mediated downregulation of transport activity and endocytosis, and accelerated basal trafficking. In this chapter, I report on my efforts to delineate mechanisms underlying the lack of AMPH actions on the DAT 615C variant. By way of introduction to these studies, I discuss aspects of AMPH-induced changes in signaling pathways that support the psychostimulant's actions to produce DA efflux and DAT trafficking.

AMPH is a DAT substrate and thereby uses DAT to enter the intracellular environment, although at high concentrations AMPH can also enter cells in a DAT-independent manner (Jones et al., 1998, Sulzer et al., 2005). Since AMPH is a weak base with the ability to be concentrated in synaptic vesicles by VMAT proteins, the psychostimulant also depletes synaptic vesicles of DA by dampening the vesicular proton gradient. AMPH also causes an increase in intracellular Ca^{2+} (Gnegy et al., 2004) that in turn activates CaMKII and promotes CaMKII-dependent DA efflux (Fog et al., 2006). With more prolonged exposure (10-30 min), AMPH produces DAT downregulation via a clathrin-mediated

endocytosis of surface transporters and a consequent reduction in DA transport activity (Saunders et al., 2000). Whether AMPH-dependent DAT internalization is dependent on CaMKII activity is unknown.

Physical association between DAT and CaMKII takes place via a three amino acid motif (RHW; amino acids 615-617) in the distal C-terminus domain of DAT and mutating these residues to alanine leads to loss of CaMKII association as well as AMPH-mediated DA efflux. Since DAT 615C lies in this motif, we speculated that altered CaMKII association could accompany or underlie the lack of AMPH action in producing net endocytosis of the mutant transporter and performed experiments to address these possibilities. Recently, Cremona and colleagues also showed that flottilin-1 interaction is essential for AMPH-mediated DA efflux, a CaMKII-dependent phenomenon. As such, I sought to investigate whether changes in flotillin-1/DAT 615C association. Finally, I sought to determine whether it is the loss of Arg *per se* or the conversion of Arg to Cys, or both, that renders the DAT 615C insensitive to AMPH treatment.

Results

DAT 615C exhibits a CaMKII-dependent state of functional inactivation.

AMPH acts to mobilize intracellular Ca^{2+}, which leads to CaMKII activation (Gnegy et al., 2004, Wei et al., 2007a). Ca^{2+} mobilization and CaMKII activation have been shown to be critical for AMPH evoked DA efflux, likely through

phosphorylation of the DAT N-terminus(Khoshbouei et al., 2004, Fog et al., 2006). Most relevant to my trafficking studies, CaMKII inhibition has been shown to preclude AMPH-mediated DAT, net surface redistribution (Wei et al., 2007a). Since DAT 615C effluxes DA in response to AMPH, yet fails to traffic, I investigated whether CaMKII/DAT interactions that depend on residues 615-617, remain intact (Fog et al., 2006). As shown in Figure 16, we detected an ~2 fold increase in recovery of DAT 615C/CaMKII vs. 615R/CaMKII complexes. Since published data is most consistent with CaMKII phosphorylation of DAT N-

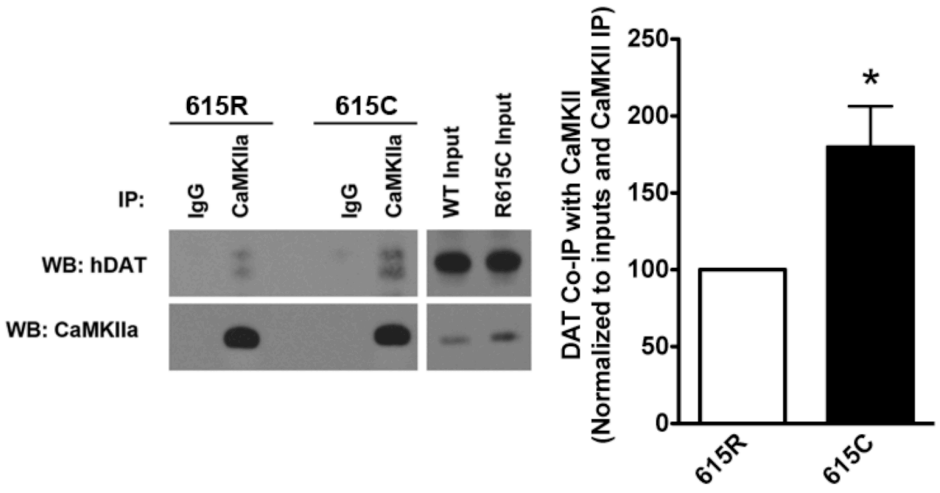


Figure 56 Increased Basal CaMKII Association of the R615C Variant: Left: Representative western blot showing increased association between DAT 615C and CaMKII compared to 615R. **Right:** Quantification of DAT co-IP with CaMKII normalized to respective inputs. Significantly larger pool of 615C is co-precipitated with CaMKII (179±26 % of 615R; n=3, $P<0.05$, Student's t test).

terminal Ser residues, after binding to the DAT C-terminus results in (Fog et al., 2006), I asked whether the increased CaMKII association of the R615C variant is

paralleled by increased transporter phosphorylation. Indeed, immunoprecipitation of DAT 615R or 615C from [^{32}P]orthophosphate labeled cells, when data were normalized by total protein input, revealed a significantly increased basal phosphorylation of the R615C variant (Figure 17). Given that, in the stable cell lines used, total DAT 615C protein levels are modestly but significantly reduced when compared to total WT DAT protein, the elevation in DAT 615C

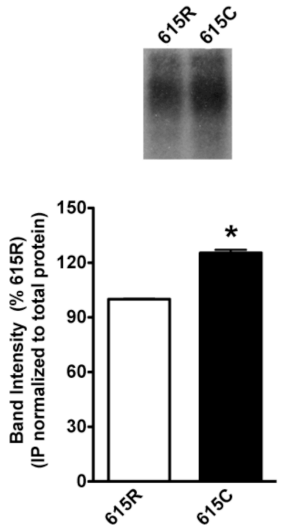


Figure 17 Increased Basal Phosphorylation of the R615C Variant: Top: Representative autoradiograph indicating increased basal phosphorylation of DAT 615C compared to 615R **Bottom:** Quantification of DAT bands from the autoradiograph showing significant increase in basal phosphorylation levels of DAT 615C (125±1.6 % of 615R; n=3, $P<0.05$, Student's t test). Equal total protein amounts were used for IP following the metabolic labeling.

phosphorylation is likely to be actually somewhat higher than illustrated. If CaMKII phosphorylates DAT at the cell surface only, the more substantial loss in DAT 615C surface expression argues that CaMKII phosphorylation of DAT may be even more elevated.

To assess whether the increased CaMKII association and basal phosphorylation associated with the DAT 615C variant has functional consequences, we treated DAT 615R or 615C cells with the CaMKII inhibitor KN-93, either during AMPH treatments, or as a pretreatment before AMPH addition. Treatment of cells with KN-93 alone failed to impact the DA transport activity of DAT 615R cells (Figure 18A). However, the same treatment blocked the reduction imposed on DA transport activity by AMPH treatment. As shown above, AMPH imposed no significant effect on DA transport activity for DAT 615C cells (Figure 18A). Surprisingly, KN-93 alone significantly decreased the DA transport activity of 615C cells, and this effect was even more pronounced when KN-93 was combined with AMPH. The specificity of these drug treatments was confirmed through the use of KN-92, a structural analog of KN-93 that does not inhibit CaMKII (Figure 18B). These data are consistent with the hypothesis that excessive, constitutive CaMKII regulation of DAT 615C produces a shift of DAT proteins to AMPH-insensitive endocytosis and recycling pathways. In support of this idea, biotinylation studies demonstrated that CaMKII blockade with KN-93 antagonized AMPH-induced reductions in cell surface levels of wildtype DAT (Figure 19), similar to its ability to block AMPH-induced reductions in DA uptake. In contrast, and distinct from its effect on DA uptake, KN-93 treatment of DAT 615C cells produced did not reverse AMPH-induced transporter internalization (Figure 19). These unexpected data reveal a CaMKII-dependent capacity for

modification of DA transport function specific to DAT 615C that occurs in a

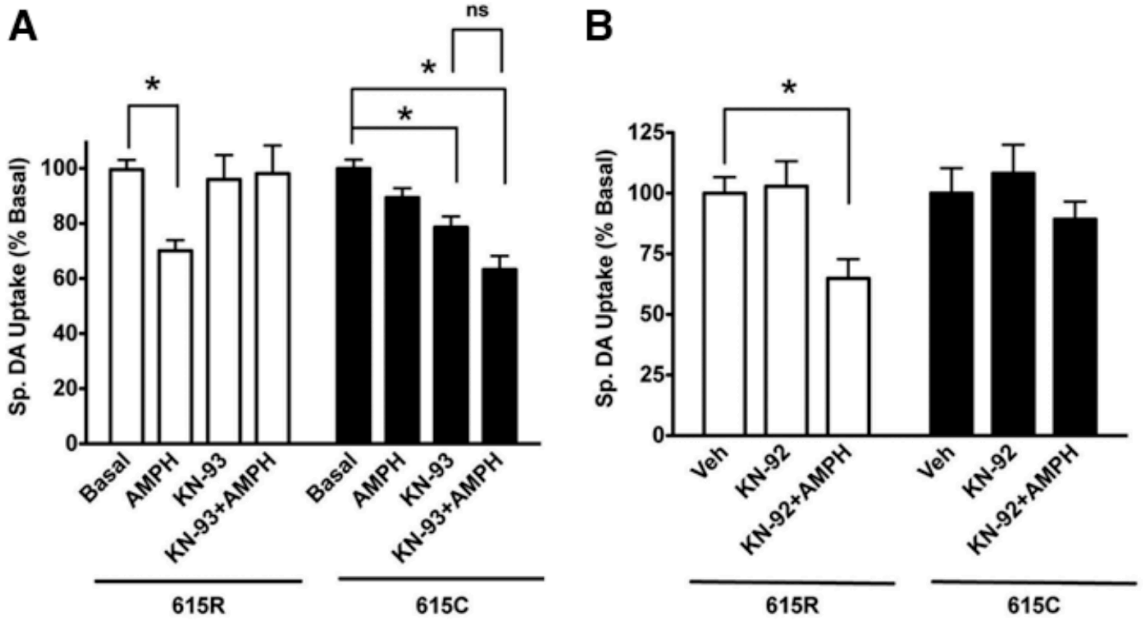


Figure 18 Effect of KN-93 and KN-92 on AMPH-mediated DA Transport Reduction: (A) KN-93 (1 μM, 30 minutes) treatment (black bar, KN-93) caused a significant reduction in the DA activity of DAT 615C (79±4% of basal) whereas no effect was seen with the same treatment of DAT 615R (white bar, KN-93, 95±9% of basal). Pretreatment with KN-93 before AMPH (10 μM, 30 minutes) addition blocked the AMPH-mediated reduction in DA transport of DAT 615R (white bars, AMPH (70±4%) vs. KN-93+AMPH (98±10%)) whereas DAT 615C showed a further, significant reduction in transport activity (black bars, AMPH (89±3%) vs. KN-93+ (63±5%)). The DA transport reduction upon KN-93+AMPH treatment was not significantly different from the reduction obtained by treatment with KN-93 alone in the R615C variant (79±4% for KN-93 vs. 63±5% for KN-93+AMPH). Data were normalized to basal DA uptake of respective cell lines. Significant differences were calculated using one-way ANOVA followed by Bonferroni's post hoc test. (n=3-6, $P<0.0001$) **(B)** The inactive analog of KN-93, KN-92 did not affect DA uptake in either DAT 615R (white bar, KN-92; 102±10% of basal) or 615C (black bar, KN-92; 108±12%of basal). Pretreatment with KN-92 (1 μM, 30 minutes) also did not block the AMPH-mediated (10 μM, 30 minutes) DA transport reduction in DAT 615R (white bar, KN-92+AMPH, 65±8% of basal) and had no effect on 615C (black bar, KN-92+AMPH, 90±7% of basal). Data were normalized to basal DA uptake of respective cell lines. Significant differences were calculated using one-way ANOVA followed by Bonferroni's post hoc test. (n=3, $P<0.05$)

trafficking-independent manner.

The ability of CaMKII to enhance DAT 615C DA transport activity could arise from either a shift of transporters to higher affinity DA recognition or from a concentration-independent increase in DA transport capacity. To address these

possibilities, we conducted saturation kinetic analysis for the DAT R615C variant, in the presence or absence of KN-93+AMPH (Figure 20). These drug treatments produced no significant change in DA transport K_m (basal, 5.4±1.5 vs. KN-93+AMPH, 3.8±0.6 μM), but generated a significant reduction in DA transport V_{max} (basal, 65±10 vs. KN-93+AMPH, 42±4 pmol/well/min, $P<0.05$, two-tailed Student's t-test).

