Effects of Human Dopamine Transporter (DAT) Mutations and Novel Allosteric Modulatory Compounds in Disrupting HIV-1 Tat-DAT Protein Interaction

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Effects of Human Dopamine Transporter (DAT) Mutations and Novel Allosteric Modulatory Compounds in Disrupting HIV-1 Tat-DAT Protein Interaction

by

Pamela Marie P. Quizon

Bachelor of Science
The University of North Carolina at Pembroke, 2011

Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Pharmaceutical Sciences

College of Pharmacy

University of South Carolina

2019

Accepted by:

Jun Zhu, Major Professor

Campbell McInnes, Chair, Examining Committee

James R. Fadel, Committee Member

Lorne J. Hofseth, Committee Member

Kennerly S. Patrick, Committee Member

Cheryl L. Addy, Vice Provost and Dean of the Graduate School
DEDICATION

I dedicate this work to my grandfather, the late Dr. Roberto T. Quizon for inspiring me to pursue science. *May isa na namang doktor sa pamilya.*
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Jun Zhu, whose patience, guidance, and support have helped me get to where I am today. Thank you for allowing me to have this amazing opportunity and supporting me all throughout my studies. I will always be grateful for your mentorship and training.

I am very grateful for the past and present members of the Zhu Lab who have become friends and trusted colleagues, particularly Dr. Matthew Strauss for all our lively discussions and for making the lab a fun place to be, Dr. Wei-Lun Sun for his mentorship and guidance with my research, and Dr. Narasimha Midde for his mentorship and advice throughout the years. Many thanks to Steven Lin, Richard McCain Jr., Yi Zhou, Yike Zhu, Palak Patel, YingYing Wang, Sierra McDonald, and Dr. Sona Tailor for their valuable technical contributions.

I would also like to thank my dissertation committee for their valuable guidance and critique: Dr. Campbell McInnes, Dr. Jim Fadel, Dr. Kennerly Patrick, and Dr. Lorne Hofseth. I would like to thank our collaborators, Dr. Chang-Guo Zhan from the University of Kentucky for sending us their molecular dynamics data on such a tight schedule, Dr. Sam Ananthan from Southern Research Institute for sending us the SRI compounds and for answering my many questions, and Dr. Jay McLaughlin from the University of Florida for our in vivo data. Special thanks to Dr. Kim Creek and Dr. Diego Altomare for allowing me to use their lab facilities and their assistance. Thank you to my graduate
directors, Dr. Doug Pittman, Dr. Lorne Hofseth, and Dr. Larry Reagan who have supported and believed in me throughout the ups and downs of grad school. Special thanks to Rachel McKeown for her administrative help and for giving me lots of encouragement and advice.

None of this would have been possible without the support of NIH/NIDA grants awarded to Dr. Jun Zhu. I would also like to thank the USC Graduate School for awarding me an International Travel Grant, the Society on Neuromune Pharmacology for an Early Career Investigator Travel Award, and the USC College of Pharmacy for awarding me the Charles Yandell Scholarship.

Many thanks to these special people at USC and UNC for their friendship, support, and encouragement throughout the years: Dr. Anusha Chaparala, Dr. Nicole Reilly, Chad Beneker, Jessy Varghese, Destan Kirimhan, Dr. Vinal Menon, Dr. Ran Hee Choi, Jennifer Erichsen, Maribel Vazquez, Jody Albright, Dr. Tangi Smallwood, and Dr. Meagan Wisniewski.

Last but not the least, I would like to thank my loving family who supported me throughout this journey. To my mom, Neil, and dad, Rigo, for your unconditional love, support, and sacrifices. To my siblings, Andrea and Arjay, for always being there for me. To Dr. Mark Phillips Henriksen for being a constant source of love, support, and encouragement. I am so grateful to have you in my life. To my furry little cookie monster, Coco—thanks for bringing so much joy and happiness into my life. I would not have made it without all of you.
ABSTRACT

More than 37 million people are living with HIV worldwide. Despite the widespread use of antiretroviral therapy (ART), up to 70% of HIV-positive individuals suffer from cognitive and behavioral deficits collectively known as HIV-associated neurocognitive disorders (HAND). HIV-mediated damage to the dopaminergic system is a mediating factor in HAND. Dopamine (DA) transporter (DAT)-mediated reuptake is essential for maintaining DA homeostasis. Because most ART cannot efficiently cross the blood-brain barrier (BBB), the brain serves as a viral reservoir that facilitates the spread of infection to microglia and astrocytes. Infected cells shed viral proteins such as Tat, which plays a critical role in HIV infection-induced dysregulation of the dopaminergic system by its allosteric inhibition of DAT. Because Tat does not compete with the DA uptake site, blocking the Tat-DAT interaction will have minimal effects on normal transporter function. In addition, cocaine, a DAT inhibitor, magnifies the effects of Tat on neurotoxicity, which contributes to HAND severity in cocaine-abusing HIV-infected patients. We hypothesize that Tat protein inhibits dopamine (DA) uptake by interacting with specific recognition binding residues on DAT thereby leading to dopaminergic dysregulation observed in HAND. Targeting the Tat-DAT interaction during the early stages of HIV-1 infection is therefore a potential therapeutic strategy to prevent HAND.
By utilizing integrated computational homology modeling and pharmacological validation, key recognition binding residues on human DAT that are critical to Tat-DAT interaction were identified. Mutations on these residues attenuated Tat-induced inhibition of DAT uptake while preserving (Y88F, D203L) or significantly enhancing (H547A) basal function of the transporter. H547A was found to enhance DAT function through alterations in phosphorylation, palmitoylation, and transporter conformation. Additionally, double (D206L/H547A) and triple (Y88F/D206L/H547A) combinations of these mutations blocked Tat inhibition. Introducing mutations on Tat\textsuperscript{1-86} (K19A, C22G) reversed the inhibitory effects of the protein, indicative of disrupted Tat-DAT binding.

Finally, to probe the therapeutic feasibility of developing allosteric modulators to block Tat and cocaine binding without interfering with normal DAT function, the SRI compounds, novel quinazoline-based allosteric modulators, were utilized. From the initial screening, SRI-32743 was selected due to its effective potency. SRI-32743 pharmacologically demonstrated allosteric properties and attenuated Tat inhibition in wild-type human DAT-expressing cells. When administered systemically to inducible HIV-1 Tat transgenic mice after 14 days of Tat induction via doxycycline, SRI-32743 dose-dependently ameliorated conditioned place preference for cocaine. These results demonstrate that developing allosteric modulatory molecules which attenuate cocaine and Tat binding to DAT will aid drug discovery efforts in the search for therapeutic interventions for HIV-infected patients who are concurrent abusers of cocaine.
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LIST OF ABBREVIATIONS

2-BP ................................................................. 2-bromopalmitate
3D ................................................................. Three-dimensional
AMPH ............................................................. d-Amphetamine
ANI ............................................................... Asymptomatic neurocognitive impairment
ANOVA ........................................................... Analysis of Variance
ART ............................................................... Anti-retroviral therapy
BBB ............................................................... Blood-brain barrier
BD ................................................................. Brownian dynamics
BIM-1 ............................................................. Bisindoylmaleimide-1
B_max .............................................................. Maximum binding capacity
CCR5 .............................................................. CC chemokine receptor 5
CD4 ............................................................... Cluster of differentiation 4
CFT ................................................................. (−)-2-β-Carbomethoxy-3-β-(4-fluorophenyl)tropane
CNS ............................................................... Central nervous system
Co-IP .............................................................. Co-Immunoprecipitation
CSF ............................................................... Cerebrospinal fluid
CXCR4 ........................................................... CXC-chemokine receptor 4
DA ................................................................. Dopamine
DAT .............................................................. Dopamine transporter
Dox ............................................................... Doxycycline
ERK .............................................................. Extracellular regulated kinase
FBS.......................................................... Fetal bovine serum
GBR12909... 1-[2-(bis[4-fluorophenyl]methoxy)ethyl]-4-[3-phenylpropyl] piperazine
GFAP.......................................................... Glial fibrillary acidic protein
GFP............................................................ Green fluorescent protein
GST............................................................ Glutathione S-transferase
HAD........................................................... HIV-1 associated dementia
HAND......................................................... HIV-1 associated neurocognitive disorder
hDAT.......................................................... Human dopamine transporter
hNET.......................................................... Human norepinephrine transporter
HIV-1.......................................................... Human Immunodeficiency Virus Type-1
IB............................................................... Immunoblotting
IC_{50}....................................................... The half maximal inhibitory concentration
IP............................................................... Immunoprecipitation
iTat-tg......................................................... Inducible HIV-1 Tat transgenic mouse
K_d.............................................................. Dissociation constant
K_m............................................................. Michaelis-Menten constant
KRH........................................................... Krebs-Ringer-HEPES
MAPK......................................................... Mitogen-activated protein kinase
MD............................................................. Molecular dynamics
MND.......................................................... Mild neurocognitive disorder
MPP+......................................................... 1-methyl-4-phenylpyridinium
MTSET....................................................... [2-(trimethylammonium) ethyl] methane thiosulfonate
NAc............................................................ Nucleus accumbens
NIH........................................................... National Institutes of Health
PAL........................................................... Palmitoylation
PAT ................................................................. Palmitoyl acyltransferase
PFC ................................................................. Prefrontal cortex
PKC ................................................................. Protein kinase C
PMA ................................................................. Phorbol 12-myristate 13-acetate
rTat1-86 ....................................................... Recombinant HIV-1 Tat protein (86 amino acids)
SAR ................................................................. Structure-activity relationship
SDS ................................................................. Sodium dodecyl sulfate
SEM ................................................................. Standard error of the mean
SLC6A3 .............................................................. Solute carrier family 6 member 3
Taq ................................................................. Trans-activator of transcription
V_{max} .............................................................. Maximal velocity
VTA ................................................................. Ventral tegmental area
WIN35,428 .................................................... (-)-2-β-Carbomethoxy-3-β-(4-fluorophenyl)tropane
WT ................................................................. Wild-type
CHAPTER 1
INTRODUCTION

1.1 SIGNIFICANCE

The introduction of antiretroviral therapy (ART) in the mid- to late nineties has transformed human immunodeficiency virus type 1 infection (“HIV-1”, but will also be referred to as “HIV” throughout this dissertation) from a fatal and terminal diagnosis to a manageable chronic disease. Subsequently, the number of people living with HIV has steadily risen throughout the decades. Currently, there are about 37 million people living with HIV worldwide (Pustil 2016). This reflects the dramatic reduction in mortality, increased access to ART, and improved life expectancy (Simioni et al. 2010; Bonnet et al. 2013). Untreated HIV progressively leads to the development of acquired immunodeficiency syndrome (AIDS), the final stage of HIV infection (Vahlne 2009). Opportunistic infections that were once a direct consequence of debilitating immunodeficiency have decreased overall (Carroll and Brew 2017); however, the increased prevalence of disrupted neurocognitive function has become one of the most common complications of HIV (Sanmarti et al. 2014). These neurological complications are referred to as HIV-associated neurocognitive disorders (HAND). Widespread access to ART has relegated the most severe manifestations of HAND such as HIV encephalitis and HIV-associated dementia (HAD) to rare occurrences (Heaton et al. 2010, 2011); however, the milder forms of HAND continue to persist, affecting up to
70% of HIV-positive individuals (Sacktor et al. 2001; Cysique et al. 2004; McArthur 2004; Robertson et al. 2007; Heaton et al. 2008, 2010; Wright et al. 2008; Woods et al. 2009; Simioni et al. 2010). The longer life expectancy in HIV-positive individuals has consequently increased the prevalence of HAND (Simioni et al. 2010; Brew and Chan 2014). This has dramatically altered patient demographics, as individuals over 50 years old now make up the largest percentage of persons living with HIV (Centers for Disease Control and Prevention 2017). Interestingly, it has been found that drugs of abuse such as cocaine and methamphetamine have a synergistic effect on the neurodegenerative effects found in HIV-infected patients regardless of ART treatment (Starace et al. 1998; Langford et al. 2003; Chana et al. 2006; Nath 2010; Purohit et al. 2011; Meade et al. 2011a; Meyer et al. 2014). The healthcare burden of HAND will continue to rise, as estimates from other countries have suggested that the number of HIV-positive patients with HAND will increase from 5- to 10-fold by the year 2030 (Cysique et al. 2011). There is no cure for HAND, and considering its progressive and neurodegenerative nature, an early intervention strategy is critical.

1.2 HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS (HAND)

Neurocognitive dysfunction in HIV-positive patients has been observed as early as the 1980s, where the only well-defined neurological complication of HIV was HIV-associated dementia (HAD) (Sanmarti et al. 2014). Patients with HAD were primarily severely immunocompromised and were observed to have highly advanced cognitive impairment that was accompanied by motor and behavioral
deficits (Sanmarti et al. 2014). The introduction of ART in the mid- to late 1990s has dramatically reduced the incidence of HAD (McArthur et al. 2005); however, the milder forms of HAND have become more common throughout the years (Heaton et al. 2010, 2011; Saylor et al. 2016). Despite having adequately suppressed plasma viral load (<50 copies/mL) through ART, neurocognitive impairment is still observed in these patients, which suggests that factors other than systemic viral replication are at play. In 2007, the Frascati Criteria was introduced, which further revised the HAND classification system into a spectrum of neurocognitive disorders that range from the very mild to the most severe (Antinori et al. 2007, 2013). Asymptomatic Neurocognitive Impairment (ANI), the mildest stage of HAND, affects about 33% of HIV-positive patients, and is characterized by neuropsychological impairment in at least two cognitive domains without affecting activities of daily living. Mild Neurocognitive Disorder (MND), which is a level higher than ANI, affects 20-30% of HIV-positive patients, and is defined as mild to moderate impairment in at least two cognitive areas with a disruption in activities of daily living. Currently, HAD, the most severe albeit rare form of HAND, affects about 2-8% of HIV-positive patients, and is marked by cognitive impairment in at least two cognitive domains and accompanied by significant impairment in activities of daily living. The differential diagnosis of HAND emphasizes that there must be no evidence of cognitive impairment that does not have HIV as the underlying cause (Antinori et al. 2013).

Entry of HIV into a cell requires binding to CD4 (cluster of differentiation 4) and an HIV co-receptor such as CXCR4 (CXC-chemokine receptor 4) or CCR5
(CC-chemokine receptor 5) (Albright et al. 2003; González-Scarano and Martín-García 2005; Mocchetti et al. 2012). HIV entry into lymphocytes is mediated by CXCR4, while CCR5 controls viral entry into monocytes and macrophages and is thought to play a greater role in the spread and initiation of HIV infection (Doms and Trono 2000; González-Scarano and Martín-García 2005; Anderson and Akkina 2007; Mocchetti et al. 2012). Unlike monocytes, macrophages, and microglia, neurons do not express CD4 and are therefore not susceptible to HIV infection (González-Scarano and Martín-García 2005). However, neuronal damage that stems from HAND occurs as a consequence of central nervous system (CNS) exposure to direct and indirect factors: viral proteins and neurotoxicity from ART (direct); and subsequent “bystander” trauma to neurons from inflammation, perturbation of calcium and glutamate homeostasis, as well as oxidative stress (indirect) (González-Scarano and Martín-García 2005; King et al. 2006; Letendre et al. 2010; Clifford and Ances 2013). HIV infection in the brain occurs within the first few weeks of initial infection through the “Trojan horse” mechanism (Peluso et al. 1985; Haase 1986): the virus rapidly crosses the selectively permeable blood-brain barrier (BBB) by piggybacking on infected monocytes and lymphocytes that migrate to the CNS (Clements et al. 2002; Orandle et al. 2002; Kim et al. 2003; Roberts et al. 2004; Williams et al. 2014). Upon reaching the brain, the infected monocytes mature into HIV-infected perivascular macrophages that produce more virions and facilitate the spread of the virus (Albright et al. 2003; González-Scarano and Martín-García 2005). Because most ART cannot cross the BBB, the brain serves as a viral reservoir as
the virus spreads to other key cell types in the CNS susceptible to HIV infection such as microglia and astrocytes (McArthur et al. 2010). As the infected cells shed neurotoxic viral particles, proteins, and cytokines, viral load is increased in the CNS, thereby facilitating continued infection, neurotoxicity, and subsequent neuronal injury that are central to the development of HAND (Tornatore et al. 1991; Brown et al. 2006; Thompson et al. 2011; Desplats et al. 2013).

1.3 HIV TAT PROTEIN

Since HIV does not directly infect neurons (González-Scarano and Martín-García 2005) and cognitive impairment is still observed in patients with controlled viral loads, neurodegeneration that results in HAND is only an indirect consequence of viral replication. Viral proteins that are shed by HIV-infected cells such as Tat (transactivator of transcription), gp120 (glycoprotein 120), and Vpr (viral protein R) have been found to cause neurotoxicity and have been implicated in HAND neuropathogenesis (Nath and Geiger 1998). Among the key viral proteins, the regulatory protein Tat is one of the most studied (Gaskill et al. 2017; Zhu et al. 2018). Tat is a small nonstructural polypeptide composed of six protein domains encoded by two exons. Amino acids 1-72 belong to the first five domains and are encoded from the first exon, which is critical to the transcriptional activity of Tat. These first five domains are the acidic domain (amino acid residues 1-21), cysteine-rich domain (amino acid residues 22-37), the hydrophobic core domain (38-48), a basic domain (48-57), and a glutamine-rich domain (58-72). The sixth domain is a C-terminus domain of varying lengths, usually comprised of amino acids 73-101 (but could include 58-101) and are
encoded from the second exon (Bertrand et al. 2013). Majority of Tat found isolated in HIV clinical samples is of the 101 amino acid variant (Tat\textsubscript{1-101}), while the shorter laboratory-adapted strain containing 86 amino acids (Tat\textsubscript{1-86}) is widely used in research (Gaskill et al. 2017). However, reports have shown that both Tat\textsubscript{1-101} and Tat\textsubscript{1-86} were found in clinical isolates from HIV-positive patients (Barré-Sinoussi et al. 1983; Jeang et al. 1999). Furthermore, studies comparing various forms of Tat have shown no marked differences between these types (Ma and Nath 1997; Bertrand et al. 2013; Midde et al. 2013). The studies reported in this dissertation have utilized Tat\textsubscript{1-86}.

Critical for viral replication, Tat is secreted by HIV-infected macrophages and microglia, and due to its highly penetrative nature, it is readily absorbed by uninfected cells (Frankel and Pabo 1988; Vivès et al. 1997; Frankel and Young 1998). These properties allow Tat to interact with a wide variety of cells and carry out numerous functions. For instance, Tat has been found to activate dormant T-lymphocytes (Ott 1997), induce cellular apoptosis (Li et al. 1995; Westendorp et al. 1995), modulate gene expression by disrupting intracellular signaling cascades (Westendorp et al. 1995; Nath and Geiger 1998; Jeang et al. 1999), attract monocytes (Albini et al. 1998), increase BBB permeability (Toborek et al. 2005), and even act as a growth factor for Kaposi-like cells (Trinh et al. 1999). Post-mortem studies reveal the presence of Tat protein in the monocytes, astrocytes, microglia, and dopamine (DA)-rich brain regions in samples from HIV-infected patients (Hofman et al. 1994; Bonwetsch et al. 1999; Del Valle et al. 2000). Tat has also been detected in the plasma, serum, and CSF of HIV-
infected patients, with concentrations ranging from 1 ng/mL to 40 ng/mL (Westendorp et al. 1995; Xiao et al. 2000). However, there is no clear consensus in the literature regarding the actual concentration of biologically active Tat in systemic circulation (Westendorp et al. 1995; Xiao et al. 2000); more studies and detection methods must be developed. Nevertheless, Tat has been found to possess neurotoxic properties (Sabatier et al. 1991; King et al. 2006). Though the exact mechanism is unclear, some studies suggest that this may be due to Tat-mediated excitotoxicity (Li et al. 2008), disruption of endogenous miRNA patterns (Chang et al. 2011), and degradation of proteins associated with microtubule and cytoskeletal formation in neurons (Aprea et al. 2006), all leading to neuronal damage observed in HIV-infected patients. Exposure to Tat results in perturbations to the dopaminergic system, which has been implicated in the development of HAND (Purohit et al. 2011; Gaskill et al. 2017; Zhu et al. 2018). The interplay of HIV infection and Tat protein in the perturbation of dopaminergic neurotransmission in relation to HAND will be discussed in the next section.

1.4 HIV AND TAT ON DOPAMINERGIC NEUROTRANSMISSION

Dopamine (DA) is a neurotransmitter involved in the reward, cognition, and motor control centers of the brain (Wise 2004). Dopaminergic homeostasis in the brain is maintained by the dopamine transporter (DAT), which rapidly sequesters extracellular DA back into the presynaptic neuron (Torres 2006; Hamilton et al. 2014). Dysfunctional dopaminergic neurotransmission has been implicated in the development of HAND (Purohit et al. 2011; Gaskill et al. 2017; Zhu et al. 2018).
Evidence from clinical samples and animal studies have shown that HIV-mediated damage to dopaminergic neurons occur in the asymptomatic early infection stage (Lopez et al. 1999; Koutsilieri et al. 2002b; Scheller et al. 2005). HIV infection has been shown to affect the CNS metabolism of DA (Kumar et al. 2009, 2011; Horn et al. 2013; Meulendyke et al. 2014), as well cause disruption in the brain’s dopaminergic system (Berger and Nath 1997; Kumar et al. 2009; McIntosh et al. 2015). Significant neuronal damage to DA-rich areas in the brain has been observed in HIV-infected patients who are undergoing ART (Kumar et al. 2009, 2011; Scheller et al. 2010; Horn et al. 2013; Meulendyke et al. 2014) as well as those who are not (Kieburtz et al. 1991; Berger et al. 1994; Sardar et al. 1996; Koutsilieri et al. 1997, 2002b, a; Di Rocco et al. 2000; Silvers et al. 2006). In patients with HIV-associated dementia (HAD), neuroimaging studies have revealed evidence of decreased levels of DAT in the basal ganglia, demonstrating the key role DAT plays in the neuropathogenesis of HAND (Wang et al. 2004). Furthermore, HIV-positive patients with a history of cocaine abuse showed a further reduction in DAT density compared with HAD patients, correlating with diminished performance in a battery of neurocognitive tests (Chang et al. 2008). These studies clinically demonstrate the connection between HIV infection, drugs of abuse, and the dopaminergic system.

Drugs of abuse, which alter dopamine levels in the brain, have been found to accelerate HIV infection and HAND neuropathology (Dahal et al. 2015; Dash et al. 2015). Cocaine, one of the most widely abused drugs in the HIV-positive population, has been found to disrupt macrophage and lymphocyte function
(Klein et al. 1993; Mao et al. 1996; Baldwin et al. 1997; Eisenstein and Hilburger 1998; Friedman et al. 2003), promote HIV infection in these target cells (Peterson et al. 1990; Bagasra and Pomerantz 1993; Roth et al. 2002; Steele et al. 2003), compromises BBB integrity (Dahal et al. 2015), and even accelerate disease progression into AIDS (Dahal et al. 2015). Most importantly, as a very potent and addictive CNS stimulator, cocaine, directly inhibits DA reuptake by blocking DAT, leading to extracellular DA overflow. Consequently, elevated levels of dopamine were found to promote HIV viral replication and infection of macrophages and lymphocytes (Scheller et al. 2000; Gaskill et al. 2009), thereby accelerating HAND neuropathogenesis. In the presence of HIV viral protein, Tat, which is also an inhibitor of DAT, the effects of cocaine are magnified. Tat protein and cocaine act synergistically to exacerbate HIV-related neurotoxicity, leading to HAND. This creates a feedback loop wherein increased levels of dopamine due to DAT inhibition interact with the dopamine receptors expressed on HIV-infected macrophages (Gaskill et al. 2013, 2014), stimulating the release of more viral proteins such as Tat (Rappaport et al. 1999), which not only accelerates viral replication, but leads to neurotoxicity (Nath and Geiger 1998). Oxidative stress characteristic of catecholamine breakdown due to the increased levels of dopamine also contributes to damage observed in dopaminergic neurons in the more advanced stages of HIV (Nath et al. 2000; Dahal et al. 2015). As the disease further progresses, dopaminergic neurodegeneration occurs, leading to more advanced clinical manifestations of HAND.
1.5 THE HUMAN DOPAMINE TRANSPORTER (hDAT)

1.5.1 Structure and function

Dopaminergic dysregulation has been associated with the development of HAND. At the center of HIV infection, viral protein release, and drugs of abuse that contribute to HAND neuropathogenesis lies the dopamine transporter (DAT). Human DAT, cloned in 1992, contains 620 amino acids and consists of 12 transmembrane (TM) domains with five intracellular and six extracellular loops (Giros et al. 1992). DAT is a sodium-coupled symporter protein that is responsible for maintaining dopamine (DA) homeostasis and terminating DA neurotransmission through rapid sequestration of DA back into the neuron. DAT carries extracellular DA against its concentration gradient back into presynaptic neurons by deriving energy from coupling substrate translocation to existing Na\(^+\) and Cl\(^-\) ionic gradients. Intracellular DA that has been through the reuptake process gets recycled for later use or is enzymatically broken down by mitochondrial monoamine oxidases (MAO-A and MAO-B) as well as catechol O-methyltransferase (COMT) (McHugh and Buckley 2015; Sitte and Freissmuth 2015). Because dopamine reuptake is the most effective way to terminate dopaminergic neurotransmission, any disruptions to DAT function and binding would significantly affect the strength and duration of dopaminergic signaling in the brain (Goodwin et al. 2009).

1.5.2 The neurotransmitter: sodium symporter family

DAT, also known as solute carrier protein SLC6A3, is a member of the Neurotransmitter: Sodium Symporter (NSS) family, which includes other
plasmalemmal transporters for monoamines such as the catecholamine norepinephrine (NET; SLC6A2) and indolamine serotonin (SERT; SLC6A4), as well as transporters for amino acids glycine and GABA (Chen and Reith 2000; Sitte and Freissmuth 2015). DAT, NET, and SERT are also known as the biogenic amine transporters (Wang et al. 2015). These transporters contain intracellular amino and carboxyl termini that help control uptake and substrate recognition via multiple putative post-translational modification sites (Foster et al. 2002; Fog et al. 2006; Wu et al. 2015; Moritz et al. 2015; Bermingham and Blakely 2016) as well as variations in amino acid sequences that result in distinct properties (Wang et al. 2015). These sequences, while highly conserved across different species, vary greatly between the different types of transporters, which suggests that transporter-specific regulation is influenced by these residues (Eriksen et al. 2010).

1.5.3 Transporter regulation

DAT is regulated by complex and overlapping signaling systems that require further exploration in order to fully harness its therapeutic potential. Three of the most studied are through posttranslational modifications, ligand-transporter interactions, and protein-protein interactions (Zhu and Reith 2008; Eriksen et al. 2010; Kristensen et al. 2011; Vaughan and Foster 2013). Majority of these regulatory mechanisms influence transporter trafficking and surface expression on the plasma membrane (Eriksen et al. 2010). This is because the strength of dopaminergic signaling is highly dependent on the number of available DAT on the surface. For the purposes of this dissertation, I will focus on these three
regulatory mechanisms and discuss how these relate to the development of neurocognitive dysfunction that is characteristic of HAND.

1.5.4 Posttranslational modifications regulate DAT

Among signaling systems that regulate DAT, phosphorylation by protein kinase C (PKC) is one of the most well characterized (Vaughan et al. 1997; Foster et al. 2002, 2012; Guptaroy et al. 2009). Activation of PKC dampens DA reuptake by increasing DAT endocytosis, which has been considered to be one of the key players of transport downregulation (Ramamoorthy et al. 2011; Vaughan and Foster 2013). The use of PKC activator PMA (phorbol 12-myristate 13-acetate) to promote DAT phosphorylation results in decreased DA uptake $V_{\text{max}}$ due to the increased endocytic trafficking of active transporters into the cytosol (Melikian 2004). In contrast, palmitoylation, a reversible lipid modification that regulates proteins in a similar manner to that of phosphorylation/dephosphorylation, increases DA uptake (Linder and Deschenes 2007; Foster and Vaughan 2011). Through mutational and pharmacological techniques, it has been found that the interplay between phosphorylation and palmitoylation is a critical mechanism that governs DAT function (Moritz et al. 2015; Rastedt et al. 2016). This novel mechanism for DA uptake and control sheds light on how DAT is regulated and offers a potential new mechanistic target for the treatment of diseases involving dysfunctional dopaminergic neurotransmission such as HAND.
1.5.5 Ligand-transporter interactions influence DAT function

Drugs such as cocaine and the amphetamines target DAT and elevate extracellular levels of DA, thereby interfering with dopaminergic reward pathways and facilitating addiction (Zhu and Reith 2008). For instance, cocaine binds to the central binding site of DAT and blocks the reuptake of DA by locking the transporter in an outward-facing conformation, leading to significant potentiation of dopaminergic neurotransmission in the brain (Giros and Caron 1993; Mortensen and Amara 2003; Navratna et al. 2018). On the other hand, amphetamines structurally resemble DA and therefore act as substrates of DAT (Chen and Reith 2000). However, amphetamines competitively inhibit DA uptake (Rothman and Baumann 2003; Han and Gu 2006) and promote the rapid efflux of DA via reverse transport through DAT (Jones et al. 1998; Sitte et al. 2002; Sitte and Freissmuth 2015; Hasenhuetl et al. 2018). The increased DA levels due to drug use are magnified by HIV infection as well as the exposure to viral proteins such as Tat. Tat protein directly interacts with DAT (Midde et al. 2013; Yuan et al. 2015) and inhibits DAT-mediated DA reuptake through an allosteric manner (Zhu et al. 2011). This mechanism will be discussed in the next section.

1.5.6 Protein-protein interactions

Protein-protein interactions are one of the major regulatory mechanisms that govern DAT function (Eriksen et al. 2010; Kristensen et al. 2011). For instance, it has been found that DA D2 receptors and G-protein βγ subunits participate in direct protein-protein interactions with DAT (Lee et al. 2007; Garcia-Olivares et al. 2013). Other proteins found to interact with DAT include
syntaxin1A, α-synuclein, parkin, and synaptogyrin-3, among many others (Eriksen et al. 2010). In the context of HAND, the HIV envelope glycoprotein gp120 has been found to impair DAT function in dopaminergic neurons by decreasing DA uptake $V_{\text{max}}$ (Hu et al. 2009). Through surface plasmon resonance (SPR) studies, glutathione-S-transferase (GST) pulldown assays, co-immunoprecipitation (Co-IP), and molecular dynamics modeling, it has been previously found that Tat engages in a direct protein-protein interaction with DAT (Midde et al. 2013; Yuan et al. 2015, 2016a). This interaction is through an allosteric manner, where Tat protein does not directly compete for the DA binding site (Zhu et al. 2009, 2011). Given the critical role Tat plays in DAT-mediated dysfunction of dopaminergic neurotransmission in HAND patients, disrupting the protein-protein interaction between Tat and DAT is therefore a potential approach to prevent HIV-induced neurodegeneration. Identifying key residues that are responsible for Tat-DAT interaction will provide potential targets for preventing the development of HAND in HIV-positive patients, especially those who are concurrent abusers of illicit drugs such as cocaine.

1.5.7 Computational studies, pharmacological validation, and allosteric modulation

Currently, the treatment of disorders of monoamine signaling target either one of these four ways: 1) the binding of neurotransmitters to their respective receptors, 2) the packaging of monoamine neurotransmitters into synaptic vesicles by vesicular monoamine transporters (VMATs), 3) the enzymatic degradation of neurotransmitters via the MAO and COMT enzymes, and 4) the
reuptake of neurotransmitters back into presynaptic neurons by DAT, NET, and SERT (Hasenhuetl et al. 2019). Because the Tat protein binds to DAT, the studies presented in this dissertation utilize the last approach. Therefore, characterizing the binding pocket of Tat and hDAT will aid in the development of small molecular probes that would either block Tat binding to DAT or decrease DAT’s affinity for Tat, effectively preventing HAND neuropathogenesis.

The DAT reuptake process follows the alternating access model wherein the transporter cycles through an outward-facing to an inward-facing pose (Hasenhuetl et al. 2019). Out of the 12 transmembrane (TM) domains of DAT, TM1, TM6, and TM10 have been found to play a critical role in regulating the conformational transition from outward-facing to inward-facing (Zhu et al. 2018). Knowledge of the transporter’s conformation is important because certain drugs and proteins bind favorably to DAT when it is at a specific conformational state. Structural studies of monoamine transporters require x-ray crystal structures. However, the field has struggled with solving the x-ray crystal structures of mammalian NSS structures because obtaining sufficient amounts of stable purified proteins has proven to be difficult (Navratna et al. 2018). While the x-ray crystal structure of human DAT (hDAT) has yet to be solved, other homologous transporters have been utilized to probe the structure and function of NSSs. For instance, most homology studies conducted in the past several years on biogenic amine transporters have utilized the x-ray crystal structure of bacterial orthologue leucine transporter (LeuT) from *Aquifex aeolicus* as well as the *Drosophila melanogaster* dopamine transporter (dDAT) (Piscitelli et al. 2010; Penmatsa et
al. 2013, 2015; Penmatsa and Gouaux 2014; Wang et al. 2015). These structures, along with advances in structural biology, computational modeling, and medicinal chemistry, paved the way for the development of various computational models of monoamine transporters, which includes the model of the Tat-hDAT binding complex that is reported in this dissertation (Yuan et al. 2015, 2016a, 2018). This binding model, which is based on the dDAT x-ray crystal structure, was determined using protein-protein docking techniques as well as molecular dynamics (MD) simulations (Pierce et al. 2011; Case et al. 2012; Yuan et al. 2015).