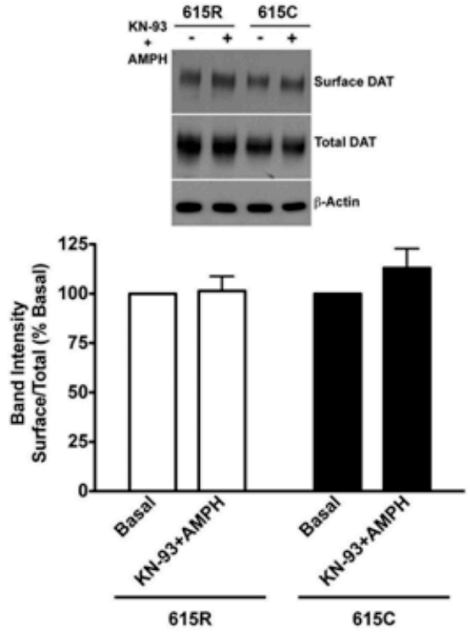


Figure 19 Effect of KN-93 on AMPH-induced DAT Internalization: Top: Representative western blot showing KN-93+AMPH treatment reduction in DAT surface levels for both DAT 615R and 615C. **Bottom:** Quantification showing KN-93+AMPH (1 μM KN-93 for 30 minutes followed by 10 μM AMPH for 30 minutes) treatment rescued the reduction in the DAT surface expression of DAT 615R (101±7% of basal) similar to results obtained in DA uptake assays. KN-93+AMPH treatment showed no change (113±10% of basal) in the surface levels of DAT 615C. The differences obtained from the KN-93+AMPH treatment were not statistically significant from their basal correlates assessed by Student's t-test. (n=7, $P>0.05$)

These findings reveal that CaMKII activity is required to sustain R615C in an active state. In the absence of CaMKII activity, DAT 615C switches to a state

incapable of DA transport but does not move to a state with increased net endocytic capacity.

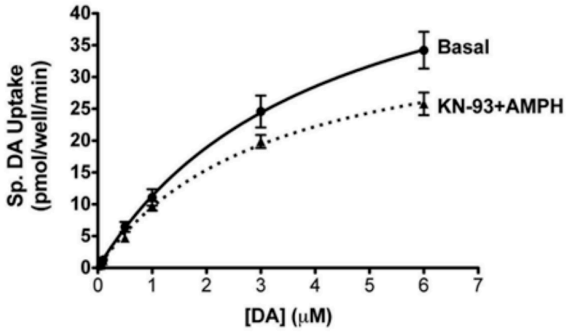


Figure 20 Effect of KN-93+AMPH Treatment of DA Transport Kinetics of DAT 615C: KN-93+AMPH (1 μM KN-93 for 30 minutes followed by 10 μM AMPH for 30 minutes) treatment resulted in significant reduction in DA transport V_{max} for the R615C variant. DA transport V_{max} and K_m are expressed as pmol/well/min and μM respectively. A significant decrease in the DA transport V_{max} was observed after KN-93+AMPH treatment (65±10, basal vs. 42±3, KN-93+AMPH; n=4, *P*<0.05, Student's t test) without a significant change in K_m (5.4±1.5, basal vs. 3.8±0.6, KN-93+AMPH; n=4, *P*>0.05, Student's t test).

DAT 615C demonstrates altered localization to membrane microdomains.

As noted above membrane microdomains have been reported to associate with and influence DAT conformations, to constrain DAT lateral mobility, to be required for PKC-mediated internalization, and to dictate CaMKII-mediated DA efflux. DAT has also been shown to localize to membrane microdomains that are enriched for the protein flotillin-1. Our co-immunoprecipitation studies revealed reduced levels of flottilin-1 in extracts of cells stably transfected with DAT 615C as compared to extracts from cells transfected with DAT 615R (Figure 21). Since reduced association of flotillin-1 could reflect diminished surface expression of DAT (no data has been published

as to whether DAT/flotillin-1 associations are present in recycling endosomes or limited to the plasma membrane), as opposed to a change in microdomain localization, we used confocal imaging to compare the differential localization of YFP-tagged DAT 615R and 615C proteins with Alexa 647-conjugated cholera toxin B subunit (CTxB). The latter probe detects GM1 ganglioside, a molecule known to localize to cholesterol-rich, membrane microdomains (Simons and Ikonen, 1997). In transfected N2a cells, DAT

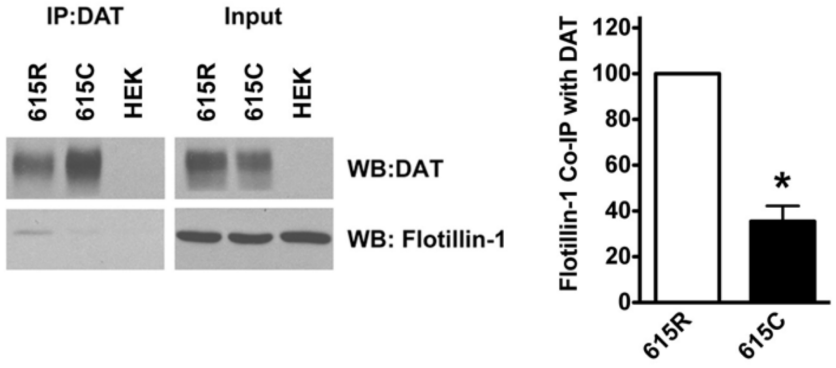


Figure 21 Flotillin-1 Association is Reduced for DAT 615C: Left: Representative western blot showing reduced association of DAT 615C with flotillin-1. **Right:** Quantification of flotillin-1 co-IP with DAT normalized to respective inputs. DAT 615C displays significantly reduced association with flotillin-1 (33±8 % of 615R; n=5, $P<0.001$, Student's t test).

has been found to exhibit a significant localization to CTxB-labeled membranes, in contrast to the transferrin receptor (Adkins et al., 2007) which was excluded from these domains. As shown in Figure 22, localization of DAT 615C to CTxB-labeled membranes was significantly reduced compared to DAT 615R.

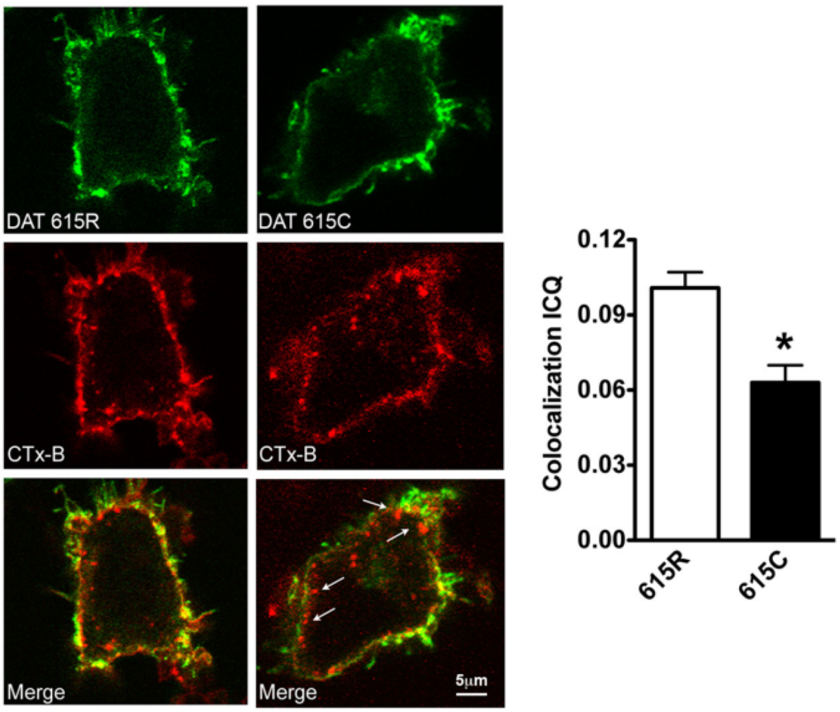


Figure 22 Decreased Localization of DAT 615C to GM1-containing Membrane Microdomains: Left: HEK 293T cells were transiently transfected with YFP-HA-DAT or YFP-HA-DAT 615C (green). Alexa 647 conjugated cholera toxin B is used to mark GM1 ganglioside (red). Representative cells are shown with white arrows indicating lack of DAT association with the raft fraction of R615C variant. **Right:** ICQ analysis was performed to define quantify colocalization of YFP-DAT with CTx-B labeled membranes. Although both 615R and 615C DAT are significantly colocalized with CTx-B, 615C exhibits a significantly reduced ICQ value compared to 615R (615R, 0.1±0.006 vs. 615C, 0.06±0.006; n=22, $P<0.01$, Student's t test).

DAT 615C acts dominantly via generation of local negative charge to disrupt AMPH actions

The ADHD subject with whom we initiated our studies is heterozygous for the R615C variant, suggesting that if the variant is a significant risk determinant for the disorder, it likely acts dominantly to alter DAT function. One mechanism by which dominance could be established is from an ability of the DAT R615C variant to preclude association of molecules needed for normal DAT trafficking and function, possibly as a consequence of the transporter's ability to dimerize

(Sorkina et al., 2003a, Torres et al., 2003). To explore this idea, we synthesized peptides that comprise the last C-terminal 24 amino acids of either DAT 615R or 615C attached to the membrane permeable TAT sequence (Schwarze et al., 1999). Incubation of DAT 615R cells with the TAT-C24^{615R} peptide failed to impact AMPH-mediated downregulation of DA uptake (Figure 23A). However, incubations of these cells with the TAT-C24^{615C} peptide eliminated the effects of AMPH on the wildtype transporter (Figure 23A). Addition of either TAT-C24 ^{615R} or TAT-C24^{615C} peptide (or no peptide) produced no effects on either basal or AMPH-modulated DA transport of DAT 615C cells (Figure 23B). These findings suggest that rather than attracting molecules that can drive DAT 615C out of GM1 and flotillin-1 containing membrane microdomains, the 615C-substituted C-terminus may preclude interactions needed for successful residency in these compartments.

Next I sought to determine whether the 615C substitution *per se* perturbs DAT regulation, or whether the loss of Arg residue at this position confers AMPH insensitivity. Following site-directed mutagenesis of the wildtype DAT C-terminus at residue 615, we found that cells transfected with DAT 615A presented a pattern of AMPH regulation indistinguishable from those of DAT 615R (Figure 24), indicating that Cys addition, rather than Arg loss, determines AMPH insensitivity. Cytoplasmic Cys residues can be modified by palmitoylation or nitrosylation (Nagahara et al., 2009), modifications that have both been

suggested to occur with catecholamine transporters (Kaye et al., 2000, Foster and Vaughan, 2011).

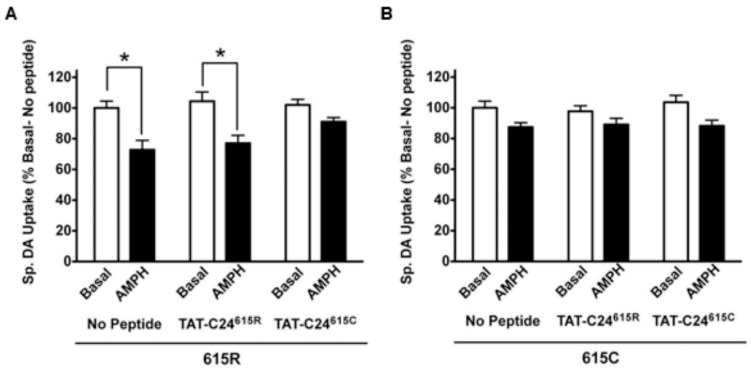


Figure 23 Effect of TAT-C24^{615R} and TAT-C24^{615C} peptides on AMPH-mediated DA Transport Reduction: (A, B) Exogenous addition of TAT-C24^{615C} peptide blocks AMPH-mediated reduction in the DA transport for DAT 615R and had no effect on 615C. Addition of either TAT-C24^{615R} or TAT-C24^{615C} peptide (10 μM, 18hrs) did not alter basal DA uptake compared to no peptide control for both DAT 615R and 615C (for DAT 615R, 104±6% with TAT-C24^{615R} and 102±3% with TAT-C24^{615C}; for DAT 615C, 96±3% with TAT-C24^{615R} and 103±4% with TAT-C24^{615C}). Addition of TAT-C24^{615R} peptide or no peptide control produced a significant reduction in DA uptake for DAT 615R upon AMPH (10 μM, 30 min) treatment (72±6% of basal no peptide for TAT-C24^{615R} and 77±5% of basal no peptide for TAT-C24^{615C}) and had minimal effect on DAT 615C (84±2% of basal no peptide for TAT-C24^{615R} and 85±1% of basal no peptide for TAT-C24^{615C}). Addition of AMPH (10 μM, 30 minutes) to TAT-C24^{R615C} peptide treated DAT 615C cells also did not produce a significant reduction in DA uptake (88±4% basal no peptide control). AMPH addition to TAT-C24^{615C} treated WT-DAT cells significantly blocked AMPH-mediated reduction in the DA transport seen in TAT-C24^{615R} treated and no peptide control conditions (91±3% basal no peptide control). Significant differences were calculated using Student's t-test. (n=3-4, P<0.05).

However, we detected no differences in palmitoylation between wildtype DAT and the R615C variant, and NOS inhibition did not restore AMPH regulation (data shown in appendix). Cys residues also participate in disulphide bond formation, though this seems unlikely given the reducing environment of the cytosol where the DAT C-terminus resides. Cytoplasmic Cys residues can form acidic thiolates (Nagahara et al., 2009) and as such could confer a charge inversion compared to the wildtype 615R residue. Therefore, we asked whether

DAT 615E would also confer insensitivity to AMPH. Indeed, DAT 615E displayed no reductions in DA uptake in response to AMPH treatment (Figure 24). Since the mutants 615K, 615A, 615Q, and 615S all respond to AMPH (Figure 24), we suggest that a negative thiolate arising from Cys substitution at the 615 position is responsible for the shift of the DAT R615C variant to AMPH insensitivity.

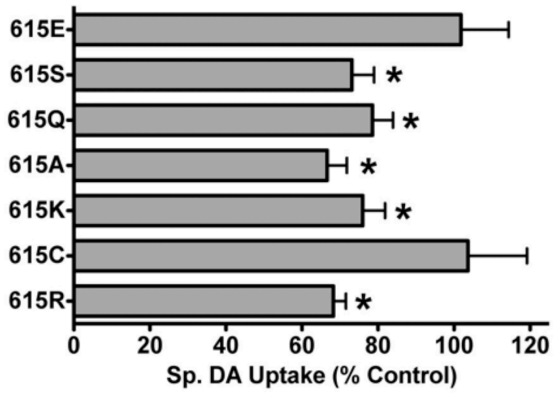


Figure 24 Effect of Amino Acid Substitutions at R615 residue on AMPH-mediated DA Transport Reduction: Presence of DAT 615C or a negatively charged aspartic acid (613E) blocked AMPH-mediated reduction in the DA transport. Specific DA uptake values are expressed as % of basal control. Only DAT 615C (103±13% of basal) and 615E (102±12% of basal) mutations did not display reduction in the DA uptake upon AMPH-treatment (10μM, 30 min). All other mutations at R615 position and DAT 615R showed a significantly reduced DA uptake upon AMPH treatment (615R-68±3%, 615K-75±6%, 615A-66±5%, 615Q-78±5%, 615S-73±5% of basal). Significant differences were calculated using Student's t-test for each mutation. (n=3-4, $P<0.05$).

The Arg residue at 615 of DAT forms a canonical phosphorylation site for multiple kinases with Thr 613 (Amanchy et al., 2007). However, since the 615A substitution is still AMPH-sensitive, phosphorylation at 613T is not required for the psychostimulant's impact on DAT regulation. Consistent with this finding, cells transfected with the T613A mutant retained AMPH sensitivity (Figure 25). However, cells transfected with a T613E mutant on the wildtype 615R

background to mimic the charge that would be induced by Thr613 phosphorylation lost AMPH sensitivity (Figure 25). These findings suggest that Thr613 may need to remain dephosphorylated to sustain psychostimulant (and PKC) regulation and that a nearby 615C thiol/thiolate may sufficiently mimic the structure or negative charge of phosphorylated Thr613 to preclude DAT regulation by AMPH.

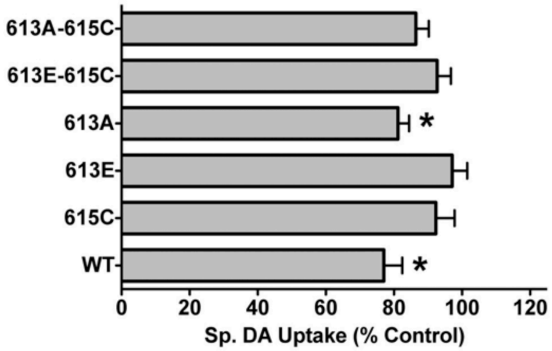


Figure 25 Importance of T613 Residue in AMPH-mediated DA Transport Reduction: Introduction of a negative charge by T613E mutation abolishes AMPH-mediated reduction in the DA transport. AMPH treatment (10 μM, 30 minutes) caused a significant reduction in the DA transport for DAT 615R and 613A (DAT 615R-77±5%, 613A-81±3% of basal) T613E mutation completely blocked AMPH-mediated reduction in the DA transport (97±4% of basal). Mutation of T613 to either A or E on DAT 615C background and 615C alone eliminated reductions in DA uptake upon AMPH treatment (10 μM, 30 minutes) (DAT 615C-92±5%, 613A-615C-86±3%, 613E-615C-92±4% of basal). Significant differences were calculated using Student's t-test for each mutation. (n=5, $P<0.01$)

Discussion

DAT proteins have been found to reside within cholesterol and GM1 ganglioside-enriched membrane microdomains, often referred to as "lipid" or "membrane rafts" (Sandvig and van Deurs, 2000, Adkins et al., 2007, Foster et al., 2008), and to associate with the raft-associated protein flotillin-1 (Cremona et al., 2011). Adkin and coworkers demonstrates that DAT proteins that are

localized to CTxB-labeled membrane microdomains demonstrate restricted mobility but can be mobilized by membrane cholesterol extraction (Adkins et al., 2007). We found a reduced colocalization of DAT 615C with CTxB-labeled membranes, as well as a reduced association of the membrane raft-associated protein flotillin-1. Since quantitation of our co-immunoprecipitation data was normalized for total DAT protein, the reduced flotillin-1 association of the R615C variant may reflect, at least in part, the reduced surface expression of the transporter. However, at present we do not know whether DAT and flotillin-1 have constitutive or dynamic interactions during normal transporter recycling. In the context of our immunofluorescence findings of a surface redistribution of R615C between GM1+ and GM1- compartments, we should also consider DAT targeting to a compartment where opportunities for flotillin-1 interactions are lost versus a direct effect on flotillin-1 associations is involved.

Cremona et al. (2011) provided evidence that interactions with flotillin-1 are required for both PKC-dependent DAT trafficking and AMPH-induced DA efflux. Consistent with these findings, we observed a loss of PKC-induced transporter trafficking with DAT 615C, though AMPH-induced DA efflux was maintained. These findings indicate that DAT/flotillin-1 associations support, but may not be necessary, for AMPH-triggered DA efflux. Since CaMKII associates with DAT through C-terminal sequences that overlay the R615 residue and is critical for AMPH-triggered DA efflux (Fog et al., 2006), the constitutively elevated DAT/CaMKII association may compensate for the loss of flotillin-1 associations,

hyperphosphorylating DAT under basal conditions and thereby enhancing the efflux-competence of DAT 615C. Alternatively, since DAT responds rapidly to AMPH action via increased surface expression (Furman et al., 2009a), the 615C substitution may also enhance DAT levels transiently in response to AMPH, during the period of DA efflux measurements. Regardless, these findings raise the possibility that flotillin-1 and CaMKII interactions may be exclusive, with their exchange being a key feature of dynamic DAT regulation. Recently, a Ras-like GTPase, Rin, was shown to associate with DAT C-terminus in membrane rafts and regulate PKC-mediated DAT downregulation (Navaroli et al., 2011). The mislocalization and regulatory perturbations we observe with DAT 615C could therefore be derived from altered Rin associations, an issue that deserves further study.

As we further explored the role of CaMKII in the insensitivity of the R615C variant to AMPH-induced internalization, we discovered a capacity for the kinase to support a trafficking-independent mode of transporter functional regulation. We hypothesize that CaMKII activity maintains basal activity of DAT 615C when transporters are localized away from flotillin-1 rich membrane microdomains. Although we did not obtain evidence for this process in DAT 615R cells, CaMKII associations may provide a mechanism whereby more physiological stimuli could enhance DAT activity, possibly during states of high DA release. In this regard, both NET and SERT proteins exhibit trafficking-independent catalytic regulation (Apparsundaram et al., 2001, Steiner et al., 2008). Moreover, a future study with

the DAT V382A variant, which also exhibits trafficking independent DAT modulation after PKC activation, should evaluate membrane microdomain associations to address this conundrum.

Studies examining the physical requirements for AMPH regulation of wildtype DAT in relation to the R615C variant provide key insights into mechanisms supporting transporter regulation. Using TAT-C24^{615R} and TAT-C24^{615C} peptides, we demonstrated that the mutant C-terminus acts dominantly to eliminate AMPH actions on wildtype DAT. These findings are important given the hemizygous status of our DAT 615C proband. These findings also indicate that DAT 615C may compete in the hemizygous state with the wild-type DAT C-terminus for key interactions needed for AMPH-induced transporter regulation. Additionally, we found that whereas 615C precludes AMPH-induced transporter downregulation, Ala, Lys, Gln, and Ser substitutions at R615 fail to perturb regulation. In further pursuit of the structural basis for the 615C effect, we found that 615E recapitulated the impact of 615C, suggesting that the generation of negative charge in the distal C-terminus may play a role in disrupting transporter regulation. Cys residues can exist as thiolates and thereby create a local negative charge (Nagahara et al., 2009). Whereas the pKa of a free Cys thiol is estimated at ~8, leaving the Cys side chain of 615C largely protonated, the pKa of proteinaceous Cys residues is known to be quite sensitive to its local environment, where the thiolate anion can be stabilized by surrounding residues (Netto et al., 2007).

Since R615 forms a potential phosphorylation site that includes T613, we considered the possibility that 615C gains its capacity to disrupt regulation by interfering with T613 phosphorylation. However, we found that a 613E mutation, created to produce the negative charge associated with Thr phosphorylation, like 615C eliminates DAT responsivity to AMPH. These findings suggest that dephosphorylation of T613 may be a critical step in sorting DAT into regulated versus constitutive endocytic pathways. Further studies are needed to determine whether, and under what conditions, kinases and phosphatases may target this residue, either constitutively or as part of a rapid, regulatory mechanism, or whether other mechanisms, such as charge-dependent protein associations, impart sorting decisions.

In toto, our findings lead us to propose a two-compartment model for the functional impact of DAT 615C (Figure 26), one that also has implications for the regulation of DAT 615R. We propose that DAT exists at the cell surface in GM1/flotillin-1 enriched microdomains that restrict transporter lateral mobility and access to key endocytic proteins that provide for regulated trafficking. Additionally, the transporter may reside in membrane depleted of these raft components and, if so, traffics largely via constitutive endocytosis and rapid recycling. Decisions dictating the localization and trafficking of DAT are dependent on sequences in the distal C-terminus of DAT, which we suspect involves phosphorylation/dephosphorylation reactions at T613. With the DAT 615C substitution, transporters are biased to traffic into and recycle from GM1-

and flotillin-1 depleted microdomains, leading to constitutive trafficking at higher

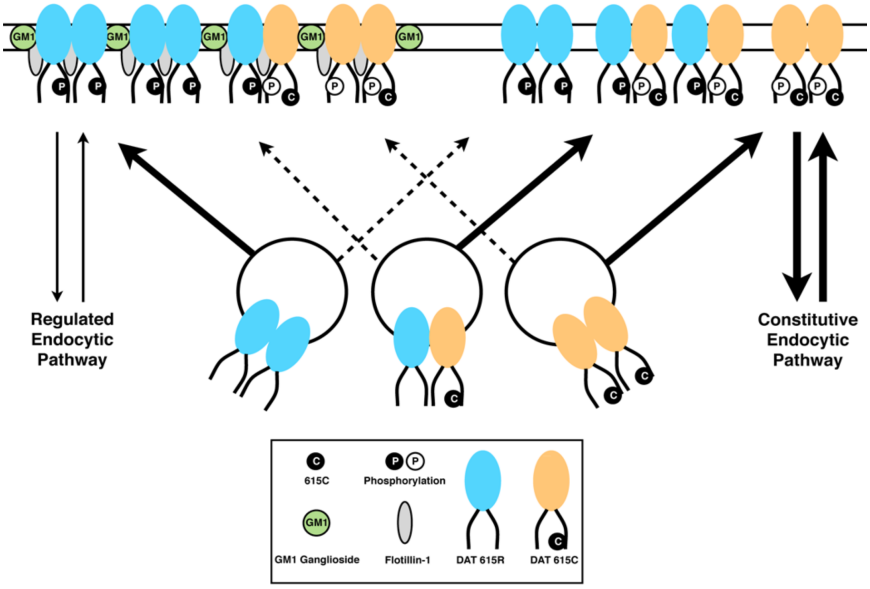


Figure 26 Model Describing Differential Trafficking of DAT 615R and DAT 615C to the Regulated and Constitutive Endocytic Pathways and Biased Localization Toward GM1/flotillin-1 rich or Depleted Membrane Microdomains: DAT molecules are depicted as located in trafficking vesicles or at the plasma membrane as homomeric or heteromeric dimers (though other configurations are acceptable). Transporters localize to two types of membrane domains, one domain enriched for GM1 ganglioside and flotillin-1, with the other domain relatively depleted of these molecules. Homo-multimers of WT DAT (615R) subunits target preferentially to GM1 ganglioside/flotillin-1 enriched compartment as compared to DAT 615C subunit containing dimers. The heterodimer is shown preferentially targeting domains that are depleted of GM1 and flotillin-1, in keeping with the dominant action of the 615C mutation in the ADHD proband and the dominant action of the DAT C-terminal peptide containing 615C. DAT 615C subunits are diminished in surface levels relative to WT subunits. Microdomains enriched for GM1 and flotillin-1 support regulated trafficking of DATs, and under basal conditions exhibit slow rates of endocytosis, whereas membranes depleted of GM-1 and flottilin-1 support more rapid, constitutive endocytosis and recycling.

rates than seen for transporters in GM-1 and flotillin-enriched microdomains.

Our model has parallels with the differential trafficking of insulin-responsive (GLUT4) and nonresponsive (GLUT1) glucose transporters. GLUT4 and GLUT1 equivalently support glucose uptake, but GLUT4 traffics through a limited capacity, regulated pathway whereas, GLUT1, traffics in the same cells through higher-capacity, constitutive mechanisms (Zorzano et al., 1997). Wildtype DAT has the capacity to occupy either a regulated or constitutive

pathway, but in our model systems is biased normally toward GM1/flotillin-1 enriched domains. As shown by our studies with the DAT^{615C} peptide, the DAT-C terminus can play a dominant role in the transporter's capacity to enter a regulated trafficking pathway. The DNA encoding DAT 615C and the other mutants generated in this study, as well as the DAT^{615C} peptide should be useful in further elucidating the mechanisms by which DAT sorts between surface trafficking pathways. Although the 615C variant is rare, our findings suggest that a more intensive analysis of proteins that sustain DAT membrane compartmentalization and recycling kinetics may provide important insights for idiopathic ADHD.