Based on this Tat-DAT computational model, it has been found that Tat favorably binds to the outward-facing state of hDAT at specific residues other than the central DA binding site (Midde et al. 2013, 2015; Bucci 2015; Yuan et al. 2015, 2016a). Tat does not directly compete with the DA binding and therefore inhibits DAT uptake by locking the transporter into an outward-facing conformation. MD simulations using this Tat-hDAT model revealed several key recognition binding residues on hDAT that are critical for binding to Tat: Tyrosine88 (Y88), Lysine92 (K92), Tyrosine470 (Y470). Amino acid substitutions on these residues resulted in the decrease (Y470A, Y470H, K92M) or preservation (Y470F, Y88F) of DA uptake $V_{\text{max}}$ in cells expressing these hDAT mutants (Midde et al. 2013, 2015). In cells expressing wild-type hDAT (WT hDAT), the addition of recombinant HIV-1 Tat$_{1-86}$ (rTat$_{1-86}$) results in a 20-30% decrease in DA uptake (Midde et al. 2013, 2015). However, Y88F, K92M, and
Y470H attenuated Tat’s inhibitory effect on DA uptake, which validates the predictions made in the Tat-hDAT computational model.

Tat-induced inhibition of DAT is mediated by binding of Tat to allosteric binding site(s) on DAT, without directly interacting with the DA uptake site. This provides a basis for a novel approach to address the problem by developing compounds to attenuate Tat binding to DAT by an allosteric mechanism while having minimal influence on physiological DA transport. A novel series of quinazoline structure-based compounds (the SRI compounds) have been developed, which function as allosteric modulators of monoamine transporters and act as partial antagonists of DA uptake without the full inhibitory profile that is typical of classic competitors of DAT (Pariser et al. 2008; Rothman et al. 2015). It has been recently found that these SRI compounds attenuate the inhibitory effects of Tat as well as cocaine on DA uptake and binding (Zhu et al. 2011; Sun et al. 2017). Additionally, the computational model of the Tat-hDAT complex reveals that SRI-30827 interferes with the interaction of Y470 and Y88 with the EL6 region of hDAT, which most likely interferes with Tat binding on hDAT via an allosteric mechanism (Sun et al. 2017). It is therefore of great clinical and scientific interest to utilize pharmacological probes that could block Tat binding to hDAT, especially in HIV-positive patients who are concurrent abusers of cocaine.

1.7 SIGNIFICANCE REVISITED

The overarching hypothesis of this dissertation is as follows: HIV-1 Tat protein allosterically perturbs transporter function by interacting with specific
recognition binding residues on DAT thereby leading to dopaminergic dysregulation as seen in HAND. This dissertation is divided into several chapters:

Chapter 2 identifies additional recognition binding sites for Tat in DAT. Through a combination of molecular dynamics predictions, site-directed mutagenesis, and pharmacological validation, the predicted key binding sites and the subsequent mutant DAT constructs were evaluated whether they (1a) alter the functional and kinetic properties of DAT, (1b) are critical to Tat-DAT binding. These mutant constructs were evaluated for the ability to preserve or improve DAT function as well as to block Tat’s inhibitory effect on DAT.

Chapter 3 showcases mutational analysis studies on DAT constructs with multiple mutations to elucidate the relationship between key recognition binding residues. By using computational modeling predictions and experimental validation via in vitro screening systems, multiple mutations in DAT were created (as opposed to single residue substitutions) in order to (2a) determine the pharmacological profiles of the mutant constructs, and (2b) identify the conformational and structural alterations that occur when these multiple mutations are combined. Mutants identified in Chapter 2 that preserve or improve DAT function as well as attenuate Tat inhibition were identified as the best candidates and were used to generate multiple mutations in the form of double and triple mutant DAT constructs.

Chapter 4 evaluates whether novel allosteric ligands have the potential to attenuate the inhibitory effects of Tat and cocaine. Novel quinazoline allosteric ligands, which are partial antagonists for monoamine transporters, were
pharmacologically characterized \textit{in vitro} using cells expressing human DAT. This chapter will (3a) identify their kinetic characteristics and potency as well as their allosteric modulatory effects on DAT. Out of the series of ligands, the most potent and ideal candidates were selected and then (3b) determined whether they can attenuate the inhibitory effects of Tat on DAT \textit{in vitro}. These were used to modify structure-activity relationship data to guide the optimization of these ligands as well as to identify one promising candidate. Finally, (3c) the candidate ligand’s ability to improve behavioral phenotypes in the inducible Tat transgenic (iTat-tg) mouse model was evaluated.

This project will provide novel insights regarding the regulation of the dopamine transporter, which could aid in the field’s current search for more DAT modulators to stabilize dopaminergic dysregulation as seen in HAND patients.
CHAPTER 2

MOLECULAR MECHANISM: THE HUMAN DOPAMINE TRANSPORTER HISTIDINE 547 REGULATES BASAL AND HIV-1 TAT PROTEIN-INHIBITED DOPAMINE TRANSPORT

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**ABSTRACT:** Abnormal dopaminergic transmission has been implicated as a risk determinant of HIV-1-associated neurocognitive disorders. HIV-1 Tat protein increases synaptic dopamine (DA) levels by directly inhibiting DA transporter (DAT) activity, ultimately leading to dopaminergic neuron damage. Through integrated computational modeling prediction and experimental validation, we identified that histidine547 on human DAT (hDAT) is critical for regulation of basal DA uptake and Tat-induced inhibition of DA transport. Compared to wild type hDAT (WT hDAT), mutation of histidine547 (H547A) displayed a 197% increase in DA uptake. Other substitutions of histidine547 showed that DA uptake was not altered in H547R but decreased by 99% in H547P and 60% in H547D, respectively. These mutants did not alter DAT surface expression or surface DAT binding sites. H547 mutants attenuated Tat-induced inhibition of DA transport observed in WT hDAT. H547A displays a differential sensitivity to PMA- or BIM-induced activation or inhibition of DAT function relative to WT hDAT, indicating a change in basal PKC activity in H547A. These findings demonstrate that histidine547 on hDAT plays a crucial role in stabilizing basal DA transport and Tat-DAT interaction. This study provides mechanistic insights into identifying targets on DAT for Tat binding and improving DAT-mediated dysfunction of DA transmission.

**2.1 INTRODUCTION**

An estimated thirty-four million people worldwide are living with HIV. More than 50% of HIV-1 positive individuals suffer from neurological complications collectively referred to as HIV-1-associated neurocognitive disorders (HAND)
HAND is a spectrum of disorders generally divided into three main groups: asymptomatic neurocognitive impairment (ANI; 33%), mild neurocognitive disorders (MND, 20-30%), and the more severe albeit rare HIV-associated dementia (HAD; 2-8%) (McArthur et al. 2010; Heaton et al. 2010). Majority of HAND patients experience deficits in memory, concentration, and decision-making. HAND patients present neuropathological conditions that emerge from the continued exposure of the central nervous system (CNS) tissues to HIV-1, viral proteins, immune inflammation, and cART (King et al. 2006; Clifford and Ances 2013). Currently, there are no promising therapeutic strategies for HAND. Considering the progressive and neurodegenerative nature of HAND, establishing an early intervention strategy would be beneficial to the preservation of neurocognitive function in HIV-infected individuals.

Converging lines of clinical observation, supported by imaging (Wang et al. 2004; Chang et al. 2008), neuropsychological performance testing (Meade et al. 2011b; Kumar et al. 2011), and postmortem examinations (Gelman et al. 2012), have implicated dopamine (DA) dysregulation with the abnormal neurocognitive function observed in HAND (Berger and Arendt 2000; Purohit et al. 2011). DA-rich brain regions (basal ganglia and related structures) are highly susceptible to the effects of both HIV infection and substance use. In the early stage of HIV infection, increased levels of DA and decreased DA turnover are found in the cerebrospinal fluid of therapy-naïve HIV patients with asymptomatic infection (Scheller et al. 2010), which may contribute to decreased levels of DA in DA-rich brain regions (Sardar et al. 1996; Kumar et al. 2009, 2011) in the
advanced stages of HIV infection. Importantly, HIV-induced elevated levels of extracellular DA in the CNS can stimulate viral replication in human macrophages within DA-rich brain regions (Gaskill et al. 2009, 2013, 2014), resulting in viral protein release. It is commonly accepted that viral replication and proteins within the CNS are correlated with the persistence of HIV-related neuropathology and subsequent neurocognitive deficits (Frankel and Young 1998; Power et al. 1998; Brach-Werner 1999; Johnston et al. 2001). Among HIV-1 viral proteins, transactivator of transcription (Tat) plays a crucial role in the neurotoxicity and cognitive impairment evident in neuroAIDS (Rappaport et al. 1999; King et al. 2006). Tat can be detected in DA-rich brain areas (Del Valle et al. 2000; Hudson et al. 2000; Lamers et al. 2010) and in the sera (Westendorp et al. 1995; Xiao et al. 2000) of HIV-1 infected patients. Long-term viral exposure can accelerate damage in the mesocorticolimbic DA system (Nath et al. 1987; Berger and Arendt 2000; Koutsilieri et al. 2002b) and to the brain pathways controlling motivation (Wise and Bozarth 1987; Everitt and Robbins 2005; Berridge 2007). DA transporter (DAT)-mediated DA reuptake is critical for normal DA homeostasis. Human DAT (hDAT) activity is strikingly reduced in HIV-1-infected cocaine-using patients, correlating with the severity of HIV-1 associated cognitive deficits (Wang et al. 2004; Chang et al. 2008). In vitro, the interplay of Tat and cocaine augments synaptic DA levels and Tat release by inhibiting DA transporter (DAT) activity (Zhu et al. 2009; Ferris et al. 2010). By producing oxidative stress-induced damage to dopaminergic neurons, prolonged exposure
to Tat protein eventually causes DAT-mediated dysregulation of DA to accelerate the progression of HAND (Purohit et al. 2011).

Through integrated computational modeling prediction and experimental validation, we have identified key residues in hDAT with which Tat interacts, which are critical for Tat-induced inhibition of DAT and transporter conformational transitions (Midde et al. 2013, 2015; Yuan et al. 2015). Our studies provide mechanistic insights into identifying residues on DAT for Tat binding, which allows the exploration of the molecular targets on DAT for therapeutic interventions, improving neurocognitive function of HAND. Our recent computational modeling study demonstrated that mutation of histidine to alanine at hDAT 547 (H547A) increases DA transport and attenuates Tat inhibitory effect on DAT function (Yuan et al. 2016b). Basal DAT activity is regulated by several protein kinases and phosphatases (Vaughan et al. 1997; Daniels and Amara 1999; Foster et al. 2002; Vuorenpää et al. 2016). Activation of protein kinase C (PKC) induces downregulation of DA reuptake (Vaughan et al. 1997; Foster et al. 2002; Melikian 2004). In the present study, we pharmacologically determined the potential contribution of PKC phosphorylation to H547A-induced enhancement of DA transport by promoting or inhibiting of PKC activation. We also evaluated the functional influence of other substitutions of Histidine547 (His547) and its associated residues, tyrosine548 and tyrosine551, in DA transport as well as Tat-induced inhibition of DA uptake.
2.2 MATERIALS AND METHODS

2.2.1 Predicting the site for hDAT binding with Tat

The binding structure of hDAT with HIV-1 clade B type Tat was modeled and simulated based on the nuclear magnetic resonance (NMR) structures of Tat (Péloponèse et al. 2000) and the constructed structure of hDAT-DA complex. The protein-protein docking program ZDOCK (Pierce et al. 2011) was used to determine the initial binding structure of the hDAT-Tat complex. A total of 220,000 potential conformations were generated based on 11 NMR structures of Tat, then all of these conformations were evaluated and ranked by ZRANK (Pierce and Weng 2007). Top-3,000 conformations selected from the protein-protein docking process were submitted to energy minimizations, and the docked structures were ranked according to the binding affinity estimated by using the Molecular Mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) method (Miller et al. 2012). Then the top-256 conformations were selected for further evaluation by performing molecular dynamics (MD) simulations. Based on the MD simulations, the most favorable hDAT-Tat binding mode (with the best geometric matching quality and reasonable interaction between hDAT and Tat) was identified, and the final hDAT-Tat binding structure was energy-minimized for analysis.

2.2.2 Construction of plasmids

All point mutations of His547, Tyrosine548 (Tyr548), and Tyrosine551 (Tyr551) in hDAT were selected based on the predictions of the three-dimensional computational modeling and simulations. Based on the favorable
hDAT-Tat binding mode (Figure 2.1), it could be expected that mutations of His547 would eliminate a hydrogen bond between D-H547 and T-R49, which impair the binding affinity of Tat with hDAT, thereby diminishing Tat-induced inhibition of DA uptake. Mutations in hDAT at His547 [Histidine to Alanine (H547A), Proline (H547P), Arginine (H547R), or Aspartic acid (H547D)], Tyr548 (tyrosine to histidine, Y548H), and Tyr551 (tyrosine to histidine, Y551H) were generated based on wild type hDAT (WT hDAT) sequence (NCBI, cDNA clone MGC: 164608 IMAGE: 40146999) by site-directed mutagenesis. Synthetic cDNA encoding hDAT subcloned into pcDNA3.1+ (provided by Dr. Haley E Melikian, University of Massachusetts) was used as a template to generate mutants using QuikChange™ site-directed mutagenesis Kit (Agilent Tech, Santa Clara CA). The sequence of the mutant construct was confirmed by DNA sequencing at University of South Carolina EnGenCore facility. Plasmid DNA were propagated and purified using a plasmid isolation kit (Qiagen, Valencia, CA, USA).

2.2.3 Cell culture and DNA transfection

PC12 cells (ATCC® CRL-1721™, American Type Culture Collection, Manassas, VA) were maintained at 37 °C in a 5% CO₂ incubator in Dulbecco’s modified eagle medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 15 % horse serum, 2.5 % bovine calf serum, 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 µg/mL streptomycin). Twenty four hours prior to transfection, cells were seeded into 24 well plates at a density of $1 \times 10^5$ cells/cm², or allowed to reach 100% confluence on plates. Cells were transfected with plasmids of WT hDAT or its mutants using Lipofectamine 2000 (Life
Technologies, Carlsbad, CA). Twenty four hours after transfection, intact cells or cell suspensions were used for experiments.

2.2.4 [$^{3}$H]DA uptake assay

To determine whether DAT mutants alter DAT function, the maximal velocity ($V_{\text{max}}$) or Michaelis-Menten constant ($K_{m}$) of [$^{3}$H]DA uptake were examined in PC12 cells transfected with WT hDAT or its mutants as previously reported (Midde et al. 2013). Intact PC12 cells in 24-well plates were rinsed twice in Krebs-Ringer-HEPES (KRH) buffer (final concentration in mM: 125 NaCl, 5 KCl, 1.5 MgSO$_4$, 1.25 CaCl$_2$, 1.5 KH$_2$PO$_4$, 10 D-glucose, 25 HEPES, 0.1 EDTA, 0.1 pargyline, and 0.1 L-ascorbic acid; pH 7.4). The cells were then preincubated for 10 min at room temperature in KRH buffer with or without nomifensine for nonspecific binding (10 µM, final concentration). Next, the cells were incubated in KRH containing one of six concentrations of unlabeled DA (final DA concentrations, 0.03–5 µM) and a fixed concentration of [$^{3}$H]DA (500,000 dpm/well, specific activity, 21.2 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) at room temperature for 8 min. Nonspecific uptake of each concentration of [$^{3}$H]DA (in the presence of 10 µM nomifensine) was subtracted from total uptake to calculate specific DAT-mediated uptake.

To determine whether H547A-induced increase in $V_{\text{max}}$ was mediated by a phosphorylation-dependent mechanism, kinetic analysis of [$^{3}$H]DA uptake was measured in the presence or absence of PKC activator PMA (Tocris, Bristol, UK) or PKC inhibitor BIM (Sigma-Aldrich, St. Louis, MO). The concentrations were chosen based on previous reports (Melikian and Buckley 1999; Navaroli et al.
In brief, intact PC12 cells transfected with WT or H547A-hDAT were preincubated with or without PMA or BIM (1 µM, final concentration) for 30 min at 37 °C or room temperature and then incubated with six concentrations of mixed [³H]DA for 8 min as described above. Nonspecific uptake of each concentration of [³H]DA (in the presence of 10 µM nomifensine) was subtracted from total uptake to calculate specific DAT-mediated uptake.

The competitive inhibition of DA uptake by DAT substrate and inhibitors was examined in intact PC12 cells transfected with WT hDAT or its mutants. Cells were preincubated in 400 µl KRH buffer containing 50 µl of one of a series of final concentrations of DA (1 nM-1 mM), GBR12909 (1 nM-10 µM), cocaine (1 nM-1 mM), or ZnCl₂ (1, 10, 100 µM) for 10 min at room temperature and then incubated for 8 min after the addition of 50 µl of [³H]DA (0.05 µM, final concentration). The reaction for DA uptake in intact cells was terminated by removing reaction reagents and washing the cells twice with ice cold 1x KRH buffer. Cells were lysed in 500 µl of 1% SDS for an hour and radioactivity was measured using a liquid scintillation counter (model Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences, Waltham, MA). V_max and K_m were determined using Prism 5.0 (GraphPad Software Inc., San Diego, CA).

To determine the inhibitory effects of Tat on [³H]DA uptake, cells were dissociated with trypsin/EDTA (0.25%/0.1%, 1 mL for one 10 cm dish) and resuspended in culture medium at room temperature. After a 10-min incubation, the dissociated cells were harvested by centrifugation at 400 × g for 5 min at 4 °C and washed once with phosphate-buffered saline followed by another
centrifugation at 400 × g for 5 min at 4 °C. The resulted cell pellets were resuspended in 1x KRH buffer. Specific [³H]DA uptake was determined in the cell suspensions prepared from WT hDAT and its mutants in the presence or absence of recombinant Tat₁⁻₈₆ (Diatheva, Fano, Italy; 140 nM, final concentration). Cell suspensions were preincubated with Tat for 20 min at room temperature and then incubated for 8 min after adding [³H]DA (0.05 µM, final concentration). Non-specific [³H]DA uptake was determined in the presence of 10 µM nomifensine. Incubation was terminated by immediate filtration through Whatman GF/B glass filters (presoaked with 1 mM pyrocatechol for 3 h). Filters were washed three times with 3 ml of ice-cold KRH buffer containing pyrocatechol using a Brandel cell harvester (model M-48; Brandel Inc., Gaithersburg, MD). Radioactivity was determined as described above.

2.2.5 [³H]WIN35,428 Binding Assay

Binding assays were conducted to determine whether mutated hDAT alters the kinetic parameters (Bₘₐₓ or Kᵅ) of [³H]WIN35,428 binding in intact PC12 cells transfected with WT hDAT or mutants. Cells were washed with sucrose-phosphate buffer (final concentration in mM: 2.1 NaH₂PO₄, 7.3 Na₂HPO₄·7H₂O, and 320 sucrose, pH 7.4) and then incubated with one of the six concentrations of [³H]WIN35,428 (84 Ci/mmol, PerkinElmer, 0.5 – 30 nM final concentrations) in a final volume of 500 µl on ice for 2 h. In parallel, nonspecific binding at each concentration of [³H]WIN35,428 (in the presence of 30 µM cocaine, final concentration) was subtracted from total binding to calculate the specific binding. For the competitive inhibition experiment, assays were performed in duplicate in
a final volume of 500 μl. Intact cells transfected with WT hDAT or its mutants were incubated in buffer containing 50 μl of [3H]WIN35,428 (final concentration, 5 nM) and one of seven concentrations of unlabeled substrate DA (1 nM – 100 μM), cocaine (1 nM – 100 μM), GBR12909 (0.01 nM – 1 μM) or ZnCl₂ (1, 10, 100 μM) on ice for 2 h. Assays were terminated by removal of reaction reagents in well and then washed three times with ice-cold assay buffer. Cells were lysed with 1% SDS for an hour. Radioactivity was determined as described above.

2.2.6 Cell surface Biotinylation

To determine whether DAT mutations alter DAT surface expression, biotinylation assays were performed as described previously (Zhu et al. 2005). PC12 cells transiently expressing hDAT or mutants were plated on 6-well plates at a density of 10⁵ cells/well. Cells were incubated with 1 ml of 1.5 mg/ml sulfo-NHS-SS biotin (Pierce, Rockford, IL) in PBS/Ca/Mg buffer (in mM: 138 NaCl, 2.7 KCl, 1.5 KH₂PO₄, 9.6 Na₂HPO₄, 1 MgCl₂, 0.1 CaCl₂, pH 7.3). After incubation, cells were washed 3 times with 1 ml of ice-cold 100 mM glycine in PBS/Ca/Mg buffer and incubated for 30 min at 4°C in 100 mM glycine in PBS/Ca/Mg buffer. Cells were then washed 3 times with 1 ml of ice-cold PBS/Ca/Mg buffer and then lysed by addition of 500 ml of Lysis buffer (Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, 250 μM phenylmethysulfonyl fluoride), followed by incubation and continuous shaking for 20 min at 4°C. Cells were transferred to 1.5 ml tubes and centrifuged at 20,000 × g for 20 min. The resulting pellets were discarded, and 100 μl of the supernatants was stored at -20 °C for determination of immunoreactive total DAT. Remaining supernatants were
incubated with continuous shaking in the presence of monomeric avidin beads in Triton X-100 buffer (100 µl/tube) for 1 h at room temperature. Samples were centrifuged subsequently at 17,000 × g for 4 min at 4°C, and supernatants (containing the non-biotinylated, intracellular protein fraction) were stored at -20°C. Resulting pellets containing the avidin-absorbed biotinylated proteins (cell-surface fraction) were resuspended in 1 ml of 1.0% Triton X-100 buffer and centrifuged at 17,000 × g for 4 min at 4°C, and pellets were resuspended and centrifuged twice. Final pellets consisted of the biotinylated proteins adsorbed to monomeric avidin beads. Biotinylated proteins were eluted by incubating with 75 µl of Laemmli sample buffer for 20 min at room temperature and stored at -20 °C if further assays were not immediately conducted.

2.2.7 Basal efflux assay

Basal DA efflux was performed at room temperature as described previously (Midde et al. 2013, 2015). Intact PC12 cells transfected with WT hDAT or its mutants were preloaded with 0.05 µM [3H]DA for 20 min and then washed 3 times with KRH buffer prior to collecting fractional efflux samples. To obtain an estimate of the total amount of [3H]DA in the cells at the zero time point, cells from a set of wells (four wells/sample) were lysed rapidly in 1 % SDS after preloading with [3H]DA. To collect factional efflux samples, buffer (500 µl) was added into a separate set of cell wells and transferred to scintillation vials after 1 min as an initial fractional efflux, and another 500 µl buffer was added to the same wells and collected after 10 min as second fractional efflux. Additional fractional efflux at 20, 30, 40, 50 min, respectively, was repeated under the same
procedure. After last fractional efflux, cells were lysed and counted as total amount of $[^3]$H]DA remaining in the cells from each well.

2.2.8 Data analysis

Results are presented as mean ± SEM, and $n$ represents the number of independent experiments for each experiment group. Kinetic parameters ($V_{\text{max}}$, $K_m$, $B_{\text{max}}$, and $K_d$) were determined from saturation curves by nonlinear regression analysis using a one-site model with variable slope. IC$_{50}$ values for substrate and inhibitors inhibiting $[^3]$H]DA uptake or $[^3]$H]WIN35,428 were determined from inhibition curves by nonlinear regression analysis using a one-site model with variable slope. For experiments involving comparisons between unpaired samples, unpaired Student's $t$ test was used to assess any difference in the kinetic parameters ($V_{\text{max}}$, $K_m$, $B_{\text{max}}$, $K_d$ or IC$_{50}$) between WT and mutant; log-transformed values of IC$_{50}$, $K_m$ or $K_d$ were used for the statistical comparisons. Significant differences between samples were analyzed with separate ANOVAs followed by post-hoc tests, as indicated in the results Section of each experiment. All statistical analyses were performed using IBM SPSS Statistics version 20, and differences were considered significant at $p < 0.05$.

2.3 RESULTS

2.3.1 Computational modeling: His547 and functional relevant residues of human DAT

Based on the constructed hDAT-Tat binding model in our previous work (Yuan et al. 2015), both the side chain and backbone of H547 forms a hydrogen bond with residue R49 of HIV-Tat (Figure 2.1B). The hydrogen bond with the
H547 side chain is expected to be broken with the H547A mutation, which is consistent with decreased inhibitory activity of Tat on hDAT-H547A. Based on the computational model proposed in our previous work (Yuan et al. 2015), the Y548-Y470-Y551 interaction motif (denoted as the YYY motif) (Figure 2.1C) could stabilize the first part of transmembrane helix 10 (TM10a) by tying down residue Y470 with extracellular loop 6. Instability of TM10a could promote the formation of a salt bridge between D476 (in TM10a) and R85 (in TM1b); however, the D476-R85 salt bridge would close the “entrance gate” of DA (Huang and Zhan 2007; Gedeon et al. 2010; Schmitt and Reith 2011; Manepalli et al. 2012; Midde et al. 2015; Yuan et al. 2015). As a result, instability of TM10a is expected to block the DA entrance in the outward-open state, which may decrease the DA uptake efficiency of hDAT. It could be observed that the three phenol rings in the YYY motif are packed in a specific face to face style, which indicates that the EL6 should also take a specific conformation for the forming of the YYY motif. Therefore, any conformation change on EL6 that occurs close to the YYY motif is expected to regulate the interaction strength of the YYY motif. As one of the nearest residue to the YYY motif on EL6, residue H547 is an appropriate candidate for validating our proposed hypothesis. The H547P, H547A, H547D, and H547R single mutations were tested because proline and alanine mutations are expected to be the most significant change in backbone conformation, while aspartic acid or arginine mutation is expected to introduce significant side-chain conformational disturbances due to their non-neutral electrostatic potentials. According to the molecular dynamics (MD) simulation
and the potential of main force (PMF) energy calculation performed in our previous work (Yuan et al. 2015), we found that the H547A mutation could enhance the strength of the YYY motif. On the contrary, the H547P mutation could weaken the strength of the YYY motif, which is consistent with the increased DA uptake efficiency for the H547A mutant and decreased DA uptake efficiency for the H547P mutant. Furthermore, the relatively weaker influence of the H547R and H547D mutations on DA uptake efficiency also support that backbone conformation of residue 547 plays a key role in the stability of the YYY motif.

2.3.2 Mutations of His547, Tyr548 and Tyr551 differentially influence DA uptake kinetics

To determine the functional influence of the His547 (H547A) mutation in DAT function, kinetic analysis of \([^3]H\)DA uptake was performed in PC 12 cells transfected with WT hDAT or H547A-hDAT. As shown in Figure 2.2A, compared to WT hDAT (12.43 ± 2.50 pmol/min/10^5 cells), H547A-hDAT displayed a 196% increase in \(V_{\text{max}}\) value [36.79 ± 7.75 pmol/min/10^5 cells, \(t(8) = 2.99, p<0.05\), unpaired Student’s \(t\) test] without changes in \(K_m\) (H547A-hDAT, 3.60 ± 1.46 and WT hDAT, 1.38 ± 0.36 nM, \(t(8) = 1.48, p = 0.08\)). To further determine whether other substitutions at His547 show differential effects on the basal DA transport, mutations at this residue (histidine to proline, arginine, and aspartate; H547P, H547R and H547D) were generated by site-directed mutagenesis. In separate experiments, the pharmacological profiles of \([^3]H\)DA uptake in PC12 cells transfected with WT hDAT or these mutants were determined. As illustrated in
Figure 2.3, compared to WT hDAT (11.93 ± 0.72 pmol/min/10^5 cells), $V_{\text{max}}$ values were not significantly changed in H547R-hDAT (11.05 ± 0.05 pmol/min/10^5 cells), but dramatically decreased in H547P-hDAT [0.16 ± 0.09 nM, $t(6) = 6.95$, $p<0.001$] and H547D-hDAT [4.80 ± 0.18 nM, $t(6) = 4.24$, $p<0.01$], respectively. $K_m$ values were not altered in H547P-, H547R-, and H547D-hDAT, compared to WT hDAT. Besides, kinetic analysis of $[^3H]$DA uptake was performed in PC12 cells transfected with WT hDAT, Y548H-hDAT, or Y551H-hDAT. As shown in Table 2.1, compared to WT hDAT (13.8 ± 2.8 pmol/min/10^5 cells), the $V_{\text{max}}$ values were decreased by 26% in Y548H-hDAT (10.2 ± 2.0 pmol/min/10^5 cells) and 76% in Y551H-hDAT [3.3 ± 1.0, $t(6) = 3.53$, $p<0.05$]. On the contrary, there was no significant change in $K_m$ in these mutants (Y548H-hDAT, 0.63 ± 0.1, Y551H-hDAT, 0.80 ± 0.3, and WT hDAT, 0.55 ± 0.1 μM).

To determine whether the H547A-induced increase in $V_{\text{max}}$ is associated with altered subcellular distribution of DAT, biotinylation and immunoblot assays were performed. Three subcellular fractions were prepared from PC12 cells transfected with WT hDAT and H547A-hDAT. DAT immunoreactivity in both total fraction and cell surface fraction (biotinylated) were examined (Figure 2.2B). No differences between WT hDAT and H547A-hDAT were found in the ratio of surface DAT (biotinylated DAT) to total DAT (biotinylated/total: WT hDAT, 0.75 ± 0.10; and H547A, 0.97 ± 0.17, $t(17) = 1.18$, $p=0.25$), indicating that increased $V_{\text{max}}$ in H547A-hDAT is not due to alteration of the available DAT on the cell surface. With regard to H547P, neither surface nor total DAT signal was detectable (data not shown). The WIN35,428 binding site shares pharmacological identity with the
DA uptake carrier (Pristupa et al. 1994). To determine whether other substitutions at His547 residue alter DA binding sites, kinetic analysis of $[^3$H]WIN35,428 binding was performed in intact PC12 cells transfected with WT hDAT, H547P, H547R, or H547D. As shown in Figure 2.3B, in comparison to WT hDAT, His547 mutants did not alter $B_{\text{max}}$ values of $[^3$H]WIN35,428 binding. However, $K_d$ values were increased in H547P [9.29 ± 2.79 pmol/10$^5$ cells, $t(6) = 2.48$, $p<0.05$], H547R [3.55 ± 0.28 pmol/10$^5$ cells, $t(6) = 2.65$, $p<0.05$] and H547D [22.33 ± 7.23 pmol/10$^5$ cells, $t(6) = 2.76$, $p<0.05$] relative to WT hDAT (2.32 ± 0.37 pmol/10$^5$ cells).

2.3.3 Mutations of His547, Tyr548 and Tyr551 differentially alter DA uptake inhibition potency of substrate and inhibitors

To determine whether mutations of His547, Tyr548, and Tyr551 influence selective binding sites on hDAT for DA, cocaine, and GBR12909, we tested the ability of substrate and DAT inhibitors to inhibit $[^3$H]DA uptake in WT hDAT and its mutants (Table 2.2). In H547A, the apparent affinity ($IC_{50}$) for DA was significantly decreased in H547A-hDAT [5356 ± 978 nM, $t(15) = 4.3$, $p<0.05$, unpaired Student’s $t$ test] relative to WT hDAT (1720 ± 206 nM). There were no changes in the potencies of cocaine and GBR12909 for inhibiting $[^3$H]DA uptake in H547A-hDAT compared to WT hDAT. We also tested whether H547A-hDAT alters the potencies of DA, cocaine, and GBR12909 for inhibiting $[^3$H]WIN35,428 binding. As shown in Table A.1, the $IC_{50}$ value of cocaine for inhibiting DA uptake was decreased in H547A-hDAT [156 ± 36 nM, $t(8) = 2.70$, $p<0.05$] compared to WT hDAT (308 ± 55 nM). However, H547A-hDAT did not alter the potencies of
DA and GBR12909 for inhibiting DA uptake. We determined whether H547P, H547R, and H547D differentially alter the potencies of DA, cocaine, and GBR12909 for inhibition of $[^3]$HDA uptake (Table 2.2). The apparent affinity (IC$_{50}$) for DA was significantly increased in H547P-hDAT [300 ± 49 nM, $t_{(15)} = 4.30$, $p<0.001$], compared to the respective WT hDAT control (DA, 1720 ± 206 nM). No changes were observed in the potencies of cocaine and GBR12909 in H547P-, H547R-, and H547D-hDAT for inhibiting $[^3]$HDA uptake compared to WT hDAT. In Y548H-hDAT and Y551H-hDAT, the affinity for DA was increased in Y551H [663 ± 70 nM, $t_{(17)} = 2.60$, $p<0.001$] relative to WT hDAT (1720 ± 206 nM) while Y548H showed no change. In addition, the affinity for cocaine was increased in Y548H [77 ± 9 nM, $t_{(17)} = 3.80$, $p<0.01$], and Y551H [59 ± 7 nM, $t_{(17)} = 4.10$, $p<0.001$], respectively, relative to WT hDAT (294 ± 34 nM).