CHAPTER V

Hyperphosphorylation and Lack of AMPH Action in the human DAT A559V Coding Variant

Introduction

In Chapter I, I introduced the DAT A559V variant and here I summarize briefly its properties so that the reader may be reminded of key aspects of the variant's biology, leading to my contributions. The A559V variant was initially identified in a female bipolar disorder subject (Grunhage et al., 2000), but further analyses were not pursued. The lack of effort to characterize the variant was likely due to the limited pedigree from which it was derived, and the fact that transmission could not be verified as the parent likely transmitting the variant was deceased. However, it must be said that the rarity of the variant also likely factored into this decision as the investigators pursuing this effort were searching for more common contributions to bipolar disorder. In 2005, our lab identified a second occurrence of DAT A559V in two male siblings with ADHD (Mazei-Robison et al., 2005). The pedigree of the A559V cohort is shown in Figure 27. All members of the pedigree that possess the A559V variant are hemizygous for this substitution, though they are homozygous for a noncoding 3'UTR variant that has been associated with ADHD (Mazei-Robinson and Blakely, 2006). The A559V variant is located in TMD 12, whose function remains largely unknown, though crystallographic studies of a bacterial homolog (Yamashita et al., 2005),

and structure function studies of SERT (Just et al., 2004) suggest that TM12 contributes to a homo-oligomeric interface. In the latter case, it is reasonable to speculate that a dominant-negative action of the mutant transporter could arise through perturbation of oligomerization. Such an action would help explain a contribution to ADHD (or bipolar) risk in subjects who are hemizygous for the variant.

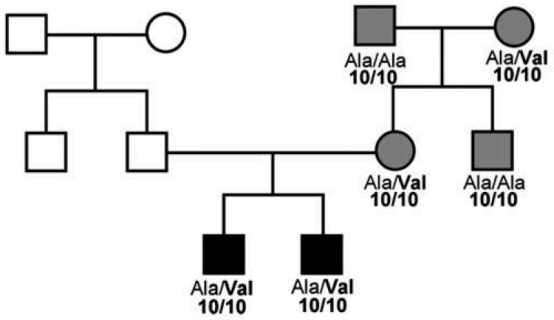


Figure 27 Pedigree of the A559V Variant: Siblings with ADHD are indicated by black boxes and were both hemizygous for the A559V variant. Transmission of the allele occurred from the mother and grandmother. Family members who did not suffer from ADHD are shown in gray. Open symbols indicate that these family members were not evaluated for ADHD symptoms (Mazei-Robison et al., 2005).

Our previous report indicates that the anomalous DA efflux (ADE) that is detected in the A559V variant under basal conditions increases in a voltage-dependent manner when monitored in voltage-clamped, transfected HEK-293T cells. Remarkably, basal ADE was completely blocked by AMPH, whereas AMPH produces DA efflux from cells expressing WT DAT. As I reviewed earlier in this thesis, evidence indicates that AMPH-mediated DA efflux is regulated by CaMKII-dependent phosphorylation of the DAT N-terminus phosphorylation (Fog et al.,

2006). My studies with the A559V variant specifically address the following three questions:

1) Is ADE of the A559V variant associated with increased basal phosphorylation?
2) Are the expected AMPH-mediated DA transport reductions and net DAT surface internalization observed in A559V-transfected cells, similar to my prior findings with the DAT 615C variant?
3) Are any changes in AMPH actions on A559V-transfected cells also observed with β-PMA treatments?

The ADE exhibited by the A559V variant was observed in transiently transfected HEK 293T cells. To explore whether changes in basal phosphorylation arise under the same conditions, I employ the same expression methods as used for ADE measurements. To investigate AMPH- and β-PMA-mediated regulation, I utilize WT and DAT A559V stable lines produced with Flp-In™ HEK cells that provide for equivalent, stable expression from the same genomic locus.

Results

To explore whether ADE from the DAT A559V variant is accompanied by increased transporter phosphorylation, we utilized [^{32}P]orthophosphoric acid metabolic labeling of transiently transfected HEK-293T cells, followed by immunoprecipitation with a DAT-specific antibody. Immunoprecipitated DATs

were resolved by SDS-PAGE and phosphorylation was detected by exposure of gel transfers to X-ray film. I found that the A559V variant displayed a significant increase in basal phosphorylation compared to WT DAT or from non-transfected cells (Figure 28). Similar results were obtained by the Javitch lab using site-specific phosphor-antibodies, which indicated that A559V expressing cells display a significant increased phosphorylation at Ser 7 and Ser 13 residues and a trend towards increased basal phosphorylation at Ser12 (Bowton et al., 2010). In parallel with these studies, the Galli lab (Bowton et al., 2010) demonstrated that ADE from the A559V variant was dependent on D2S receptor activity and dependent on sites likely to mediate CaMKII-mediated DAT phosphorylation.

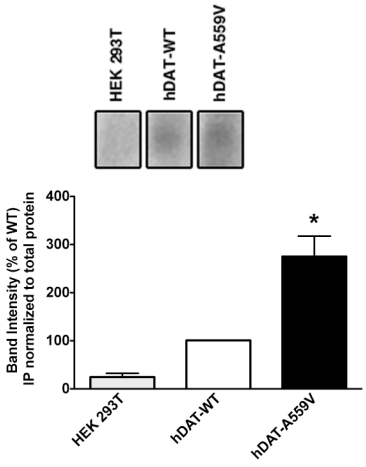


Figure 28 Increased basal phosphorylation of the A559V variant: Top: Representative autoradiograph from immunoprecipitations of [^{32}P] PO_4 labeled cells. **Bottom:** Quantification of DAT bands from the autoradiograph showing significant increase in basal phosphorylation levels of A559V (275±42 % of WT; n=4, *P*<0.001, One-way ANOVA followed by Bonferroni's post hoc test.). Data represent protein labeling normalized by protein loading.

In the Blakely lab's initial report that described the ADE phenotype of the A559V variant, they showed that ADE could surprisingly be blocked by AMPH treatment whereas AMPH caused DA efflux from WT DAT expressing cells.

These finding lead me to investigate the actions of AMPH on DAT A559V activity and surface expression. Although transiently transfected HEK 293T cells offer an excellent, single cell readout of basal DA leak, population-based assays such as those monitoring DA transport or cell surface biotinylation benefit stably-transfected cells. Conventional stable cell methods utilize genes or cDNAs randomly integrated into host cells. Clonal lines derived from these methods often produce different levels of expression due to different sites of integration and a lack of control over the copy number of genes integrated. To minimize these issues, we used Flp-In™ HEK cells to produce stable WT DAT and DAT A559V cells as these cells provide for single copy expression from the same genomic locus.

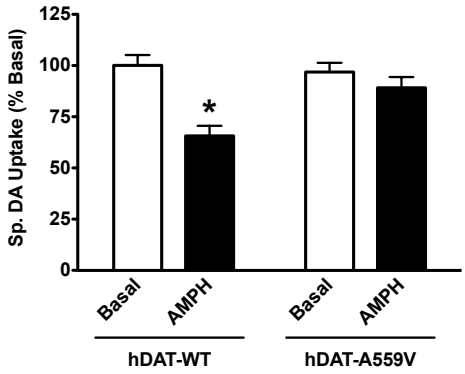


Figure 29 Effect of AMPH on DA Transport Reduction: AMPH-mediated (10 μM, 30 minutes) reduction in DA transport seen in WT DAT (66±5 % of basal) whereas no significant changes were detected for A559V cells (89±5 % of basal). Basal DA transport levels were normalized to uptake in cells treated with vehicle. Significant differences were calculated using Student's t-test. (n=4, $P<0.001$)

As mentioned previously, AMPH normally causes a reduction in DA transport activity and a loss of transporters from the cell surface via clathrin-mediated endocytosis (Saunders et al., 2000). Consistent with these findings and

as noted in a prior chapter, our stable cells that express WT DAT displayed a significant reduction in DA transport upon a 30 min, 10 μM AMPH treatment. In contrast, DAT A559V variant expressing cells failed to exhibit a reduction in DA

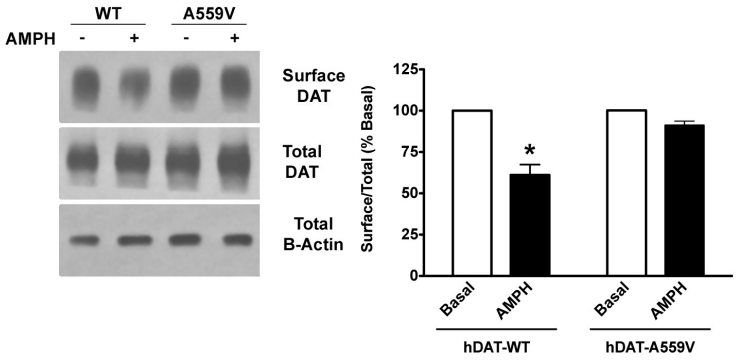


Figure 30 Effect of AMPH on DAT Surface Expression: Left: Representative western blot showing surface DAT protein levels after AMPH treatment (10 μM, 30 minutes) for WT DAT and DAT A559V transfected cells. Total β-actin is used as an intracellular loading control. **Right:** Quantification of AMPH-induced decrease in DAT surface levels expressed as surface/total. Data is normalized to basal DAT surface/total expressions as 100%. AMPH treatment caused a significant (63±7%) decrease in wt DAT expression while no change was detected in A559V expression (92±3%). Significant differences were calculated using one-way ANOVA followed by Bonferroni's post hoc test. (n=6, P<0.0001)

transport (Figure 29). Loss of DA transport, as previously documented, was found to be paralleled by a net reduction in surface expression of WT DAT. As with the insensitivity of DAT A559V-mediated DA transport to AMPH, the variant displayed no AMPH-induced reduction in surface expression (Figure 30). These findings were replicated in transiently transfected HEK 293T cells (E. Bowton, unpublished data).

Previous studies conducted in transiently transfected COS-7 and SH-SY-5Y cells that assessed the impact of PKC activation on DA transport by WT DAT and the DAT A559V variant indicated no significant differences (Mazei-Robison and Blakely, 2005). The lack of AMPH regulation of DAT A559V regulation in the

Flp-In HEK stable cell lines prompted me to verify these observations in this cell background. Consistent with the transiently transfected model studies, β-PMA induced a significant reduction in DA transport by both WT DAT and DAT A559V

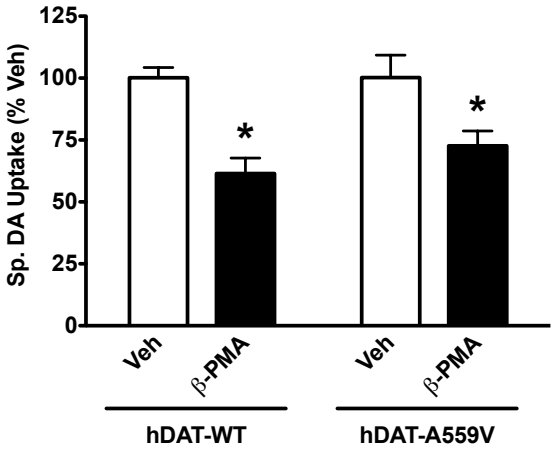
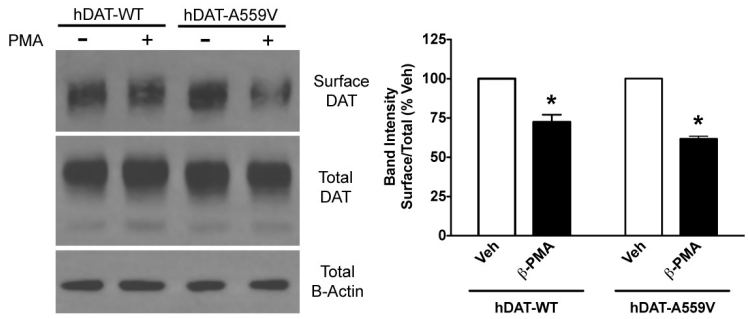


Figure 31 Effect of β-PMA on DA Transport Reduction: β-PMA-mediated (200 nM, 30 minutes) reduction in the DA transport seen in both WT DAT and the A559V variant. Basal DA transport levels were normalized to respective cell lines and β-PMA-mediated reduction was calculated. β-PMA caused a significant decrease in DA transport of WT DAT (63±5 % of Veh) as well as in the A559V variant (71±6 % of veh). Significant differences were calculated using Student's t-test. (n=3, $P<0.001$)

stable cells (Figure 31). β-PMA treatments also produced a significant loss of surface expression for both WT DAT and DAT A559V cells (Figure 32). Therefore, the lack of transport and trafficking sensitivity to AMPH possessed by the A559V variant, unlike findings with the R615C variant, does not translate to activated PKC-triggered DAT regulation.

Recently it was shown that AMPH-induced DA efflux was dependent on DAT/flotillin-1 interaction (Cremona et al., 2011). Following the analogy, one can speculate that ADE must be occurring from flotillin-1 rich membrane microdomains and A559V/flotillin-1 interaction could be increased under basal

conditions, a hypothesis, that still remains to be investigated. However, when the colocalization of the A559V variant with ganglioside GM1 was measured, a



Figure

32 Effect of β-PMA on DAT Surface Expression: Left: Representative western blot showing surface DAT protein levels after β-PMA treatment (200 nM, 30 minutes) for both WT and A559V DAT. Total β-actin was used as an additional loading control. **Right:** Quantification of β-PMA-induced decrease in DAT surface levels expressed as surface/total. Data is normalized to basal DAT surface/total expressions as 100%. β-PMA treatment caused a significant decrease in both WT DAT (72±5 % of Veh) and DAT A559V (60±0.4 % of Veh) expression. Significant differences were calculated using Student's t-test. (n=3, $P<0.0001$)

significant difference in the colocalization ICQ of DAT-WT/GM1 and DAT-A559V/GM1 was not observed (Figure 33). Flotilin-1 and GM1 colocalize in membrane microdomains, often referred to as lipid rafts. This finding itself is very interesting as it points to the possibility of differential targeting of transporters to specialized membrane microdomains and heterogeneity of cell membrane. Further studies are required to address this question.