2.3.4 Mutations of His547 attenuate Tat-induced inhibition of DA transport

Based on our computational prediction, it could be expected that mutations of His547 would eliminate a hydrogen bond between D-H547 and T-R49 (Figure 2.1), impairing Tat binding on hDAT, thereby inducing an attenuation of Tat-induced inhibition of DA uptake. We examined the specific $[^3]$HDA uptake in WT hDAT and the His547 mutants in the presence or absence of recombinant Tat$_{1-86}$. Due to the difference in the specific $[^3]$HDA uptake in WT hDAT and H547 mutants was shown in Figures 2.2 and 2.3, the inhibitory effect of Tat on DAT function in WT, H547A, H547P, H547R, H547D, and Y551H were presented as the ratio of Tat-mediated $[^3]$HDA uptake to their respective controls (in the absence of Tat, Figure 2.4). One-way ANOVA revealed a significant main effect
of genotype \( F(3, 28) = 5.72; p<0.01 \). Post hoc analysis showed that Tat (140 nM, final concentration) produced a 31% decrease in the specific \(^3\text{H}\)DA uptake in WT hDAT relative to its control [in DPM: Tat (4794 ± 989) vs control (6858 ± 1393), \( t(7) = 5.03, p<0.01 \); however, no effect of Tat on DA uptake was observed in H547A [in DPM: Tat (6190 ± 1474) vs control (5799 ± 1408)], H547R [in DPM: Tat (5026 ± 1097) vs control (4698 ± 1257)], H547D [in DPM: Tat (2159 ± 557) vs control (2021 ± 565)], and Y551H [in DPM: Tat (662 ± 107) vs control (642 ± 108)] (\( ps > 0.01 \), Bonferroni \( t \)-test), suggesting that mutations of H547 attenuate Tat-induced inhibition of DA uptake.

2.3.5 Effects of H547A and H547D on Zinc regulation of DAT conformational transitions and basal DA efflux

We have demonstrated that Tat protein regulates DA transport allosterically (Zhu et al. 2009, 2011). To determine whether His547 residue acts as a potential site for the allosteric modulation of hDAT-Tat interaction, we examined the effects of the respective mutations of His547 on Zn\(^{2+}\) modulation of \(^3\text{H}\)DA uptake and \(^3\text{H}\)WIN35,428 binding. In general, the conformational changes in DA transport processes involve conversions between outward- and inward-facing conformations (Zhao et al. 2010). Occupancy of the endogenous Zn\(^{2+}\) binding site in WT hDAT stabilizes the transporter in an outward-facing conformation, which allows DA to bind but inhibits its translocation, thereby decreasing DA uptake (Loland et al. 2003) but increasing \(^3\text{H}\)WIN35,428 binding (Norrregaard et al. 1998). Addition of Zn\(^{2+}\) is able to partially reverse an inward-facing state to an outward-facing state (Norrregaard et al. 1998; Loland et al.
On the basis of this principle, the addition of Zn$^{2+}$ to WT hDAT would inhibit DA uptake, whereas in a functional mutation in DAT Zn$^{2+}$ might diminish the preference for the inward-facing conformation and thus enhance DA uptake. As shown in Figure 2.5, two-way ANOVA on the specific $[^3]$H]DA uptake in WT and H547A-hDAT and H547D-hDAT revealed a significant main effect of zinc [F(3, 48) = 51.1; p<0.001] and a significant mutation × zinc interaction [F(6, 48) = 4.90; p<0.01]. The addition of Zn$^{2+}$ significantly decreased [H]DA uptake in WT and mutants in a zinc concentration-dependent manner (Figure 2.5A). Compared to control (in absence of Zn$^{2+}$), the addition of Zn$^{2+}$ (100 µM) decreased [H]DA uptake in WT (37%), H547A-hDAT (38%) and H547D-hDAT (60%), respectively (p< 0.01, unpaired Student’s t test), suggesting these mutants do not affect the highest concentration of Zn$^{2+}$-mediated regulation of DA transport. However, the addition of Zn$^{2+}$ (10 µM) decreased [H]DA uptake in WT (25%) and H547D-hDAT (53%), respectively (p< 0.01, unpaired Student’s t test) but not in H547A-hDAT (10%), suggesting an attenuation of Zn$^{2+}$-mediated regulation of DA transport by H547A-hDAT. In contrast, as shown in Figure 2.5B, a two way ANOVA on the specific [H]WIN35,428 binding in WT and H547A and H547D revealed significant main effects of mutation [F(2, 12) = 5.55; p<0.05], zinc [F(3, 36) = 19.19; p<0.001] and a significant mutation × zinc interaction [F(6, 36) = 7.76; p<0.001]. The addition of Zn$^{2+}$ (10 and 100 µM, final concentration) significantly increased [H]WIN35,428 binding in WT hDAT (10 µM, 80% and 100 µM, 82%) and H547A-hDAT (100 µM, 27%), respectively. The Zn$^{2+}$ (10 and 100 µM)-
induced increase in [3H]WIN35,428 binding was significantly diminished in H547A-hDAT and H547D-hDAT.

To further determine the effects of His547 mutants on transporter conformational transitions, we examined the basal efflux levels of [3H]DA in WT hDAT, H547A-hDAT, and H547D-hDAT. With regard to H547A-hDAT (Figure 2.5C), after preloading with 0.05 µM [3H]DA for 20 min at room temperature, PC12 cells transfected with WT hDAT and H547A-hDAT were washed and fractional DA efflux samples were collected at the indicated time. Two-way ANOVA on the basal efflux of [3H]DA indicated a significant main effect of time [F(5, 30) = 115.59; p<0.001]. No significant main effects of mutation and mutation × time interaction were found. With regard to H547D-hDAT (Figure 5D), a two-way ANOVA on the basal efflux of [3H]DA revealed significant main effects of mutation [F(1, 8) = 17.38; p<0.01] and time [F(5, 40) = 252.09; p<0.001] and mutation × time interaction [F(5, 40) = 11.39; p<0.001]. Post-hoc analyses showed that compared to WT hDAT, DA efflux levels were elevated at 1 and 10 min in H547D-hDAT (ps<0.05, Bonferroni t-test).

2.3.6 Effects of H547A on basal PKC-mediated regulation of DAT function

To determine whether H547A-induced enhancement of V_{max} is associated with basal levels of DAT phosphorylation, kinetic analysis of [3H]DA uptake was performed in WT and H547A-hDAT in the presence or absence of a PKC activator phorbol 12-myristate 13-acetate (PMA). As shown in Figure 2.6, a two-way ANOVA on the V_{max} values revealed significant main effects of mutation [F(1, 32) = 14.67; p<0.001] and PMA treatment [F(1, 32) = 13.31; p<0.001] and mutation
× PMA treatment interaction \(F_{(1,32)} = 4.8; p<0.05\). In the absence of PMA, the \(V_{\text{max}}\) \(^{3}\text{H}\)DA uptake was higher in H547A-hDAT than WT \(F_{(1,16)} = 10.69; p<0.01\). The addition of 1 µM PMA produced a 40% and 60% decrease in the \(V_{\text{max}}\) in WT and H547A-hDAT, respectively, compared to the respective control \(F_{(1,16)} = 4.43; p<0.05\). With regard to the \(K_{\text{m}}\) values, two-way ANOVA revealed significant main effects of mutation \(F_{(1,32)} = 20.73; p<0.001\) and PMA treatment \(F_{(1,32)} = 7.36; p<0.05\). No significant mutation × PMA treatment interaction \(F_{(1,32)} = 3.62; p = 0.066\) was found. Post hoc tests showed that the \(K_{\text{m}}\) value was lower in WT hDAT (2.18 ± 0.24) than H547A-hDAT (7.89 ± 1.30, \(t_{(15)} = 4.06; p< 0.01\) in the absence of PMA. After addition of PMA, the \(K_{\text{m}}\) value was still lower in WT hDAT (1.89 ± 0.23) than H547A-hDAT (4.08 ± 0.88, \(t_{(15)} = 4.06; p< 0.01\), however, PMA significantly decreased \(K_{\text{m}}\) values in H547A-hDAT (4.08 ± 0.88 µM, \(t_{(16)} = 2.42; p< 0.05\) but not in WT hDAT \(p>0.05\) compared to the respective control.

In a separate experiment, we determined the specific \(^{3}\text{H}\)DA uptake in the WT and H547 mutants in the presence or absence of a PKC inhibitor bisindoylmaleimide-I (BIM). As shown in Figure 2.6 (C and D), a two-way ANOVA on the \(V_{\text{max}}\) values revealed significant main effects of mutation \(F_{(1,20)} = 14.24; p<0.01\) and BIM treatment \(F_{(1,20)} = 4.55; p<0.05\). No significant interaction of mutation × BIM treatment was observed. Addition of 1 µM BIM produced a 98% increase in the \(V_{\text{max}}\) in WT \(F_{(1,10)} = 6.96; p<0.05\) but not in H547A relative to their controls. The \(K_{\text{m}}\) value was lower in WT hDAT (0.65 ± 0.07) than H547A-hDAT (1.37 ± 0.31, \(t_{(10)} = 2.24; p< 0.05\) in the absence of BIM.
This difference was not observed between WT hDAT and H547A-hDAT after BIM.

2.4 DISCUSSION

Our recent computational-experimental study demonstrated that an alanine mutation to hDAT His547 (H547A) plays a crucial role in the hDAT-Tat binding and DA uptake by hDAT (Yuan et al. 2016b). The present study aims to pharmacologically characterize His547 and its functional influence in Tat-induced inhibition of DA uptake. There were two main findings. First, H547A enhances DA transport in a PKC-dependent manner, and other substitutions of His547 (H547P, H547R, H547D) differentially altered DA uptake. Second, Tat inhibited DA uptake in WT hDAT, which was attenuated in His547 mutants. In addition, H547A attenuated zinc ion modulation of $[^3]H$DA uptake and $[^3]H$WIN35,428 binding, indicating that H547A leads to altered conformational transporter transitions. Overall, these results suggest potential therapeutic effects of targeting hDAT His547 on Tat-induced dysfunction of dopaminergic transmission observed in HAND patients.

Both His547 and the YYY motif (Tyr548-Tyr470-Tyr551) are found in the extracellular loop 6 (EL6) region of DAT. There was no significant change in the YYY motif structure unit during the conformational conversion of hDAT from the outward-open state to the outward-occlude state and then to the inward-open state, according to our computational simulation (Yuan et al. 2016b). This observation suggests the possibility of promoting hDAT activity by increasing the stability of EL6 via enhancing the strength of the YYY motif (Yuan et al. 2016b).
Y470H mutation is expected to destabilize the YYY motif by impairing both the Y470-Y548 and Y470-Y551 interactions, whereas Y548H and Y551H mutations are expected to partially reproduce the effect of Y470H, as they only impair one of the Y470-Y548 and Y470-Y551 interactions. The Y470H mutant was reported to retain only \( \sim 18\% \) \cite{midde2013,yuan2016} compared to WT hDAT, whereas the present study shows that Y548H and Y551H mutants retain 24\% and 74\% \( V_{\text{max}} \) compared to WT hDAT, respectively (Table 2.1). Interestingly, based on the YYY motif model, the combined effect of the Y548H and Y551H mutations may be expected to retain 18\% (24\%×74\%) \( V_{\text{max}} \) compared to WT hDAT, which is consistent with the effect of the Y470H single mutation on \( V_{\text{max}} \). This observation confirms our computational prediction that the YYY motif could stabilize the critical transmembrane 10 (TM10) by tying down Tyr470 with EL6 for enhanced transporter stability \cite{yuan2016}. Disrupting this motif will result in the perturbation of transport function, as evidenced by the decrease in \( V_{\text{max}} \) in the mutants Y548H, Y470H, and Y551H. According to our previous work \cite{midde2013, midde2015, yuan2015}, the center residue of YYY motif, i.e., residue Y470, is critical for Tat-induced inhibition of DA uptake, which indicates that stability of the YYY motif is also involved in hDAT/Tat binding. Thus, mutating tyrosine551 to histidine may not only change the transporter’s capacity for uptake but also inflict a structural change of hDAT/Tat binding. Our results show that the Y551H mutation attenuates Tat-induced inhibition of DA uptake (Figure 2.4), which is also consistent with our computational model.
To investigate the effect of the H547 mutation on influencing the YYY motif and hDAT transport, experiments were performed to obtain the kinetics data for four mutations, including H547A, H547P, H547R, and H547D. Alanine has the highest helix propensity, on the contrary, proline and glycine are generally known as “helix breakers” (O’Neil and DeGrado 1990; Pace and Scholtz 1998). Therefore, H547A and H547P mutations suggest two distinct directions of disturbing the backbone conformation. Besides, mutation to other residues such as arginine or aspartic acid would introduce the most significant side-chain disturbance. Consequently, the H547R and H547D mutations would be reasonable probes for investigating the role of the side chain of residue 547.

The present study shows that an alanine mutation on His547 increases the \( V_{\text{max}} \) by ~3-fold compared to WT hDAT, indicating that H547A is a critical residue that mediates the enhancement of DA transport, which supports our computational prediction (Yuan et al. 2016b). Furthermore, the H547A-induced enhancement of DA uptake was not observed in other substitutions of H547, in which \( V_{\text{max}} \) was significantly decreased in H547P but not altered in H547R, whereas the effect of the H547D mutation on \( V_{\text{max}} \) is relatively weaker than that of the H547P mutation.

The hDAT activity shows higher sensitivity to backbone disturbances induced by H547A or H547P rather than side chain disturbances induced by H547D or H547R. Furthermore, the distinct helix propensity of H547A and H547P correspond to the up-regulation and down-regulation of hDAT activity. Thus, the backbone conformation of residue His547 is expected to play an important role in hDAT function, which also supports our proposed hypothesis based on
computational prediction (Yuan et al. 2016b). Notably, the baseline $K_m$ values for WT hDAT vary in the current study, which may be affected by the experimental conditions and individual experiments performed across different times. For example, the $K_m$ values are relatively higher in either WT or H547A in Figure 2.6B than others; this is because PMA-mediated DA uptake assay was conducted at 37 °C. Previous studies have reported that DAT expression in plasma membrane is increased in a temperature-dependent manner (Loder and Melikian 2003), which may cause increased $V_{\text{max}}$ and decreased $K_m$ for DA uptake. However, the $K_m$ value of H547A hDAT was always compared to its respective WT hDAT control within each individual experiment. Importantly, our results demonstrate that H547A-hDAT displays an increased $K_m$ value for DA uptake relative to WT hDAT, indicating that mutating the His547 residue may alter the binding site of substrate DA. Given that DA transport efficiency by DAT is largely governed by surface DAT expression (Zhu and Reith 2008), our results show that mutations of these residues do not alter either cell surface DAT expression or $[\text{H}]\text{WIN35,428}$ binding sites in intact cells expressing these mutants, indicating that the altered $V_{\text{max}}$ in these mutants is not due to changing surface DAT expression. Interestingly, mutant H547P displays a dramatic decrease in DA uptake, which is accompanied by decreased total DAT expression (data not shown). Considering that site-directed mutagenesis studies on DAT typically result in a decrease in DA uptake (Chen and Reith 2000; Loland et al. 2001; Midde et al. 2013, 2015), the enhancement of DA transporter as evidenced by the H547A mutant suggest that targeting this residue may provide
an exciting knowledge basis for the development of novel concepts for therapeutic treatment of HAND.

Consistent with our previous reports (Midde et al. 2013, 2015), the current results show that mutations of His547 completely eliminate the inhibitory effect of Tat on DA transport, further suggesting that the Tat molecule is associated with DAT through intermolecular electrostatic attractions and complementary hydrophobic interactions. Our computational study predicts that the side chain of H547 forms hydrogen bond with residue R49 of HIV-Tat and that alanine mutation of H547 would be expected to change the local backbone conformation of hDAT-H547 and eliminate the hydrogen bond between hDAT-H547A/Tat-R49 (Figure 2.1). These findings indicate the important role of His547 in Tat-DAT interaction. The allosteric modulation of DAT is responsible for conformational transitions via substrate- and ligand-binding sites on DAT (Zhao et al. 2010; Shan et al. 2011). Given that Tat protein regulates DAT function allosterically (Zhu et al. 2009, 2011), we tested whether mutated His547 attenuates Tat effect through a conformational transporter transition. First, our results show that H547A and H547D attenuate zinc-mediated decrease in DA uptake and increase in WIN binding. Second, H547D but not H547A enhances basal DA efflux, which further supports our previous report showing Tat protein enhancing DA efflux in WT hDAT (Pascoli et al. 2011). These results are consistent with our previous reports (Midde et al. 2013, 2015), suggesting that disrupting the intermolecular interaction of Tat and DAT influences Tat-induced inhibition of DA uptake. Further understanding the functional relevance of additional residues in Tat for
DAT modulation will provide useful feedback for further refining the computationally predicted binding model of DAT with Tat.