Discussion

DAT dictates the duration and magnitude of DA signaling by removing the catecholamine from the synapse and thereby terminating DA actions on pre- and

postsynaptic DA receptors. The psychostimulant AMPH alters DAT activity by modulating DAT function and surface expression (Saunders et al., 2000). AMPH, being a weak base substrate of VMAT1 and 2, is known to deplete the synaptic

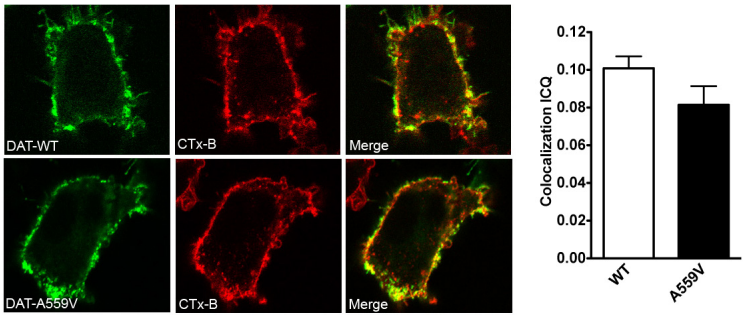


Figure 33 Colocalization of DAT with Ganglioside GM1: Left: HEK 293T cells are transiently transfected with YFP-HA-DATs (green). Alexa 647 conjugated cholera toxin B (CTxB) is used to mark GM1 ganglioside (red). Representative cells are shown with yellow merge indicating DAT association with the raft fraction. **Right:** ICQ analysis is performed to define colocalization of YFP-DAT with CTx-B labeled lipid rafts. Quantification demonstrated that both WT and A559V DAT are significantly colocalized with CTx-B. The differences between colocalization ICQ are not significantly different. (DAT-WT, 0.1±0.006 vs. DAT-A559V, 0.08±0.01; n=22, *P*>0.05, Student's t test).

vesicles of DA and, with activation of CaMKII, produces DAT-dependent DA efflux *in vitro* and *in vivo* (Sulzer et al., 2005). In addition to producing non-vesicular DA release, AMPH also has CaMKII-dependent effects on the surface expression of DAT, triggering net relocation of DAT to intracellular compartments (Wei et al., 2007b).

Our previous report demonstrated a novel A559V-associated ADE that could be blocked by both AMPH and MPH, two drugs used to treat ADHD. The similarity here between the actions of AMPH and methylphenidate were unexpected given that 1) AMPH causes, not blocks, DA efflux with WT DAT and 2) AMPH and methylphenidate act in opposition with WT DAT, one a substrate

and the other an antagonist that can actually block the actions of the other. Since both compounds now act in a similar manner *in vitro*, as they do therapeutically *in vivo*, the occurrence of an enhanced leak state needs to be considered more broadly than just a feature of the A559V variant.

As noted above, AMPH-induced DA efflux is dependent on CaMKII-activation and appears to involve DAT phosphorylation (Fog et al., 2006). Thus, basal DA leak could be a result of increased basal DAT phosphorylation. When we assessed basal levels of phosphorylation of the A559V variant, we found it to be true confirming the former hypothesis. Moreover, PKC activation is also linked to DA efflux (Kantor and Gnegy, 1998, Johnson et al., 2005b) and PKC-mediated DAT internalization has been reported to occur through lipid raft microdomains (Cremona et al., 2011). A normal response of DAT A559V variant to PKC activation as well as WT like association with lipid raft component, GM1, suggests that A559V-associated ADE is likely to be a consequence of changes arising from within lipid rafts, rather than the altered domain localization seen with DAT 615C.

To explore further the effects of AMPH on the A559V variant, we developed stable lines using Flp-In HEK cells to capture the opportunity to have a homogenous population of cells expressing either WT DAT or DAT A559V from the same genomic locus. Here, we showed that the A559V variant lacks the ability to internalize following AMPH treatment. This property was not due to a global loss of the A559V regulation, as PKC activation lead to a reduction in DA

transport, as well as a loss of the A559V variant from the cell surface similar to WT DAT. Since AMPH acts as an antagonist at DAT A599V in the ADE paradigm, it is possible that AMPH is not transported inside the cell (although it readily transports DA) and therefore cannot activate signaling pathways required for transporter internalization. Previous studies have shown that intracellular injection of AMPH was sufficient to cause trafficking from an uptake-impaired Y335A DAT mutant (Kahlig et al., 2006). Alternatively, DAT A559V, in assuming a conformation that produces ADE, may have entered a state that cannot receive intracellular signals that produce DAT endocytosis. Additional studies with DAT A559V that assess 1) AMPH internalization, 2) impact of injected intracellular AMPH on transporter trafficking, 3) changes in transporter conformations, as with Cys modifying reagents, and 4) altered protein associations may be able to select among these possibilities.

Although, like DAT 615C, the DAT A559V variant is rare. Nonetheless, studies of the mutant may more broadly inform how ADE contributes to ADHD as well as defining the exact mechanisms by which AMPH and methylphenidate effectively treat ADHD. Additionally, study of rare coding variants such as DAT A559V can help us define a broader network of genes that regulate DA neurotransmission and help search for additional determinants of risk for ADHD and its comorbid disorders. An important step in this direction is the generation and characterization of DAT A559V knock-in mice, currently underway in the Blakely lab.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

DA signaling is of critical importance in multiple behaviors including motor control, reward, working memory, and mood. Dopaminergic dysfunction has been linked to many neuropsychiatric disorders including addiction and schizophrenia, as well as to the movement disorders dystonia and Parkinson's disease. An important player in maintaining DA tone in the brain is DAT. DAT knock-out mice display tonically-elevated extracellular DA levels, reduced presynaptic DA stores and extreme hyperactivity. In contrast to the mouse findings, which could suggest a link between altered DAT and ADHD, human subjects with IPD were found to possess a full genetic loss of DAT function (Kurian et al., 2009). Thus, prior to the Blakely lab's efforts to further pursue DAT in ADHD, neuropsychiatric disorders directly linked to DAT dysfunction were unknown. Possibly, functional compensations *in vivo* could mask the phenotype of low penetrance DAT dysfunction, or simply that a correct clinical phenotype had yet to be identified.

In large part, the fact that DAT is the site of action for the most commonly prescribed ADHD medications, the Blakely lab pursued ADHD as a disorder enriched in penetrant gene variants. Since DAT coding exons had already been screened for variants in a large number of subjects (Grunhage et al., 2000,

Vandenbergh et al., 2000), it was clear that common coding polymorphisms were unlikely to exist that would implicate structural changes in the transporter for population ADHD risk. Nonetheless, the identification of highly penetrant, rare DAT coding variants could help solidify the case for DA dysfunction in ADHD, much as the occurrence of rare mutations in genes linked to protein ubiquitination in Parkinson's disease have opened new opportunities for progress in this disorder. Parallels to this position can also be made with the goal of forward genetic studies, where the identification of single mutant alleles can lead to the identification of undiscovered pathways that underlie broader phenotypes as well as disease states (e.g. the discovery of *ced-1* (Horvitz, 2003) and signaling leading to apoptosis in *C. elegans*).

Using a high-throughput, polymorphism discovery approach our lab has identified four nonsynonymous (or coding), single nucleotide polymorphisms (SNPs) in the DAT gene (*SLC6A3*). These four variants (V24M, L167F, A559V, and R615C) are distributed throughout the transporter sequence and are present on intracellular as well as extracellular sides and in a transmembrane domain (Figure 34). The first of these variants to be identified, and a subject of study in this thesis is A559V. The fact that the A559V variant was originally identified in a bipolar disorder subject (Grunhage et al., 2000), and then was rediscovered in two siblings with ADHD (Mazei-Robison et al., 2005), a disorder comorbid with bipolar disorder, seems unlikely to be a chance finding. In this regard, children of subjects with bipolar disorder are three times more likely to have ADHD

compared to children of unaffected subjects (Faraone and Tsuang, 2003). Likewise, subjects with an ADHD diagnosis are more likely to have a relative with bipolar disorder than non-ADHD subjects (Kunwar et al., 2007).

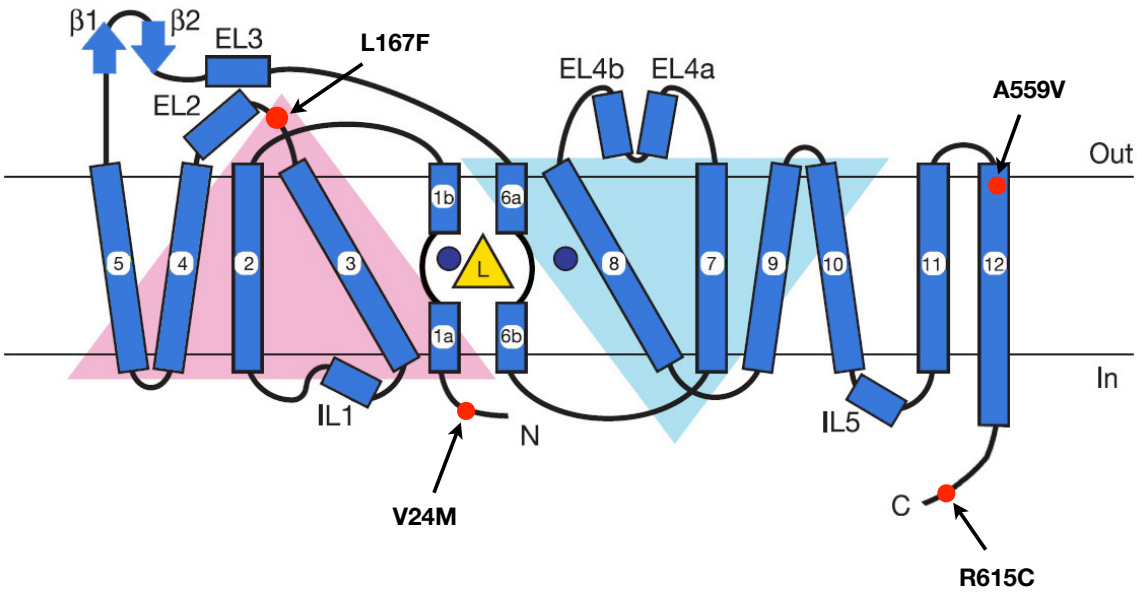


Figure 34 Localization of ADHD-associated DAT Coding Variants: Schematic Topology of DAT Based on $LeuT_{aA}$ crystal structure is depicted. TMDs1-5 and 6-10 form inverted triangle configuration and fold over forming substrate translocation pathway. TMDs 11 and 12 stick out from other TMDs, possibly contributing to the oligomerization. Adapted from (Yamashita et al., 2005).

The allele encoding the A559V mutation is present in single copy in all carriers in the pedigree within which it was found, and was transmitted to the two affected boys from the mother and the maternal grandmother. Unfortunately, given the male:female bias of ~4:1 in ADHD, no other male relatives could be identified as carriers of the variant. The two female carriers did not possess symptoms that qualify for an ADHD diagnosis, though the mother reported a mild

learning disability, and the grandmother scored above the 95th percentile for impulsivity traits on the Connor's instrument (Mazei-Robison et al., 2008).

Under basal conditions, the DAT A559V variant displays anomalous DA efflux (ADE) that can be blocked by methylphenidate, cocaine and AMPH (Mazei-Robison et al., 2008). More recently, ADE has been found to be supported by tonic signaling of DA D2 receptors that are expressed on the same cells and that act via a CaMKII-mediated mechanism (Bowton et al., 2010). CaMKII is suspected to phosphorylate DAT at N-terminal Ser residues known to be required for AMPH-induced DA efflux (Khoshbouei et al., 2004, Fog et al., 2006). In this thesis, I presented evidence demonstrating that DAT A559V is hyperphosphorylated relative to WT DAT, suggesting that the mutation may trigger ADE by permitting the transporter to achieve conformations like those seen following AMPH treatments. Since, at the present, DAT A559V is not known to have a cell-autonomous action in elevating intracellular Ca^{2+} levels and in activating CaMKII, we cannot conclude that this conformation permits the phosphorylation of the DAT N-terminus by CaMKII. Indeed, unpublished data from the Galli lab (Bowton et al., manuscript in preparation) links the PKCβ signaling to the ADE associated with the A559V variant. Thus, treatment of cells with a PKCβ-specific inhibitor eliminates the ADE of the A559V variant and rescues the ability of AMPH to produce nonvesicular DA release. Comparable studies involving kinase inhibitors, in the context of [^{32}P] orthophosphoric acid metabolic labeling studies, will be important in further clarifying this mechanism.

Studies are also currently underway in the Blakely lab to identify the phosphorylation sites responsible for the hyperphosphorylation of DAT A559V.

Due to the evidence of a functional perturbation of the A559V variant with *in vitro* studies, the Blakely lab recently generated a DAT A559V knock-in mouse line using transgenic technology (Mergy, 2011). Biochemical and behavioral analyses of these mice are in progress, though preliminary findings demonstrate a novel darting (hyperactive) phenotype, as well as blunted DA release from striatal slices *in vitro*, and a reduced locomotor response to AMPH *in vivo*. Studies are also needed that investigate signaling network alterations, particularly those mediated by CaMKII- and PKCβ, with respect to DAT regulation in this model. Using microdialysis and chronoamperometry approaches *in vivo*, basal extracellular DA levels and DAT-mediated DA clearance should be explored. Finally, compensatory changes in DA target genes (e.g. DA receptors) and signaling proteins should be determined as evidence of alterations here may open opportunities for evaluation of other potential determinants of ADHD risk.