An important finding from the current study is that promoting PKC phosphorylation of DAT with PMA resulted in 40% and 60% reduction of DA uptake in WT hDAT and H547A, respectively. Similarly, preventing PKC phosphorylation of DAT with BIM produces a 98% and 42% increase in DA uptake in WT hDAT and H547A, respectively. This suggests a differential sensitivity to PMA- or BIM-induced activation or inhibition of DAT function between WT and H547A. It has been well characterized that PKC-dependent phosphorylation of DAT regulates DA uptake velocity (Vaughan et al. 1997; Zhu et al. 1997; Huff et al. 2002). One possibility is that mutation of His547 alters basal levels of PKC-mediated phosphorylation of DAT, thereby resulting in the enhanced DA uptake. Recent studies demonstrate that the serine7 (S7) DAT residue is critical for PKC-dependent DAT phosphorylation (Moritz et al. 2013), and the alanine mutation of S7 results in an increase in DA uptake relative to WT DAT (Moritz et al. 2015). As the PKC phosphorylation sites on cytoplasmic domain (intracellular side) of hDAT are structurally far away from the residue H547 on the extracellular side of hDAT (Midde et al. 2013; Yuan et al. 2016b), the H547A mutation is likely to regulate the PKC-mediated phosphorylation by allosteric effect. Therefore, future studies will be necessary to investigate the double mutant S7A/H547A as an approach to identify the site of reduced PKC phosphorylation is on H547A-hDAT.
The current finding showing an unusual hDAT mutant capable of both enhancing DA transport and preventing Tat inhibitory effect on DAT is of general interest in therapeutic treatment of drug addiction. The interplay of Tat and cocaine augments synaptic DA levels and Tat release by inhibiting DAT activity (Zhu et al. 2009; Ferris et al. 2010), which may contribute to the progression of HAND underlying the cognitive deficits in HIV-1 positive cocaine-using individuals (Wang et al. 2004; Chang et al. 2008). Similarly, conditioned expression of Tat in the mouse brain further potentiates cocaine rewarding in vivo (Paris et al. 2014). Together, these results suggest a synergistic effect of cocaine and Tat on DA transmission contributing to cognitive dysfunction and elevating the cocaine addictive effects. The current findings might provide novel insight into developing small molecule compounds that could bind to the unique residue on hDAT (His547), thereby not only preventing Tat interaction with DAT but also enhancing DA transport function. Ideally, the effectiveness of early intervention for HAND may combine such compound(s) with anti-retroviral therapy, which would be beneficial to the preservation of neurocognitive function in HIV-infected individuals.
Table 2.1 Kinetic properties of [³H]DA uptake in WT hDAT, Y548H-hDAT and Y551H-hDAT

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ (pmol/min/10⁵ cells)</th>
<th>$K_{\text{m}}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT hDAT</td>
<td>13.8 ± 2.8</td>
<td>0.55 ± 0.1</td>
</tr>
<tr>
<td>Y548H</td>
<td>10.2 ± 2.0</td>
<td>0.63 ± 0.1</td>
</tr>
<tr>
<td>Y551H</td>
<td>3.3 ± 1.0*</td>
<td>0.80 ± 0.3</td>
</tr>
</tbody>
</table>

* $p<0.05$ compared with WT hDAT
Table 2.2 Summary of inhibitory activities in [$^3$H]DA uptake assay in WT and mutated hDAT in the presence of DA, cocaine and GBR12909

<table>
<thead>
<tr>
<th>IC$_{50}$ (nM)</th>
<th>WT hDAT</th>
<th>H547A</th>
<th>H547P</th>
<th>H547R</th>
<th>H547D</th>
<th>Y548H</th>
<th>Y551H</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>1720 ± 206</td>
<td>5356 ± 978</td>
<td>300 ± 48</td>
<td>2454 ± 366</td>
<td>1168 ± 154</td>
<td>1744 ± 105</td>
<td>663 ± 70</td>
</tr>
<tr>
<td>Cocaine</td>
<td>294 ± 34</td>
<td>338 ± 26</td>
<td>238 ± 470</td>
<td>198 ± 32</td>
<td>77 ± 9</td>
<td>59 ± 7</td>
<td></td>
</tr>
<tr>
<td>GBR12909</td>
<td>262 ± 42</td>
<td>270 ± 85</td>
<td>332 ± 98</td>
<td>344 ± 18</td>
<td>162 ± 44</td>
<td>279 ± 46</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. values from five to seven independent experiments performed in duplicate. * $p$<0.05 compared with WT hDAT (unpaired Student’s $t$ test)
Figure 2.1  (A) Computational model of human dopamine transporter (hDAT) and HIV-1 Tat binding. hDAT and Tat are represented as cyan surface and golden ribbon, respectively. (B) A local view of DAT residue H547 and its direct interaction with Tat residue R49, hDAT is represented as a cyan ribbon. The residues are represented in sticks, with hydrogen bonds between the two residues represented with dashed lines labeled with their corresponding coordination distances. (C) Structural details of the residues Y470, Y551, H547, D476, and R85 on hDAT. hDAT is represented as a cyan ribbon, while the first part of transmembrane helix 10 (TM10a) and extracellular loop 6 (EL6) are colored in green and orange, respectively. Dopamine is represented as a ball-and-stick molecule in purple. Hydrogen bonds between D476 and R85 are indicated by dashed lines with coordinating distances labeled.
Figure 2.2 DA transport and DAT surface expression in WT hDAT and mutant. (A) Kinetic analysis of $[^3]$H]DA uptake in WT hDAT and H547A-hDAT. PC12 cells transfected with WT hDAT or H547A-hDAT were incubated with one of 6 mixed concentrations of $[^3]$H]DA as total rate of DA uptake. In parallel, nonspecific uptake of each concentration of $[^3]$H]DA (in the presence of 10 µM nomifensine, final concentration) was subtracted from total uptake to calculate DAT-mediated uptake. The $V_{\text{max}}$ and $K_m$ values were estimated by fitting the data to the Michaelis-Menten equation and represent the means from five independent experiments ± S.E.M. * $p < 0.05$ compared to control value (unpaired Student’s t test) (n = 5). (B) Cell surface expression of WT hDAT and H547A-hDAT was analyzed by biotinylation assay. Top panel: representative immunoblots (see Appendix A) of PC12 cells expressing WT hDAT (WT) or H547A-hDAT (H547A) (n=9).
Figure 2.3 DA transport and DAT surface binding sites in WT hDAT and H547 substitutional mutants. (A) Kinetic analysis of $[^3]$H]DA uptake in WT hDAT and mutants. The $V_{\text{max}}$ and $K_m$ values were estimated by fitting the data to the Michaelis-Menten equation and represent the means from five independent experiments ± S.E.M. * $p < 0.05$ compared to WT hDAT value (unpaired Student's t test) ($n = 5$). (B) Saturation binding of $[^3]$H]WIN35,428 in intact PC12 cells transfected with WT hDAT and mutants. The $B_{\text{max}}$ and $K_d$ values were estimated by fitting one-site binding and represent the means from four independent experiments ± S.E.M. * $p < 0.05$ compared to control value (unpaired Student's t test) ($n = 4$).
Figure 2.4 Effects of Tat on kinetic analysis of $[^3$H]$\text{DA}$ uptake in WT hDAT and His547 mutants. PC12 cells transfected with WT hDAT (WT), H547A-hDAT (H547A), H547R-hDAT (H547R), H547D-hDAT (H547D), or Y551H-hDAT (Y551H) were preincubated with or without recombinant Tat$_{1-86}$ (rTat$_{1-86}$) (140 nM, final concentration) at room temperature for 20 min followed by the addition of $[^3$H]$\text{DA}$. Nonspecific uptake was determined in the presence of 10 µM final concentration of nomifensine. Data are expressed as the ratio of the specific $[^3$H]$\text{DA}$ uptake in the presence of Tat to that in the absence of Tat (in DPM: WT hDAT (Tat, 4794 ± 989 vs control, 6858 ± 1393); H547A (Tat, 6190 ± 1474 vs control, 5799 ± 1408); H547R (Tat, 5026 ± 1097 vs control, 4698 ± 1257); H547D (Tat, 2159 ± 557 vs control, 2021 ± 565); and Y551H (Tat, 662 ± 107 vs control, 642 ± 108)) n=7-8. * $p < 0.05$ compared with WT hDAT control values.
Figure 2.5 Effects of H547A and H547D mutants on transporter conformational transitions. Mutations of His547 affect zinc regulation of [(3)H]DA uptake (A) and [(3)H]WIN35,428 binding (B). PC12 cells transfected with WT hDAT (WT), H547A-hDAT (H547A) and H547D-hDAT (H547D) were incubated with KRH buffer alone (control) or ZnCl₂ (1, 10, 100 µM, final concentration) followed by [(3)H]DA uptake or [(3)H]WIN35,428 binding (n = 5-7). The histogram shows [(3)H]DA uptake and [(3)H]WIN35,428 binding expressed as mean ± S.E.M. of the respective controls set to 100% for the mutant. * p < 0.05 compared to control. # p < 0.05 compared to WT hDAT with ZnCl₂. Functional DA efflux of DA properties of H547A-hDAT (C) and H547D-hDAT (D) with their respective WT hDAT control. PC12 cells transfected with WT hDAT or mutants were preincubated with KRH buffer containing [(3)H]DA (0.05 µM, final concentration) at room temperature for 20 min. After incubation, cells were washed and incubated with fresh buffer as indicated time points. Subsequently, the buffer was removed from cells, and radioactivity in the buffer and remaining in the cells was counted. Each fractional efflux of [(3)H]DA in WT hDAT (WT) or mutants was expressed as percentage of total [(3)H]DA in the cells at the start of the experiment. Fractional [(3)H]DA efflux levels at 1, 10, 20, 30, 40 and 50 min are expressed as the percentage of total [(3)H]DA with preloading with 0.05 µM (WT hDAT: 13743 ± 3050 dpm, H547A-hDAT: 14464 ± 2547 dpm and H547D-hDAT: 1891 ± 428 dpm) presented in the cells at the start of the experiment (n = 4). ** p < 0.05 compared to WT hDAT (Bonferroni t-test).
Figure 2.6 Effects of H547A on basal PKC-mediated regulation of DAT function. Kinetic analysis of [3H]DA uptake in PC12 cells transfected with WT hDAT or H547A-hDAT in the presence or absence of PKC activator PMA (1 µM) (A and B) or inhibitor BIM (1 µM) (C and D). * p< 0.05 compared to their respective controls. # p< 0.05 compared to WT hDAT (n = 6-9).
MUTATIONS ON THE HUMAN DOPAMINE TRANSPORTER AT TYROSINE88, ASPARTIC ACID206, AND HISTIDINE547 INFLUENCE TRANSPORTER FUNCTION AND ATTENUATE TAT-INDUCED INHIBITION OF DOPAMINE UPTAKE

ABSTRACT: Dopamine transporter (DAT)-mediated dopamine reuptake is critical for normal dopamine homeostasis. HIV-1 transactivator of transcription (Tat) has a major impact on the development of HIV-1 associated neurocognitive disorders through its direct inhibition of DAT. The current study determined the effects of single (D206L, D381L), double (D206L/H547A), and triple (Y88F/D206L/H547A) mutants of human DAT (hDAT) on basal dopamine transport and Tat-induced inhibition of dopamine uptake. Compared to wild-type hDAT, the maximal velocity ($V_{max}$) of $[^3H]$dopamine was decreased in D381L and Y88F/D206L/H547A, increased in D206L/H547A, and unaltered in D206L. Recombinant Tat1-86 induced a 30% reduction of dopamine uptake in wild-type hDAT, which was attenuated in D206L, D206L/H547A, and Y88F/D206L/H547A. Introducing mutations on Tat1-86 (K19A and C22G) disrupted Tat inhibition, demonstrating perturbed Tat-DAT interaction. Differential effects of DAT mutants on transporter conformation were evidenced by attenuation of zinc-induced increased $[^3H]WIN35,428$ binding in D206L/H547A and Y88F/D206A/H547A and enhanced basal MPP+ efflux in D206L/H547A, suggesting that both D206 and H547 are critical in transport stabilization. It was then determined whether the H547A-induced increase in DA uptake is due to conformational alterations by inserting a cysteine in position 159 on a cysteine-insensitive hDAT background. In combination with the H547A mutation (E2C-I159C/H547A), the addition of MTSET increased accessibility to the inserted cysteine while decreasing dopamine uptake, indicative of an outward-open conformation. H547A showed altered palmitoylation, which may contribute to its enhanced $V_{max}$. These results
indicate that the mutants generated from Y88F, D206L, and H547A attenuate Tat inhibition while preserving DA uptake, providing insights into identifying targets for improving DAT-mediated dopaminergic dysregulation.

3.1 INTRODUCTION

HIV-1 continues to affect an estimated thirty-seven million people worldwide. Despite advances in combined antiretroviral therapy, more than 50% of HIV-1 positive individuals suffer from HIV-associated neurocognitive disorders (HAND), a variety of neuropsychological complications that range from mild impairment to severe dementia (Heaton et al. 2011; Clifford and Ances 2013). Patients with HAND exhibit cognitive deficits as well as functional impairment in activities of daily living (Clifford and Ances 2013). Because antiretroviral medications cannot efficiently cross the blood-brain barrier, chronic neuroinflammation and neurotoxicity induced by HIV-1 viral proteins that are shed from infected monocytes that enter the brain eventually lead to the development of HAND (Ensoli et al. 1993; Sulzer et al. 2005; King et al. 2006; Rayne et al. 2010). Due to the degenerative and irreversible nature of these disorders, early intervention is critical; however, there are currently no promising therapeutic strategies for the treatment of HAND.

Dopamine (DA) is involved in the control of reward, attention, motivation, and cognition centers of the brain. The dopamine transporter (DAT) regulates DA levels through a reuptake mechanism that rapidly sequesters extracellular DA back into the presynaptic neuron (Torres et al. 2003; Torres 2006). However, in HIV-infected patients, the HIV-1 viral protein Tat (transactivator of transcription)
directly inhibits DAT (Zhu et al. 2011; Yuan et al. 2015), leading to DA overflow that further stimulates viral replication and protein release in infected cells (Gaskill et al. 2009; Nolan and Gaskill 2019), eventually accelerating HAND pathogenesis. Targeting the Tat-DAT interaction during the early stages of HIV-1 infection is therefore a potential therapeutic strategy to prevent the development of HAND.

It has been recently reported that the amino acid residues tyrosine88 (Y88), lysine92 (K92), aspartic acid206 (D206), and histidine547 (H547) of human DAT (hDAT) are critical to Tat-DAT binding, as mutations on these residues attenuated Tat-induced inhibition of DA uptake while differentially affecting transporter function (Bucci 2015; Midde et al. 2015; Yuan et al. 2015, 2016b; Quizon et al. 2016). For instance, point mutations Y88F and D206L preserved basal DA uptake activity; K92M decreased DA uptake (Midde et al. 2015). Interestingly, H547A dramatically increased DA uptake by 196% (Quizon et al. 2016). Mutational analysis with hDAT constructs that bear point mutations on these key binding sites only reflect the properties of single residues. In order to account for the complex interactions between key residues involved in Tat-DAT binding, mutants Y88F/H547A and Y88F/K92M/H547A were generated. Y88F/H547A preserved the DA uptake enhancement observed in single mutant H547A, while Y88F/K92M/H547A dramatically decreased DA uptake (Sun et al. 2019). Y88F/H547A attenuated the inhibitory effects of Tat on DA uptake as well, suggestive of the key role of Y88 and H547 in DAT function as well as Tat-DAT interaction (Sun et al. 2019). To further delve into the interactions between
key recognition binding residues, other combined mutant constructs D206L/H547A and Y88F/D206L/H547A were generated and their effects on transporter function, conformation, and Tat-induced inhibition of DA uptake were characterized. A mutation on the Tat protein at position 19 (lysine 19 to alanine, K19A) was also generated to further demonstrate the key interaction between Y88 on DAT with K19 on Tat.

Lastly, in order to determine whether the dramatic increase in DA uptake in H547A is due to conformational alterations, posttranslational modifications such as palmitoylation, or a combination of both, two experiments were utilized: the substituted cysteine accessibility method using sulfhydryl-reactive and positively charged agent MTSET ([2-(trimethylammonium) ethyl] methane thiosulfonate; MTSET assay) and the 2-BP assay, which uses palmitoylation inhibitor 2-bromopalmitate (2-BP). The MTSET tested whether an engineered cysteine in position 159 in the H547A mutant renders the transporter more reactive to inactivation to MTSET. Previous reports studying changes in DAT conformation demonstrated that the accessibility of the inserted cysteine in position 159 to MTSET was highly dependent upon whether the transporter was in an outward- or inward-facing conformation (Loland et al. 2003, 2004; Pedersen et al. 2014). Accessibility of MTSET to the homologous norepinephrine transporter’s (NET) and serotonin transporter’s (SERT) corresponding residues (155 and 179, respectively) was also observed to be indicative of conformational state (Chen and Rudnick 2000), suggesting that the substituted cysteine accessibility method may be used in probing alterations to the conformational
equilibrium in other monoamine transporters. The 2-BP assay pharmacologically tested whether H547A exhibited a greater sensitivity to palmitoylation inactivation by 2-BP compared to WT. It has been previously reported that DAT kinetics is regulated by both phosphorylation and palmitoylation in a reciprocal manner: that is, an increase in phosphorylation results in a decrease in palmitoylation and vice-versa (Moritz et al. 2015). Because it has been recently reported that H547A enhanced DAT uptake in a PKC-dependent manner (Quizon et al. 2016), it was then tested whether this is true for palmitoylation as well.