On a more cellular level, TMD 12 that has been suggested to contribute to SERT oligomerization (Just et al., 2004), and as such it will be interesting to monitor changes in DAT homomeric protein associations, for example using differentially tagged transporters or FRET-based imaging approaches. As AMPH-induced DA efflux is known to be dependent on CaMKII and flotillin-1 association (Fog et al., 2006, Cremona et al., 2011), I performed a preliminary analysis of CaMKII/DAT associations, using WT DAT or the A559V variant, but failed to

detect differences (data not shown). It would be wise, however, to pursue this effort further in the DAT A559V transgenic mice as a more native environment may provide clearer evidence of a role for such associations (or not).

Flotillin-1 is a constituent of lipid raft microdomains that have been shown to harbor DAT (Cremona et al., 2011). Although I did not search for direct evidence of altered A559V/flotillin-1 interactions, I did investigate the colocalization A559V and GM1, another marker of lipid rafts. Although there was a slight (~20%) reduction in ICQ estimates of colocalization, this difference did not reach significance. The heterologous nature of these studies needs to be kept in mind. Further evaluation of DAT A559V/flotillin-1 interactions in dopaminergic nerve terminals ex vivo is now feasible with the generation of DAT A559V mice.

The central focus of this thesis is the second DAT coding variant that the Blakely lab identified, R615C. This variant lies in the distal C-terminus and is located in a well-conserved region of the transporter. The 615C variant was discovered in an Irish subject who displayed high scores for a diagnosis of combined type ADHD. The subject inherited the mutation from his mother who probably had ADHD as a child, based on a retrospective analysis. The subject is being well treated with methylphenidate. The DAT C-terminus harbors many domains known to support protein-protein interaction, and has been implicated in both constitutive and PKC-mediated, DAT regulation. The R615C variant lies within the RHW motif that has been reported to be involved in DAT export from

the ER as well as for DAT/CaMKII interaction (Bjerggaard et al., 2004, Fog et al., 2006).

Studies presented in this thesis demonstrate that the DAT 615C variant exhibits reduced total and surface expression, which results in a significantly decreased DA transport V_{max} but no change in DA transport K_M. The fact that DAT 615C surface levels are reduced more than total levels was the first indication that a trafficking alteration may be present. The lack of an impact of the DAT 615C variant on the DA transport K_M also supports basal trafficking or altered regulation as a primary contributor to any functional deficits that may be observed *in vivo*.

Although basal and AMPH-induced DA efflux of DAT 615C appeared normal, these findings, in the context of a reduced surface expression do raise the possibility that the variant does impact conformations of the transporter dictating inward versus outward substrate flux. Studies should be performed that evaluate DA efflux at a single cell DA efflux and normalize findings with surface DAT protein levels in the same cells (perhaps via DAT transient currents that are often used as a surrogate for surface expression).

As shown earlier, the DAT 615C variant lacks AMPH- and PKC-mediated DAT regulation as revealed by either trafficking or functional studies. We found that DAT 615C constitutively internalizes and recycles significantly faster than WT DAT. Moreover, unlike WT DAT, AMPH failed to accelerate the basal rate of endocytosis of the 615C variant. Like the A559V variant, which a displays similar

loss of AMPH action, DAT 615C displays hyperphosphorylation, possibly due to its demonstrated increase in CaMKII association. Additional studies that elucidate determinants of DAT C-terminus/CaMKII associations and utilize this information for functional and regulatory studies with the full length transporter should help clarify how CaMKII associations dictate DAT trafficking.

While investigating the underlying cause for the lack of AMPH action in DAT 615C, we uncovered a novel role of CaMKII in supporting DAT function, one that at present only manifests itself with the 615C variant. Previous studies report biphasic regulation of the transporter in response to AMPH. Immediately upon AMPH administration (seconds), AMPH has shown to increase DAT activity via recruitment of DAT to plasma membrane and over the period of time (minutes) reduce DAT activity by reducing cell surface expression (Johnson et al., 2005a). This increased DAT expression might be governed by CaMKII activity and perhaps WT transporters also use CaMKII to sustain DAT activity similar to that seen with DAT 615C, though perhaps not under the conditions I used in my experiments. CaMKII-mediated effects could be occurring very rapidly and as such might not be measurable with traditional methods employed in these studies. Nonetheless, the effects of CaMKII inhibition on this rapid phase of biphasic regulation of DAT will require further investigation. Another possible mechanism by which this effect on WT DAT might be masked is through protein-protein interactions required to produce the effect of AMPH. Studies delineating

protein-protein interaction requirements need to be addressed in the future studies.

The rapid, constitutive trafficking of DAT 615C may be attributed to the transporter's reduced localization to GM1- and flotillin-1 rich membrane microdomains. These microdomains are known to be rich in cholesterol and extraction of membrane cholesterol has been shown to alter DAT function (Foster et al., 2008, Hong and Amara, 2010). As demonstrated by Adkins and colleagues, these microdomains likely restrict DAT surface mobility as well as reduce rates of endocytic trafficking (Adkins et al., 2007). Alternatively, the transporter can reside in membrane depleted of these raft components and here traffics largely via constitutive endocytosis and rapid recycling. Decisions dictating the localization and trafficking of DAT are dependent on sequences in the distal C-terminus of DAT, which we suspect involves phosphorylation/dephosphorylation reactions at T613 as well as protein-protein interactions that allow or limit the motility of a protein in the raft fraction. Our model has parallels with the differential trafficking of insulin-responsive (GLUT4) and nonresponsive (GLUT1) glucose transporters. GLUT4 and GLUT1 equivalently support glucose uptake, but GLUT4 traffics through a limited capacity, regulated pathway whereas, GLUT1, traffics in the same cells through higher-capacity, constitutive mechanisms (Zorzano et al., 1997). Finally, we showed that DAT 615C could act dominantly and presence of a negative charge near DAT C-terminus could inhibit AMPH-mediated DAT regulation.

As with DAT A559V, there remain many opportunities to expand the impact of our studies with the DAT 615C variant. Our studies clearly demonstrate accelerated endocytosis and recycling of DAT 615C through a pathway lacking regulation by AMPH treatment or PKC activation.

Importantly, we are currently ignorant of the endocytic pathway along which this rapid trafficking occurs. DAT is known to shuttle between plasma membrane and recycling endosomes in PC12 cells (Loder and Melikian, 2003) and in dopaminergic neurons (Rao et al., 2011). We have yet to explore the recycling compartments that support our findings from stable Flp-In™ HEK cells and thereby determine components of the regulated versus constitutive path. Rab 11, a member of Ras superfamily of monomeric G proteins, has been shown to support constitutive trafficking of DAT in N2A neuroblastoma cells (Furman et al., 2009b) and it would be important to examine the localization of DAT 615C in relation to this small G protein, for example via sucrose or Percoll gradient fractionations. However, since Rab 11 has been suggested to support AMPH-mediated trafficking of NET (Matthies et al., 2010), a close relative of DAT, molecules other than Rab11 may play a role in the shift from a regulated trafficking pathway to one more consistent with constitutive trafficking. Possibly, the use of the term “constitutive” by Furman and colleagues was misused in simply referring to a basal recycling pathway, which may or may not provide for regulation. If DAT recycles in N2A cells via a pathway that can respond to AMPH

or PMA, then we could expect DAT 615C to recycle through a separate compartment, such as one defined by Rab8 (Furman et al., 2009b).

Another important aspect of our studies that requires further investigation is the link of CaMKII associations and DAT 615C hyperphosphorylation to intracellular Ca^{2+} alterations. Previous studies have shown that CaMKII binds to the DAT C-terminus and modulates AMPH-mediated DA efflux (Fog et al., 2006). Since my experiments revealed 1) that CaMKII exhibits an increased association with DAT 615C variant and 2) that CaMKII activity regulates DAT 615C function, it seems reasonable to question whether the chronic expression of the variant can lead to elevated basal Ca^{2+} levels. Using a calcium 3 assay kit (Molecular Devices), I obtained preliminary data that basal Ca^{2+} is elevated in cells expressing DAT 615C as compared to WT DAT transfected cells, whether using transient expression or in Flip-in HEK 293 stable cells. A more quantitative approach such as the use of the ratiometric dye Fura 2AM and transiently transfected cells could provide a more definitive answer to this question.

My studies with the DAT 615C variant revealed that this transporter could enter into an “inactive” state when present on the cell surface when CaMKII activity is pharmacologically inhibited. These findings raise the question as to the physiological significance of an inactive state, or whether the observations are an artifact of the transporter’s mislocalization. Studies with platelet SERT have demonstrated that this transporter, when treated with phorbol esters to activate PKC, internalizes minutes after the transporter moves to a less active state

(Jayanthi et al., 2005). Similarly, our group recently showed that p38 MAPK controls the intrinsic activity of surface-resident NET and SERT proteins (Steiner et al., 2008). In contrast, data from previous DAT studies (Kahlig et al., 2005) are consistent with internalization of the transporter in an active form, though such a conclusion requires knowledge of whether DAT transient currents are modified in active versus inactive states. Recently, Amara's group has demonstrated a state of lower activity in DAT that arises from cholesterol depletion. Possibly, our findings with DAT 615C reflect the relocation of DAT away from cholesterol-rich, flotillin-1 and GM-1 enriched membrane microdomains. Additional biophysical studies are needed comparing the steady-state an transient currents of DAT 615C vs. WT DAT. Regardless of the outcome, our findings clearly reveal a capacity of DAT to enter into inactive states and thus the further investigation of this process may provide a path to the development of novel strategies to reduce DAT activity for therapeutic benefit, as in ADHD.

We hypothesized that DAT 615C imparts a negative charge via formation of a thiolate with Cys substitution and that this charge, more so than the physical alteration of removing the Arg residue, blocks AMPH action. This hypothesis is supported by the finding that conversion of Arg to Glu also eliminated AMPH regulation, whereas neutral amino acid substitutions retained regulation. Although negative findings cannot be said to be conclusive, we were unable to demonstrate that other potential posttranslational modifications that occur on Cys residues such as palmitoylation or nitrosylation explain the lack of AMPH action

on the variant transporter (See Appendix Figure 37). We are currently employing LC/MS approaches to achieve a more definitive analysis of potential Cys adducts.

Finally, most of our studies with DAT 615C have been pursued to date in HEK 293 cells. Although these cells offer an excellent model for pharmacological, biochemical and electrophysiological studies, they clearly do not provide the same environment as found in native DA terminals. However, we did recapitulate the finding that DAT 615C lacks of AMPH-mediated regulation in transiently transfected noradrenergic CAD cell line. Studies using cultured DA neurons from DAT KO mice where WT DAT or DAT 615C have been virally expressed could offer important insights. Ultimately, studies using a DAT 615C knock-in mouse will be needed to validate our findings in a native setting *in vivo*. Such a model would of course also allow us to gather important data that the DAT 615C variant is causal in contributing to ADHD in the proband from which the variant was isolated.

In conclusion, our findings with the DAT A559V and R615C variants yields evidence that ADHD, the most commonly diagnosed childhood disorder, is enriched for penetrant DAT coding variants. Indeed, four of the known DAT coding variants identified to date derive from studies with ADHD subjects. Besides identifying phenotypes associated with naturally occurring DAT variation our studies suggest that mechanisms that do not provide flexibility in controlling extracellular DA may be a common feature of ADHD. DAT A559V can promote

increased extracellular DA via spontaneous, voltage-dependent DA efflux from cytoplasmic stores, thereby generating “noise” due to DA release outside of that provided by the highly regulated process of vesicular release. DAT 615C exhibits a disrupted localization to compartments that normally subserve the sensitive regulation of membrane proteins; it lacks the ability to be downregulated by PKC pathways (as well as AMPH), and displays ectopic CaMKII associations and phosphorylation. Together, these attributes suggest strongly that the normal dynamic range of DAT surface expression and function has been significantly limited. DA neurons transit between tonic and phasic patterns of excitation and DA release (Goto et al., 2007). The mechanisms by which DAT responds to the varying demands imposed on DA circuits are largely unknown. The study of naturally arising, disease associated DAT coding variants provides an important path to uncovering these mechanisms and also in penetrating the complexity of neuropsychiatric disorders linked to compromised DA signaling.

CHAPTER VII

INTRODUCTION TO APPENDICES

Further Characterization of DAT 615C-Associated Altered regulation

In addition to the data presented in Chapters III and IV, which resulted in a publication, I have pursued additional studies with the goal of increasing our understanding of regulatory perturbations resulting from the DAT 615C variant. Although, these findings are not published, they have informed previous and ongoing efforts.