This study reveals how multiple mutations on DAT and a point mutation on Tat affect Tat-DAT binding. The current study demonstrates how the H547A mutation enhances DAT uptake through a combination of conformational alterations and posttranslational modifications. These results will aid in the development of therapies that target Tat-DAT binding in order to prevent neurocognitive dysfunction in patients infected with HIV-1.

3.2 MATERIALS AND METHODS

3.2.1 Predicting the site for hDAT binding with Tat

The binding structure of hDAT with HIV-1 clade B type Tat was modeled and simulated based on the nuclear magnetic resonance (NMR) structures of Tat (Péloponèse et al., 2000) and the constructed structure of the hDAT-DA complex (Midde et al., 2015; Yuan, Huang, et al., 2016). Protein docking method and molecular dynamics simulation were employed to identify the conformation of hDAT-Tat complex. The energy-minimized complex structure used in this work was extracted from long-time equilibrated molecular dynamics simulation
trajectories in previous reports (Midde et al., 2015; Quizon et al., 2016; Yuan et al., 2015; Yuan, Huang, et al., 2016; Yuan, Quizon, et al., 2016).

3.2.2 Construction of plasmids

Mutants on hDAT were generated from the computational modeling predictions as described previously using site-directed mutagenesis based on the wild-type hDAT (WT hDAT) sequence (NCBI, cDNA clone MGC: 164608 IMAGE: 40146999) (Midde et al., 2015; Quizon et al., 2016; Yuan, Huang, et al., 2016; Yuan, Quizon, et al., 2016). The combination of hDAT mutations on Asp206 and His547 (Aspartic acid 206 to leucine and histidine 547 to alanine, D206L/H547A) were predicted to eliminate two hydrogen bonds between Tat and hDAT. On the other hand, the combination of mutations on Tyr88, Asp206, and His547 (tyrosine88 to phenylalanine and aspartic acid 206 to leucine and histidine 547 to alanine, Y88F/D206L/H547A) were predicted to eliminate three hydrogen bonds from the Tat-hDAT complex. E2C mutants on hDAT used for the MTSET assay were generated based on the same WT hDAT sequence and are designated “E2C” because two extracellular cysteines 90 and 306 were mutated to alanine, rendering the E2C hDAT background MTSET-insensitive (Pedersen et al. 2014). Synthetic cDNA encoding hDAT subcloned into pcDNA3.1+ (provided by Dr. Haley E Melikian, University of Massachusetts) was used as a template to generate mutants using site-directed mutagenesis performed by GENEWIZ (South Plainfield, NJ). DNA sequencing was also performed by GENEWIZ to confirm the sequences of the mutant constructs. Plasmid DNA were propagated
and purified using the Qiagen Hi-speed maxi prep plasmid DNA isolation kit (Qiagen, Valencia, CA, USA).

3.2.3 Cell culture, transfection, and stable cell lines

PC12 cells (ATCC® CRL-1721™, American Type Culture Collection, Manassas, VA) were maintained at 37 °C in a 5% CO2 incubator in Dulbecco’s modified eagle medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 15 % horse serum, 2.5 % fetal bovine serum, 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 µg/mL streptomycin). HEK293 cells (ATCC® CRL-1573TM, American Type Culture Collection, Manassas, VA) were used for the MTSET experiments and were maintained in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/mL streptomycin). For transient transfection, cells were seeded into 24-well plates at a density of 1×105 cells/cm², or allowed to reach 100% confluence, and were transfected 24 h later with WT hDAT or mutant hDAT plasmid DNA using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). Cells were used for experiments 24 h after transfection. In order to generate cell lines stably expressing WT or mutant hDAT, cells were transfected with Lipofectamine 2000 and cells expressing WT or mutant hDAT were selected and maintained with G418 (Sigma-Aldrich, St. Louis, MO).

3.2.4 [³H]DA uptake assay

The maximal velocity (V_{max}) and the Michaelis-Menten constant (K_m) of [³H]DA uptake were examined in intact PC12 cells transiently expressing WT or
mutant hDAT as previously described (Midde et al. 2013). In brief, the cells were washed twice in 1X Krebs-Ringer-HEPES (1X KRH) buffer, preincubated for 10 min at room temperature with buffer and/or 10 µM nomifensine (final concentration), and then incubated with the addition of one of six concentrations of unlabeled DA (final DA concentrations, 0.03-5 µM) and a fixed concentration of [³H]DA (500,000 DPM/well, specific activity, 21.2 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) at room temperature for 8 min. Specific uptake was calculated by subtracting nonspecific uptake (in the presence of 10 µM nomifensine) from total uptake.

The IC₅₀ of DA uptake in WT or mutant hDAT by DAT substrate and inhibitors was determined in intact PC12 cells seeded into 24-well plates as reported previously (Quizon et al. 2016). Briefly, cells were preincubated in 1X KRH buffer containing either DA (1 nM to 1 mM, final concentration), GBR12909 (1 nM to 10 µM, final concentration), cocaine (1 nM to 1 mM, final concentration), or ZnCl₂ (1, 10, 100 µM; final concentration) for 10 min at room temperature and then incubated for 8 min after the addition of [³H]DA (0.05 µM, final concentration). The cells were then washed twice with ice-cold 1X KRH buffer. Cells were lysed in 500 µl of 1% SDS for an hour. Radioactivity was measured the next day using a liquid scintillation counter (model Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences, Waltham, MA). The IC₅₀, Vmax and Kₘ were determined using Prism 8.0 (GraphPad Software Inc., San Diego, CA).

To determine the effects of Tat inhibition on [³H]DA uptake, cells were harvested and resuspended in culture medium and allowed to incubate at room
temperature for 10 min. The detached cells were then pelleted by centrifugation at 400 × g for 5 min at 4 °C, washed once and resuspended with phosphate-buffered saline, followed by another centrifugation at 400 × g for 5 min at 4 °C, and finally resuspended in 1X KRH buffer. Specific [³H]DA uptake was determined in the cell suspensions prepared from WT hDAT and its mutants in the presence or absence of recombinant Tat₁⁻⁸⁶ (Diatheva, Fano, Italy; 140 nM, final concentration). Cell suspensions were preincubated with Tat for 20 min at room temperature and then incubated for 8 min after adding [³H]DA (0.05 µM, final concentration). 10 µM nomifensine (final concentration) was used to determine non-specific [³H]DA uptake. The incubation was terminated by immediate filtration through Whatman GF/B glass filters (presoaked with 1XKRH buffer containing 1 mM pyrocatechol for at least 3 h). Filters were washed three times with 3 ml of ice-cold 1X KRH buffer containing pyrocatechol using a Brandel cell harvester (model M-48; Brandel Inc., Gaithersburg, MD). Radioactivity was determined as described above.

To determine whether the H547A-induced increase in Vₘₐₓ was due to a palmitoylation-mediated mechanism, [³H]DA uptake kinetic analysis was performed in the presence or absence of 15 µM 2-BP (2-bromopalmitate or 2-bromohexadecanoic acid; MilliporeSigma, St. Louis, MO), a palmitoylation inhibitor. This concentration was based on previous studies as well as pilot experiments conducted to determine the optimal concentration (data not shown). A 1500 µM 2-BP stock was prepared in 100% DMSO and was diluted to 150 µM/10% DMSO working concentration. Intact PC12 cells in 24-well plates
transiently expressing WT or H547A-hDAT were washed twice with 1X KRH buffer. To determine the effects of 2-BP exposure at the zero time point (0 h), the cells were incubated for 8 min at room temperature with 15 µM 2-BP or DMSO control (veh) and six concentrations of mixed unlabeled and labeled [3H]DA as described above. 10 µM nomifensine was added to selected wells to determine nonspecific uptake. To determine specific uptake, nonspecific uptake was subtracted from total uptake. To determine the effects of 2-BP exposure at the 2 h time point, the cells were incubated similarly as above, but allowed to incubate at room temperature for 2 h. After each incubation, cells were washed twice with ice-cold 1X KRH buffer to terminate the reaction, lysed with 500 µl 1% SDS, and allowed to shake at room temperature for an hour. Lysates were collected into scintillation vials and radioactivity was measured via liquid scintillation as above. The V_{max} and K_{m} were determined using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA).

3.2.5 [3H]WIN35,428 Binding Assay

Binding assays were conducted to determine whether mutated hDAT alters the kinetic parameters (B_{max} or K_{d}) of [3H]WIN35,428 binding in intact PC12 cells transfected with WT hDAT or mutants. Cells were washed with sucrose-phosphate buffer (final concentration in mM: 2.1 NaH2PO4, 7.3 Na2HPO47H2O, and 320 sucrose, pH 7.4) and then incubated with one of the six concentrations of [3H]WIN35,428 (84 Ci/mmol, PerkinElmer, 0.5 – 30 nM final concentrations) in a final volume of 500 µl on ice for 2 h. In parallel, nonspecific binding at each concentration of [3H]WIN35,428 (in the presence of 30 µM cocaine, final
concentration) was subtracted from total binding to calculate the specific binding. For the competitive inhibition experiment, assays were performed in duplicate in a final volume of 500 μl. Intact cells transfected with WT hDAT or its mutants were incubated in buffer containing 50 μl of [3H]WIN35,428 (final concentration, 5 nM) and one of seven concentrations of unlabeled substrate DA (1 nM – 100 μM), cocaine (1 nM – 100 μM), GBR12909 (0.01 nM – 1 μM) or ZnCl₂ (1, 10, 100 μM) on ice for 2 h. Assays were terminated by removal of reaction reagents in well and then washed three times with ice-cold assay buffer. Cells were lysed with 1% SDS for an hour. Radioactivity was determined as described above.

3.2.6 Cell surface Biotinylation

To determine whether the mutations alter DAT cell surface expression, biotinylation assays were performed as described previously (Midde et al. 2015). PC12 cells transiently expressing WT hDAT or mutants were grown to 90% confluence in 6-well plates (at a density of 1×10⁵ cells/cm²). Cells were incubated with 1 ml of 1.5 mg/ml sulfo-NHS-SS biotin (Pierce, Rockford, IL) in PBS/Ca/Mg buffer (in mM: 138 NaCl, 2.7 KCl, 1.5 KH₂PO₄, 9.6 Na₂HPO₄, 1 MgCl₂, 0.1 CaCl₂, pH 7.3). After incubation, cells were washed 3 times with 1 ml of ice-cold 100 mM glycine in PBS/Ca/Mg buffer and incubated for 30 min at 4°C in 100 mM glycine in PBS/Ca/Mg buffer. Cells were then washed 3 times with 1 ml of ice-cold PBS/Ca/Mg buffer and then lysed with 500 ml of Lysis buffer (Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μM pepstatin, 250 μM phenylmethylsulfonyl fluoride), followed by incubation and continuous shaking for 30 min at 4 °C. Cells were transferred to 1.5 ml tubes and centrifuged at 17,000 × g for 30 min at 4 °C.
The resulting pellets were discarded, and 200 µl of the supernatants was stored at -20 °C as the total DAT fraction. The remaining supernatants were incubated with continuous shaking with ImmunoPure Immobilized Streptavidin beads in lysis buffer for 1 h at room temperature. Samples were centrifuged subsequently at 17,000 × g for 4 min at 4°C, and supernatants (containing the non-biotinylated, intracellular protein fraction) were stored at -20°C. Resulting pellets containing the avidin-absorbed biotinylated proteins (cell surface fraction) were resuspended in 1 ml of 1.0% Triton X-100 buffer and centrifuged at 17,000 × g for 4 min at 4°C, and pellets were resuspended and centrifuged twice. Final pellets consisted of the biotinylated proteins adsorbed to monomeric avidin beads. Biotinylated proteins were eluted by incubating with 75 µl of Laemmlli sample buffer for 20 min at room temperature and stored at -20 °C until western blot analysis was conducted.

3.2.7 Basal and MPP+ efflux assay

Basal DA and MPP+ efflux was performed at room temperature as described previously (Midde et al. 2015). Intact PC12 cells transfected with WT hDAT or its mutants were preloaded with 0.05 μM [³H]DA for 20 min and then washed 3 times with KRH buffer prior to collecting fractional efflux samples. To obtain an estimate of the total amount of [³H]DA or [³H]MPP+ in the cells at the zero time point, cells from a set of wells (four wells/sample) were lysed rapidly in 1% SDS after preloading with [³H]DA or [³H]MPP+. To collect fractional efflux samples, buffer (500 µl) was added into a separate set of cell wells and transferred to scintillation vials after 1 min as an initial fractional efflux, and
another 500 μl buffer was added to the same wells and collected after 10 min as second fractional efflux. Additional fractional efflux at 20, 30, 40, 50 min, respectively, was repeated under the same procedure. After the last fractional efflux, cells were lysed and counted as total amount of [3H]DA or [3H]MPP+ remaining in the cells from each well.

3.2.8 MTSET assay

This assay was based on the work of Loland and colleagues (Loland et al. 2004, 2008; Pedersen et al. 2014), with a few modifications. First, a cysteine-insensitive background construct was generated based on WT hDAT, E2C, in which two external endogenous cysteines were mutated into alanines (C90A/C306A-hDAT or E2C-hDAT). This will reduce background MTSET activity and serve as the negative control. Then, a cysteine in position 159 was inserted, resulting in the second construct, C90A/C306A/I159C-hDAT or E2C/I159C-hDAT, which is the baseline upon which other mutant constructs with I159C will be compared. HEK293 cells were seeded onto 24-well plates at a density of 1×10^5 cells/cm² or allowed to reach confluence before transfection with WT, the E2C hDAT background construct, or subsequent E2C mutants. The next day, intact cells were washed twice with 1X KRH buffer, and preincubated with or without the presence of 10 μM nomifensine (for non-specific uptake) and 1 mM MTSET chloride ([2-trimethylammonium]ethyl]-methanethiosulfate) (Santa Cruz Biotechnology, Dallas, TX) for 10 min at room temperature. While previous publications reported using 0.5 mM MTSET for 10 min exhibiting a 40% inhibition on E2C-I159C (Loland et al. 2004, 2008), 1 mM MTSET was used due
to its ability to induce greater inhibition compared to the 0.5 mM concentration. Because MTSET is easily hydrolyzed in solution, the amount needed for a 10X stock was weighed out and dissolved in 1X KRH buffer just moments before adding to the assigned wells, for a 10-fold dilution (final concentration of 1 mM and final volume of 250 μl). After preincubation, [3H]DA (0.05 μM, final concentration) was added and cells were incubated for 8 min at room temperature. Cells were immediately washed twice with ice-cold 1X KRH buffer to terminate the incubation and were lysed rapidly with the addition of 1% SDS. Cell lysates were collected after incubation for 1 h at room temperature and radioactivity was determined the next day.

3.2.9 Data analysis

Data are presented as means ± SEM, and n corresponds to the number of independent experiments performed for each group. GraphPad Prism version 8.0 was used to calculate kinetic parameters ($V_{\text{max}}$, $K_m$, $B_{\text{max}}$, and $K_d$) from saturation curves using nonlinear regression via a Michaelis-Menten fit. IC$_{50}$ values for substrate and inhibitors inhibiting [3H]DA uptake or [3H]WIN35,428 binding were determined from a inhibitory curve using a one-site model with variable slope. For data involving comparisons between unpaired samples, the unpaired Student’s $t$ test was used to assess differences in kinetic parameters ($V_{\text{max}}$, $K_m$, $B_{\text{max}}$, $K_d$ or IC$_{50}$) between WT and mutant; log-transformed values of IC$_{50}$, $K_m$ or $K_d$ were used for the statistical comparisons. To determine significant differences between samples, data were analyzed with Student’s $t$ tests or separate ANOVAs followed by post-hoc tests, as indicated in the results and figure.
legends of each experiment. All statistical analyses were performed using IBM SPSS Statistics version 24, with a significance threshold of $p<0.05$.