APPENDIX A: Effect of PKCβ inhibition on AMPH-mediated reduction in DA uptake

APPENDIX B: Determination of ectopic palmitoylation due to the presence of cysteine

APPENDIX C: Effect of NOS activators and NO scavengers on AMPH-mediated reduction in DA uptake

APPENDIX D: Effect of Dynasore on DA uptake

APPENDIX E: Development and validation of a fluorescent-based assay to monitor DAT function and AMPH-mediated substrate efflux

APPENDIX F: Measurement of intracellular Ca^{2+}

APPENDIX A: Effect of PKCβ inhibition on AMPH-mediated reduction in DA uptake

Activation of PKC leads to significant downregulation of DA transport activity, as well as DAT internalization (Huff et al., 1997, Vaughan et al., 1997, Zhang et al., 1997, Zhu et al., 1997, Daniels and Amara, 1999, Loder and Melikian, 2003). An isoform of PKC, PKCβ, colocalizes with DAT in mesencephalic neurons (O'Malley et al., 2010b), and regulates AMPH-mediated DA efflux (Johnson et al., 2005b) as well as DAT trafficking (Chen et al., 2009). Furthermore, the PKCβ inhibitor (3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione) restored AMPH-mediated DAT trafficking and normalized ADE produced by the A559V variant (Erica Bowton et al, manuscript in preparation). Since the A559V and R615C variants share a common property, the inability to be regulated by AMPH, we investigated whether PKCβ inhibition would restore AMPH-mediated reduction in DA transport by the R615C variant. Here we used the same inhibitor dose (300 nM) and time (30 min) that produced alterations with the A559V variant. As shown in Figure 35, AMPH treatment produced the expected reduction in DA uptake for DAT 615R without affecting uptake by DAT 615C. PKCβ inhibition completely rescued the AMPH-mediated reduction in DA uptake for DAT 615R whereas the antagonist did not restore DA uptake capacity of DAT 615C. This result, although somewhat surprising, points towards a differential contribution of intracellular signaling networks to produce the same endo-phenotype, lack of AMPH-mediated trafficking through both DAT 615C and the A559V variant. The lack of response of DAT 615C to a PKCβ

inhibitor may relate to the lack of sensitivity to the PKC activator PMA, whereas DAT A559V exhibits PMA sensitivity. These findings, along with the localization of DAT A559V to GM1-positive microdomains, indicates that localization to these microdomains is required for PMA-induced DAT internalization whereas suppression of AMPH-induced trafficking can occur whether or not DAT is localized to these compartments.

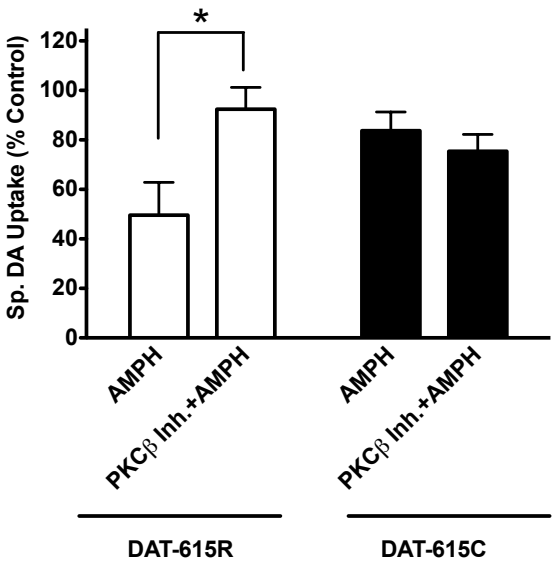


Figure 35 Effect of PKCβ Inhibitor on AMPH-induced Reduction in DA Uptake: (A) AMPH (10 μM, 30 minutes) treatment caused a significant DA transport reduction in 615R (49±13% of control) while having no effect on DAT 615C (84±8% of control). Pretreatment with the PKCβ inhibitor (300 nM, 30 minutes) before AMPH (10 μM, 30 minutes) addition blocked AMPH-mediated DA transport reduction in DAT 615R (AMPH (49±13%) vs. PKCβ inhibitor + AMPH (92±9%)) while DAT 615C showed non-significant reduction upon PKCβ inhibitor + AMPH treatment (AMPH (84±8%) vs. PKCβ inhibitor + AMPH (76±7%). Data were normalized to basal DA uptake of respective cell lines. Significant differences were calculated using Student's t-test. (n=3, $P<0.05$).

APPENDIX B: Determination of Ectopic Palmitoylation Due to the Presence of Cysteine

DAT undergoes multiple posttranslational modifications including glycosylation, ubiquitination, phosphorylation, and most recently identified palmitoylation (Patel et al., 1994, Foster et al., 2002, Li et al., 2004, Miranda et al., 2007, Foster and Vaughan, 2011). These posttranslational modifications regulate DAT activity as well as expression as discussed in Chapter I. Foster and colleagues show that rat DAT gets palmitoylated on residue C580, which is present on the junction of TMD 12 and the cytoplasmic C terminus, and possibly on one or more unidentified sites (Foster and Vaughan, 2011). Acute loss of palmitoylation is shown to impact DAT activity, without a change in surface expression, whereas chronic loss of palmitoylation leads to decreased DAT surface expression and increased degradation along with DAT activity alterations (Foster and Vaughan, 2011). A similar role of palmitoylation has not yet been shown for other neurotransmitter transporters.

Because the R615C variant introduces a cysteine, I asked the question whether this would result in ectopic palmitoylation? As palmitoylation plays a significant role in protein targeting and may be involved in altering protein-protein interactions, I hypothesized that signaling perturbations seen with the R615C variant could be due to changes in the mutant transporter's palmitoylation status. The conventional way of identifying protein palmitoylation is via metabolic labeling using [^{3}H]palmitic acid followed by IP, SDS-PAGE, and autoradiography. Due to the low beta emission of tritium, such efforts take several months to detect

the palmitoylated protein. Therefore, I decided to use a click-chemistry methodology that offers a quick and reliable way to detect DAT palmitoylation. I have described, in detail, this procedure in Chapter II. Briefly, the click-chemistry reaction utilizes a copper (I)–catalyzed azide-alkyne cycloaddition reaction followed by the Staudinger ligation with biotin-linked alkynes, and probe-modified proteins can be detected by streptavidin–horseradish peroxidase blotting (Martin and Cravatt, 2009). When we used this technique to detect changes in palmitoylation with the R615C variant, we did not find any significant increase in the palmitoylation levels of DAT 615C compared to the wildtype DAT (Figure 36). These data need to be replicated with additional controls like hydroxylamine treatment to remove palmitoylation and C580A mutant DAT to check the specificity of the technique. Nonetheless, the initial results indicate that the signaling alterations observed in the R615C variant are not due to altered palmitoylation.

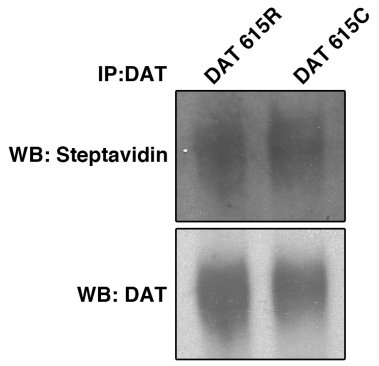


Figure 36 Determination of Ectopic Palmitoylation Due to the Presence of Cysteine: DATs were metabolically labeled with 17-ODYA and then immunoprecipitated. On the beads, click chemistry was used and the resulting product was subjected to SDS-PAGE and western blotting. The top blot shows the signal obtained from streptavidin HRP blot indicating similar palmitoylation between wildtype DAT and DAT 615C. The same membrane was stripped and re-probed with DAT antibody. The immunoblot shows equivalent expression of DAT 615R and DAT 615C. (n=1)

APPENDIX C: Effect of Nitrous Oxide Synthase (Nos) Activators and Nitric Oxide (No) Scavengers on AMPH-mediated Reduction in DA Uptake

While searching for possible answers to the conundrum of local negative charge as a reason for a lack of AMPH action with DAT 615C, my attention was drawn to the possibility that nitrosylation could explain these findings. Intracellular cysteines can get nitrosylated by action of NO and thereby impart a partial negative charge on that residue (Nagahara et al., 2009). NET, a closely related transporter to DAT, has been found to be nitrosylated (Kaye et al., 2000). Nitrosylation of NET occurs on C351 in TMD7, a residue that is absent in human DAT. Functionally, nitrosylation of NET leads to significantly decreased uptake activity (Kaye et al., 2000), similar to DAT 615C that as shown above displays a significantly lower DA transport V_{max}. Therefore, we decided to inhibit NOS, an enzyme required for NO production, thereby attempting to inhibit ectopic nitrosylation of DAT. Using two separate NOS inhibitors, S-ethylisothiourea (EIT) and S-isopropylisothiourea (IIT), I evaluated the effect of NOS inhibition on AMPH-mediated DA transport reduction. Treatment of stable cells expressing DAT 615R with EIT alone produced a significant reduction in DA uptake whereas IIT treatment alone did not affect DA uptake (Figure 37A). Neither inhibitor, when used alone, had any effect on DAT 615C activity suggesting a lack of ectopic nitrosylation (Figure 37). Interestingly, pretreatment with both EIT and IIT resulted in rescue of AMPH-mediated reduction in the DA transport from the DAT 615R cells (Figure 38). As mentioned before, AMPH action is dependent on CaMKII activity, and NO regulates CaMKII expression (Johnston and Morris, 1995),

suggesting AMPH action may also have an unappreciated NO dependency.. Pretreatment of both EIT and IIT did not produce any further decrease in DA uptake from the DAT 615C expressing cells (Figure 38). Possibly, these data may reflect the fact that CaMKII is already associated at a higher level with DAT 615C, leading to elevated basal transporter phosphorylation, and is not further stimulated by a NO-CamKII dependent manner.

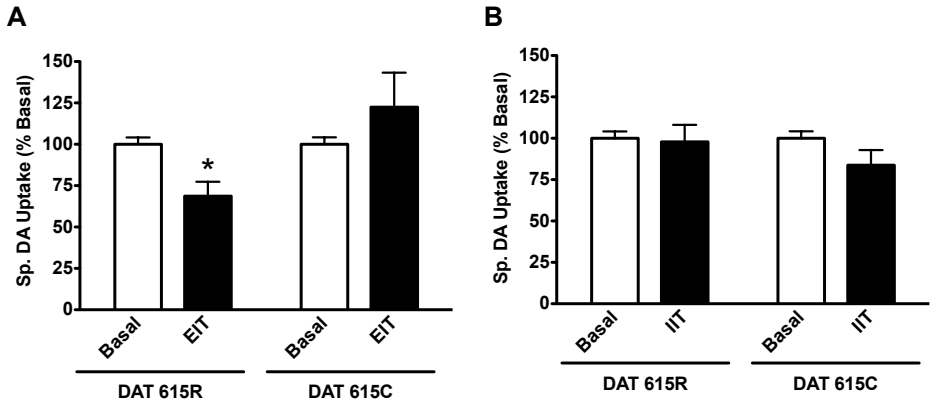


Figure 37 Effect of NOS Inhibition on DA Uptake: (A) Treatment with EIT produced a significant reduction in DA uptake for DAT 615R (69±9% of basal) without affecting the activity of DAT 615R (122±20% of basal). (B) IIT treatment did not produce any significant reduction in DA uptake for both DAT 615R (93±10% of basal) and DAT 615C (83±9% of basal).

Next we asked whether promoting nitrosylation by addition of a NO donor would impact AMPH-mediated DA transport reduction. Addition of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) did not affect basal DA uptake for either DAT 615R and the R615C variant (data not shown). Previous reports also indicate that the NO donor, SNAP, does not have any effect on DAT, but reduced NET activity as mentioned before (Kaye et al., 2000). Surprisingly, I found that SNAP reversed the AMPH-mediated DA transport reduction in DAT 615R, and

had no effect on DAT 615C (Figure 39). Both NOS inhibition and treatment with a NO donor rescued the AMPH-mediated DA transport reduction in the wildtype DAT. These similar outcomes of supposedly opposite treatments raise the

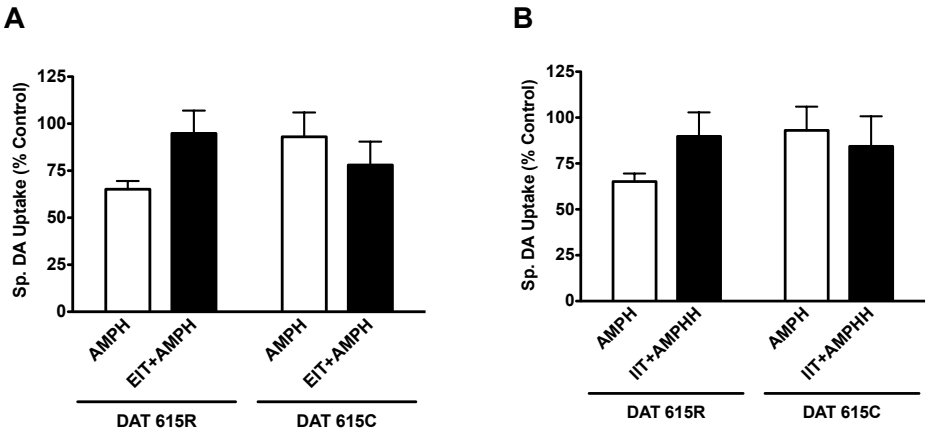


Figure 38 Effect of NOS Inhibition on AMPH-mediated Reduction in the DA Transport: Data is normalized to individual treatment controls for each cell line. **(A, B)** Treatment with AMPH produced a reduction in DA uptake for DAT 615R (65±4% of basal) without affecting DAT 615C (93±12% of basal). EIT pretreatment rescued the AMPH-mediated DA transport reduction in DAT 615R (94±12% of EIT) without affecting DAT 615C (78±12% of EIT). IIT pretreatment also rescued the AMPH-mediated DA transport reduction in DAT 615R (90±13% of IIT) without affecting DAT 615C (84±16% of IIT). (n=2)

validity of use of these compounds and may need further characterization before arriving to a conclusion. Alternatively, a possible explanation is that the NOS antagonist can block AMPH actions with DAT 615R because AMPH induces a Ca2+-NOS-CamKII dependent mechanism leading to transporter internalization. Thus, NOS blockade presents AMPH effects. When cells are pretreated with a NO donor, the need for AMPH-induced Ca2+ generation is no longer needed as the product of Ca2+ driven NOS activity is already provided. When AMPH is then added to these cells, no AMPH effect is seen because under basal conditions, the actions of AMPH have already been mimicked. These findings require that the NOS donor should produce DAT downregulation, just like AMPH. However,

no basal effects of SNAP on DAT 615R were observed. In other models (cite synaptosome studies), SNAP does act to reduce wildtype DAT activity. Thus, further studies are needed to explore the complexity of NOS contribution to both WT DAT and DAT 615C trafficking.

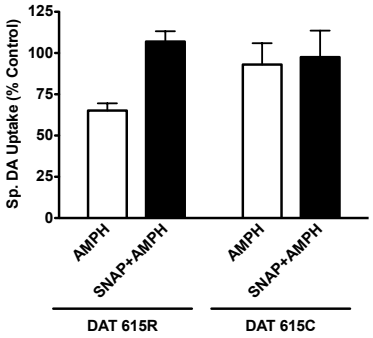


Figure 39 Effect of a NO Donor on AMPH-mediated Reduction in the DA Transport: Data is normalized to individual treatment controls for each cell line. Treatment with AMPH produced a reduction in DA uptake for DAT 615R (65±4% of basal) without affecting DAT 615C (93±12% of basal). SNAP pretreatment rescued the AMPH-mediated DA transport reduction in DAT 615R (107±6% of SNAP) without affecting DAT 615C (97±16% of SNAP). (n=2)

APPENDIX D: Effect of Dynasore on DA Uptake

AMPH-induced, as well as β-PMA-mediated, DAT internalization has been shown to occur through clathrin-mediated mechanisms (Daniels and Amara, 1999, Saunders et al., 2000). Clathrin-mediated endocytosis involves attachment of adaptor protein molecules to a membrane associated protein followed by formation of clathrin-coated pits by sequential addition of clathrin chains and invagination. Finally, clathrin-coated vesicles are pinched off from the membrane by action of the GTPase dynamin (Robinson, 1994). The role of dynamin is crucial for clathrin-mediated endocytosis, since an inactive K44A mutant of dynamin blocked the internalization of clathrin-coated vesicles (Damke et al., 1994). Recently, a small molecule inhibitor of dynamin, Dynasore, was developed, that rapidly blocks formation of coated pits within minutes of addition (Macia et al., 2006). Dynasore inhibits the GTPase activity of dynamin *in vitro* (Macia et al., 2006). Since AMPH-mediated DAT internalization is supposed to be clathrin-dependent, I tested whether blocking clathrin-mediated internalization could rescue the AMPH effects on wildtype DAT. Based on the literature, I chose a Dynasore concentration of 20 and 40 μM (Macia et al., 2006) and monitored the effect of AMPH in a DA uptake assay. As shown in Figure 40 treatment with either 20 μM or 40 μM dynasore alone did not alter DA uptake in the cell line stably-expressing WT DAT. Treatment with AMPH alone caused a significant decrease in DA uptake. Surprisingly, pretreatment with Dynasore before AMPH caused a further reduction in DA uptake, rather than a rescue. It is evident from

my studies with the CaMKII inhibitor, KN-93, that the transporter can be inactivated on the surface. Therefore, it is possible that inactive transporters may be retained on the surface, thereby reducing uptake even though clathrin-dependent internalization of the transporter is blocked. Due to these incongruent data, we decided not to pursue these studies, with the possibility of reinvestigating this interesting phenomenon of transporter inactivation in future.

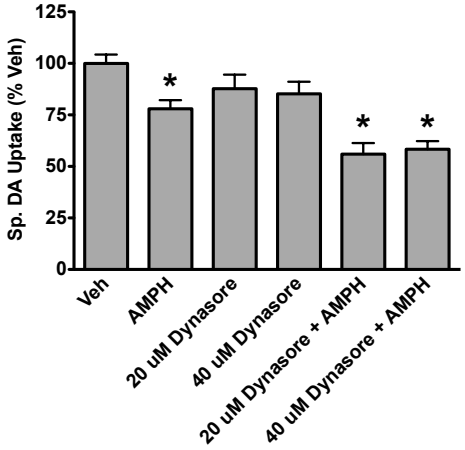


Figure 40 Effect of Dynasore on AMPH-mediated Reduction in the DA Transport: Wildtype DAT Flp-In™ HEK cells were used. Data was normalized to Vehicle treatment as 100%. Treatment with AMPH (10 μM, 30 min) alone caused a significant reduction in DA transport (78±4% of Veh). Treatment with dynasore alone (20 or 40 μM, 30 min) did not cause any significant reduction in DA uptake. Pretreatment with 20 μM or 40 μM dynasore before AMPH addition caused significant reduction in DA uptake (56±5% for 20 μM dynasore+AMPH and 58±4% for 40 μM dynasore+AMPH). Significant differences were calculated using one-way ANOVA followed by Bonferroni's post hoc test. (n=4, $P<0.01$)

APPENDIX E: Development And Validation of a Fluorescent-based Assay to Monitor DAT Function and AMPH-mediated Substrate Efflux

Historically DAT function has been monitored using [^{3}H]DA uptake assays. Using radiolabeled DA, though more physiological, creates tritiated waste and limits extensive experimental manipulations due to the high cost associated with using radioactivity. Even more important, standard uptake assays do not permit a real-time assessment of transport kinetics, and they have the confound that the substrate, DA in this case, can activate cell-surface receptors during the transport assay (D2 receptors in this case). To circumvent these issues, in collaboration with Dr. Sandra Rosenthal, our lab developed fluorescent DAT substrates as molecules that can be monitored in real-time and easily adapted for high-throughput screening of DAT function.

I have used one of these fluorescent compounds (IDT 307) to monitor the function of wildtype DAT and compared it to the function of DAT coding variants. The fluorescence of IDT 307 is not fluorescent in the extracellular medium. IDT 307, similar to DA, uses DAT to enter the cell, and fluorescence intensity increases once transported inside the cell due to substrate immobilization on intracellular proteins. This increased fluorescence can be measured as a function of time using a kinetic fluorescence plate reader. The commercial form of IDT 307 is available from the Molecular Devices as “Neurotransmitter Transporter Uptake Assay Kit”.

Saturation kinetic analysis using [^{3}H]DA uptake assays using DAT 615C showed reduced DA transport V_{max} without a significant change in K_m (Chapter

III). I performed similar saturation kinetic analysis using IDT 307 and established decreased IDT 307 transport V_{max} associated with the R615C variant (DAT 615R, 32279±6110 vs. DAT 615C, 22079±2827 RFU/μg protein) (Figure 41). Interestingly, the affinity (K_M) of DAT 615C for IDT 307 also appears to be increased (DAT 615R, 2.6±1.7 vs. DAT 615C 6.1±2.1 μM), possibly owing to structural differences between natural substrate, DA, and a synthetic substrate, IDT 307. These findings may point to D2 modulation of DAT to place the transporter in a high affinity state, which could be tested by studies using DA as a substrate in conventional transport assays +/- the D2 receptor antagonists haloperidol or sulpiride or by testing IDT307 uptake via fluorescence methods using the D2 receptor agonist quinpirole.

Next, I tested whether IDT 307 can be used to detect substrate efflux, similar to the DA efflux detected upon AMPH treatment. One thing to note here is that IDT 307 appears to need intracellular immobilization to show increased fluorescence intensity and this interaction may be strong enough to block the efflux of IDT 307 upon AMPH administration. Flp-In HEK stable cells lines were loaded with IDT 307 for 10 min, and excess IDT 307 was washed away before addition of AMPH. Change in fluorescence was measured and, as shown in Figure 42, AMPH did cause a detectible change in IDT 307-mediated fluorescence, whereas vehicle control and GBR 12909-treated cells showed a minimal decrease in the fluorescence (data not shown). I was also able to detect diminished IDT 307 efflux activity in the DAT 615C transfected cells as compared

to DAT 615R. These findings are consistent with the reduced surface expression of DAT 615C, but are at odds with the data previously reported for AMPH-mediated DA efflux. These findings may reflect the role of the D2 receptor in maintaining the efflux of DA, despite reduced DAT 615C surface expression, an activity that IDT 307 will not produce. Overall, these findings indicate that the use of IDT 307 provides a novel paradigm for studying DAT-dependent substrate efflux and receptor modulation of transport and efflux processes.

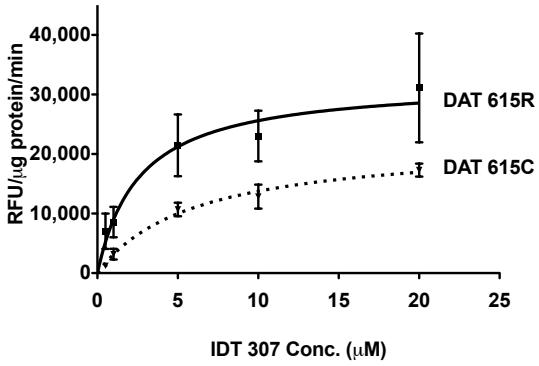


Figure 41 Saturation Kinetic Analysis Using IDT 307 Uptake: IDT 307 uptake V_{max} and K_m are expressed as RFU/μg protein/min and μM respectively. K_m and V_{max} values are expressed as ± SEM. Compared to DAT 615R; both V_{max} and K_m are significantly lower for DAT 615C. (n=4)

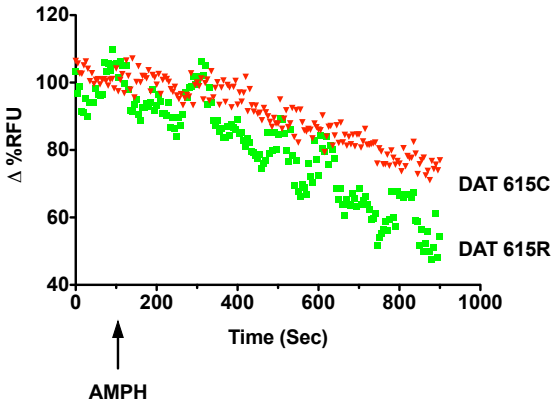


Figure 42 AMPH-mediated IDT 307 Efflux: Flp-In HEK Stable lines expressing DAT 615R, and DAT 615C were preloaded with IDT 307 for 10 min and AMPH-induced IDT 307 efflux was measured. Both wildtype DAT and the R615C variant display significant reduction in fluorescence upon AMPH treatment, which may indicate IDT 307 efflux. DAT 615R may exhibit greater efflux compared to DAT 615C. (n=1)

APPENDIX F: Impact of DAT Mutation on Intracellular Ca^{2+}

AMPH-mediated DA efflux is dependent on intracellular Ca^{2+}. Chelation of Ca^{2+} using 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) or thapsigargin blocked AMPH-mediated DA efflux measured by amperometry (Gnegy et al., 2004). The AMPH-induced increase in intracellular Ca^{2+} is thought to activate CaMKII and regulate DA efflux (Fog et al., 2006). The DAT 615C variant displays a normal AMPH-induced DA efflux, but also exhibits loss of AMPH-mediated downregulation. This prompted me to investigate levels of intracellular Ca^{2+} in our stable cell lines. Here I used a commercially available, single wavelength Ca^{2+} measuring dye (Calcium 3 Assay Kit, Molecular Devices) to determine relative Ca^{2+} levels between DAT 615R and 615C expressing cells. Under basal conditions, DAT 615C displayed a significantly higher intracellular Ca^{2+} compared to the WT DAT (Figure 43). Measurement of Ca^{2+} over a period of 10 min using FlexStation also showed a sustained increase in the basal Ca^{2+} levels in cells expressing DAT 615C (data not shown). Unfortunately, treatment with either AMPH or a Ca^{2+} ionophore, A23187, produced similar increases in both WT DAT and the R615C variant. There are a few caveats to using this type of single wavelength determination of Ca^{2+} levels. It is very difficult to determine absolute levels of Ca^{2+} concentration and variability between cell lines (size, volume and density) is not accounted in the estimate. To circumvent these problems, I also measured intracellular Ca^{2+} using a dual wavelength Fura 2AM dye. Fura 2AM, is a membrane permeable, Ca^{2+} sensitive dye, which when

excited at 340 nm shows increased fluorescence upon Ca^{2+} binding and at 380 nm shows decreased fluorescence upon Ca^{2+} binding. The ratio between 340 and 380 gives a better estimate for free intracellular Ca^{2+}. Our instruments are unfortunately not sensitive enough to rapidly shift between the 340 and 380 nm

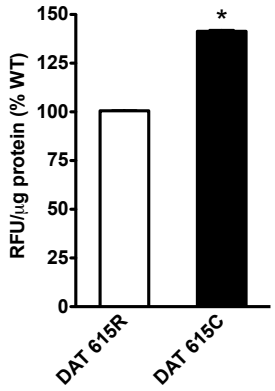


Figure 43 Measurement of Intracellular Ca^{2+}: Flp-In HEK stable lines were used to determine intracellular Ca^{2+} using calcium 3 assay kit. Compared to DAT 615R, the R615C variant displays significantly elevated basal intracellular Ca^{2+}. Data is normalized to basal intracellular Ca^{2+} from DAT 615R as 100% and expressed as ± SEM. (n=3; Student's t-test , P<0.001)

excitation making it impossible to generate any usable data. Nonetheless, results obtained from the calcium 3 assay kit strongly indicate altered basal intracellular Ca^{2+} in our stable lines and as such further studies using single cell Ca^{2+} imaging might reveal interesting underlying phenotypes, which might be pertinent to loss of AMPH action on the R615C variant. Should these findings be reproduced and validated, they would suggest that DAT 615C can elevate intracellular Ca^{2+}, for which a mechanism would need to be elucidated. Possibilities include 1) DAT 615C fluxes Ca^{2+} instead of Na^{+} under basal conditions,, 2) DAT 615C tonically depolarizes the host cell, leading to altered Ca^{2+} homeostasis, or 3) DAT interacts ectopically with molecules normally controlling intracellular Ca^{2+}stores.

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