3.3 RESULTS

3.3.1 Computational modeling: Mutations on DAT and Tat attenuate Tat-DAT interaction

Based on the constructed outward-open hDAT-Tat complex structure previously reported (Midde et al. 2015; Yuan et al. 2015, 2016a), it has been found that key molecular interactions between Tat and hDAT include a hydroxyl group of the Y88 side chain on DAT forming a hydrogen bond with K19 on Tat (Midde et al. 2015), and the side chain and backbone of H547 on DAT forming a hydrogen bond with R49 on Tat (Yuan et al. 2016b; Quizon et al. 2016). Single mutations on these residues (Y88F and H547A) resulted in the significant disruption of the binding between Tat and DAT (Midde et al. 2015; Yuan et al. 2016b; Quizon et al. 2016). Using the same computational model, it has been determined that the negatively charged side chain of D206 in DAT forms a hydrogen bond with the positively charged side chain of R57 in Tat (Figure 3.1A, 3.1B). Therefore, the mutation of these residues (D206L or D381L) in DAT is expected to impair the hydrogen bond between DAT and Tat (Figure 3.1) and attenuate the inhibitory effect of Tat on DAT activity. Furthermore, this computational model has revealed that D206 is a solvent exposed residue in the extracellular side of the hDAT structure, which resides far away from the dopamine binding site on the transporter (Figure 3.1). Therefore, D206L is unlikely to directly interfere with the binding of dopamine with DAT, making this
residue an ideal target for interfering with Tat-DAT binding without significant disruption to transporter function. On the other hand, this computational model reveals that DAT residues Y88, D206, and H547 independently interact with Tat (Figure 3.1B). This suggests that the combination of these mutants into double and triple mutant constructs may additively result in greater interference to Tat-DAT binding. As shown in Figure 3.1C, two hydrogen bonds between Tat and DAT were eliminated by the double mutation D206L/H547A, while Figure 3.1D shows that three hydrogen bonds were eliminated by the triple mutation Y88F/D206L/H547A.

3.3.2 Tat mutant K19A disrupts Tat-DAT interaction

It has been previously reported that key recognition residue Y88 on DAT directly interacts with residue K19 on Tat and that the mutation Y88F successfully disrupts the Tat-induced inhibition of DA transport in vitro (Midde et al., 2015; Yuan et al., 2015; Yuan, Huang, et al., 2016). The current study tested whether a mutation on Tat protein, K19A, would result in a similar disruption of Tat-DAT interaction by performing co-immunoprecipitation on mouse striatal synaptosomes as well as DA uptake and WIN35,428 binding experiments on PC12 cells transiently expressing WT hDAT. The mutant C22G was used as a negative control because any mutation on the cysteine-rich domain of recombinant Tat protein results in loss of functionality (Bertrand, Aksenova, Mactutus, & Booze, 2013; Midde et al., 2013). In the current study, wild-type and mutant forms of recombinant Tat1-86 bound to Tat antibody was able to immunoprecipitate hDAT in varying degrees as shown in Figure 3.2A. C22G
completely disrupted the interaction while K19A showed decreased density compared to the wild-type recombinant Tat1-86 (rTat1-86). To determine whether the K19A mutation on Tat disrupted [\(^3\)H]DA uptake and [\(^3\)H]WIN35,428 binding in PC12 cells expressing WT hDAT, [\(^3\)H]DA uptake and [\(^3\)H]WIN35,428 binding assays were conducted in the presence of 140 nM WT, K19A, or heated rTat1-86. The addition of WT rTat1-86 resulted in a 40.0% decrease in [\(^3\)H]DA uptake (59.97 ± 4.30%, \(t(6)=4.34, p<0.01\)) and a 25.6% decrease in [\(^3\)H]WIN35,428 binding (76.20 ± 2.06%, \(t(6)=4.16, p<0.01\)) compared to untreated control. The WT Tat-induced decrease in [\(^3\)H]DA uptake and [\(^3\)H]WIN35,428 binding was attenuated by both K19A mutant and heated Tat (\(p>0.05\)). This demonstrates that the K19A mutant on Tat successfully disrupts Tat-DAT interaction.

3.3.3 Mutants on DAT differentially alter DA uptake kinetics and DAT binding

To determine whether the mutations on hDAT at Asp206 and Asp381 influence DAT function, kinetic analysis of [\(^3\)H]DA uptake was performed on PC12 cells transiently transfected with WT hDAT, D206L, or D381L. As shown in Table 3.1 and Figure 3.3A, compared to WT hDAT (12.43 ± 2.50 pmol/min/10^5 cells), the maximal velocity (\(V_{max}\)) values were decreased by 63% in D381L (4.54 ± 0.95, \(t(8)=2.95, p<0.05\)), while D206L did not alter the \(V_{max}\). Both mutants did not alter \(K_m\) values. In a separate study, the additive effects of D206L/H547A and Y88F/D206L/H547A on DAT function was determined. As shown in Table 3.3 and Figure 3.3B, compared to WT hDAT (26.62 ± 3.87 pmol/min/10^5 cells), the \(V_{max}\) values were increased by 57% in D206L/H547A (41.82 ± 2.96 pmol/min/10^5 cells, \(t(6)=3.12, p<0.05\)) and decreased by 60% in Y88F/D206L/H547A (10.78 ±
0.98 pmol/min/10^5 cells, \( t_{(6)} = 3.97, p<0.01 \) respectively. Compared to WT hDAT (0.45 ± 0.21 µM), \( K_m \) value in Y88F/D206L/H547A was increased by 324% (1.92 ± 0.37 µM, \( t_{(6)} = 3.44, p<0.05 \)) but not altered in D206L/H547A.

In the hDAT, \([^3]H\)WIN35,428 binding sites share pharmacological identity with the DA uptake carrier and is part of the cocaine binding domain (Coffey and Reith 1994; Pristupa et al. 1994). It was then determined whether single point or multiple mutations of hDAT alter DA binding sites. As shown in Table 3.2 and Figure 3.4, compared to WT hDAT (3.23 ± 0.58 pmol/10^5 cells), the maximal binding sites (\( B_{\text{max}} \)) of \([^3]H\)WIN35,428 were not altered in D206L and D381L (ps>0.05); however, the \( K_d \) values of D381L significantly increased (17.52 ± 6.57 nM, \( t_{(16)} = 2.50, p<0.05 \)) in comparison to WT hDAT (5.98 ± 0.73 nM). As shown in Figure 3.4, although the \( B_{\text{max}} \) values of \([^3]H\)WIN35,428 were not altered in D206L/H547A and Y88F/D206L/H547A relative to WT hDAT, the \( K_d \) was significantly decreased in Y88F/D206L/H547A (6.90 ± 1.26 nM, \( t_{(6)} = 2.49, p<0.05 \)) in comparison to WT hDAT (12.53 ± 1.88 pmol/min/10^5 cells).

To determine whether single or multiple mutations on hDAT alter the subcellular distribution of DAT, biotinylation and subsequent immunoblot assays were performed in both total and cell surface (biotinylated) fractions from PC12 cells transiently transfected with either WT hDAT or mutants (Figure 3.3C and 3.3D). Compared to WT hDAT, D206L did not alter total and cell surface DAT expression, which corresponds to the unaltered \( V_{\text{max}} \) values as shown in Figure 3.3A. Biotinylation was also performed on D381L (Figure 3.3C). Compared to their respective controls (100%), total DAT and surface DAT were decreased by
48.4 % \( t(8)=3.04, p<0.05 \) and 40.9 % \( t(8)=3.44, p<0.05 \), respectively, which correspond to the decreased \( V_{\text{max}} \) compared to WT hDAT (Table 3.1 and Figure 3.3A). Interestingly, although the increased \( V_{\text{max}} \) was observed in D206L/H547A, this mutant did not alter total and surface DAT relative to WT hDAT. The DAT expression in Y88F/ D206L/H547A was significantly decreased by 58.4 % in total \( t(6)=2.76, p<0.05 \) and 72.5 % in surface \( t(8)=4.38, p<0.05 \) fractions, respectively, compared to WT hDAT (100%, Figure 3.3D), which corresponds to the significant decrease in \( V_{\text{max}} \) (Figure 3.3B and Table 3.3).

3.3.4 DAT mutants alter the inhibition potency of DA uptake and DAT binding by substrates and inhibitors

The ability of DAT substrates (DA) and inhibitors (cocaine and its analog GBR12909) to inhibit \(^3\)HDA uptake (Table 3.1) was performed in PC12 cells transiently transfected with WT hDAT, D206L, and D381L. Compared to WT hDAT (1810 ± 490 nM), D381L exhibited a decrease in IC\(_{50}\) for DA (340 ± 40 nM, \( t(11)=2.70, p<0.05 \), unpaired Student’s \( t \) test), while D206L did not alter the IC\(_{50}\) value. In addition, both D206L and D381L did not alter the IC\(_{50}\) values for cocaine and GBR12909 relative to WT hDAT. In separate experiments, we determined the IC\(_{50}\) values of \(^3\)HWIN35,428 binding for DA, cocaine, and GBR12909 in WT hDAT and mutants. As shown in Table 3.2, both D206L and D381L did not alter the IC\(_{50}\) values for DA; however, D381L decreased the IC\(_{50}\) values for cocaine (1480 ± 276, \( t(8)=5.70, p<0.001 \), unpaired Student’s \( t \) test) and GBR12909 (2620 ± 404, \( t(8)=3.50, p<0.01 \), unpaired Student’s \( t \) test) compared to WT hDAT (308 ± 55 and 770 ± 193, respectively). Compared to WT hDAT, D206L/H547A
preserved potencies for DA, cocaine, or GBR12909 for inhibiting \(^{3}\text{H}\)DA uptake (Table 3.3), while Y88F/D206L/H547A increased IC\(_{50}\) values for DA (1830 ± 587, \(t\)(9)=2.53, \(p<0.05\), unpaired Student’s \(t\) test) and decreased IC\(_{50}\) values for GBR12909 (180 ± 18, \(t\)(8)=5.58, \(p<0.001\), unpaired Student’s \(t\) test) compared to WT hDAT (467 ± 85 and 660 ± 84 nM, respectively). Both double and triple mutants did not alter the IC\(_{50}\) values for cocaine.

3.3.5 D206L, D206L/H547A, and Y88F/D206L/H547A attenuate Tat-induced Inhibition of DA Transport

The computational model predicted that altering either Asp206 or Asp381 to Leucine, as well as the combination of mutants Y88F, D206L, and H547A would interfere with the hydrogen bonds between Tat and DAT (Figure 3.1), thereby affecting Tat-induced inhibition of DA transport. It has also been previously confirmed that mutants Y88F and H547A attenuate the inhibitory effects of Tat on DA uptake (Midde et al. 2015; Yuan et al. 2016b; Quizon et al. 2016). In order to validate the computational predictions experimentally, the specific \(^{3}\text{H}\)DA uptake and \(^{3}\text{H}\)WIN35,428 binding was measured in the presence or absence of 140 nM recombinant Tat\(_{1-86}\) in PC12 cells transiently expressing WT hDAT and mutants. As shown in Figure 3.5A, two-way ANOVA analysis showed that a significant main effect of treatment (\(F\)(1, 18) = 7.44; \(p<0.05\)); however, no main effects of genotype and interaction were found. Post-hoc analysis showed that the addition of Tat significantly decreased DA uptake by 28.5% in WT hDAT (71.5 ± 3.3 %, \(F\)(1, 6) = 19.2; \(p<0.01\)) and by 43% in D381L (68.0 ± 8.9%, \(F\)(1, 6) = 9.80, \(p<0.05\)), while Tat did not alter DA uptake in D206L
relative to WT hDAT. In Figure 3.5B, two-way ANOVA analysis showed that a significant main effect of genotype ($F(2, 18) = 13.0; \ p<0.001$) and interaction of genotype x treatment ($F(2, 18) = 13.0; \ p<0.001$). Post-hoc analysis showed that the addition of Tat significantly decreased DA uptake by 40.3% in WT hDAT (59.97 ± 4.30%, $F(1, 6) = 18.9, \ p<0.001$), which was attenuated by D206L/H547A and Y88F/D206L/H547A. As shown in Figure 3.5C, two-way ANOVA analysis showed no significant main effect of genotype/treatment and their and interaction; however, Tat significantly decreased DA uptake by 25.6% in WT hDAT (74.4 ± 2.59%, $F(1, 6) = 17.3, \ p<0.01$).

3.3.6 Effects of Mutants on zinc-induced DAT conformational transition and basal DAT-mediated efflux

WT hDAT contains endogenous zinc (Zn$^{2+}$) binding sites that serve to stabilize the transporter in the outward-facing conformation when bound to Zn$^{2+}$. This promotes DA binding (thereby increasing [$^{3}$H]WIN35,428 binding) while inhibiting DA translocation (decreasing [$^{3}$H]DA uptake) (Norregaard et al. 1998; Loland et al. 2003). The addition of Zn$^{2+}$ partially reverses the transporter from the inward-facing state to the outward-facing state, making this technique useful for determining alterations to the transporter’s conformational equilibrium. As shown in Figure 3.6A, the addition of Zn$^{2+}$ to WT hDAT resulted in a concentration-dependent decrease in [$^{3}$H]DA uptake, with 10 and 100 µM Zn$^{2+}$ resulting in a 26.93%, and 37.58% decrease in [$^{3}$H]DA uptake, respectively. A similar concentration-dependent effect was observed in both D206L (10 µM Zn$^{2+}$: 23.53% decrease, 100 µM Zn$^{2+}$: 35.11% decrease) and D381L (10 µM Zn$^{2+}$:...
23.86% decrease, 100 µM Zn$^{2+}$: 33.79% decrease) as well. Two-way ANOVA analysis on WT, D381L, and D206L revealed a significant main effect of zinc concentration ($F_{(3,45)}=66.0$, $p<0.0001$) and zinc concentration × mutation interaction ($F_{(6,45)}=9.23$, $p<0.0001$). In Figure 3.6B, the addition of Zn$^{2+}$ to WT hDAT resulted in a dose-dependent increase in [$^3$H]WIN35,428 binding, with 10 and 100 µM Zn$^{2+}$ resulting in an 80.9%, and 82.9% increase in [$^3$H]WIN35,428 binding, respectively. A similar effect was observed in both D381L and D206L mutants. Two-way ANOVA analysis on WT, D381L, and D206L revealed a significant main effect of zinc concentration ($F_{(3,33)}=39.63$, $p<0.0001$) and zinc concentration × mutation interaction ($F_{(6,33)}=6.68$, $p<0.0001$). In Figure 3.6C, the addition of Zn$^{2+}$ to WT hDAT resulted in a dose-dependent decrease in [$^3$H]DA uptake, with 10 and 100 µM Zn$^{2+}$ resulting in a 55.8%, and 68.2% decrease in [$^3$H]DA uptake, respectively. A similar dose-dependent effect was observed in both D206L/H547A (10 µM Zn$^{2+}$: 70.35% decrease, 100 µM Zn$^{2+}$: 84.4% decrease) and Y88F/D206L/H547A (10 µM Zn$^{2+}$: 63.73% decrease, 100 µM Zn$^{2+}$: 88.77% decrease), as well. Two-way ANOVA analysis on WT, D206L/H547A, and Y88F/D206L/H547A specific [$^3$H]DA uptake revealed a significant main effect of zinc concentration ($F_{(3,27)}=92.61$, $p<0.0001$) and zinc concentration × mutation interaction ($F_{(6,27)}=21.89$, $p<0.0001$). On the other hand, Figure 3.6D shows that the addition of Zn$^{2+}$ to WT hDAT resulted in a dose-dependent increase in [$^3$H]WIN35,428 binding, with 10 and 100 µM Zn$^{2+}$ resulting in a 39.2%, and 52.0% increase in [$^3$H]WIN35,428 binding, respectively. However, D206L/H547A and Y88F/D206L/H547A attenuated the Zn$^{2+}$-induced increase in [$^3$H]WIN35,428
binding (Figure 3.6D). Two-way ANOVA analysis on $[^3]$HWIN35,428 binding in WT, D206L/H547A, and Y88F/D206L/H547A revealed a significant main effect of zinc concentration × mutation interaction only ($F_{(6,36)}=2.99, p<0.05$), suggestive of altered binding sites in both mutants.

To further examine whether the mutants altered transporter conformational transitions, basal efflux of DA and MPP$^+$ was examined and found no significant differences between WT hDAT and mutants D206L or D381L (Figure 3.7A, 3.7B, and 3.7C). As shown in Figure 3.7D, compared to WT, D206L/H547A had a higher overall MPP$^+$ efflux activity. Two-way ANOVA analysis reveals a main effect of genotype ($F_{(2,126)}=29.04, p<0.001$) and time ($F_{(5,126)}=10.17, p<0.001$). No significant interaction of genotype × time was found ($F_{(10,126)}=1.78, p = 0.072$). Post hoc analyses showed that compared to WT hDAT, the MPP$^+$ efflux levels were elevated at 1 and 10 min in D206L/H547A ($p<0.05$, Bonferroni t-test). This suggests that the double mutant D206L/H547A may have altered transporter conformational transitions.

3.3.7 H547A mutant renders the transporter into a more outward-facing conformation and alters basal palmitoylation

It is interesting to note that the $V_{\text{max}}$ was not altered in D206L but increased in H547A (Quizon et al. 2016), suggesting that the H547A mutant plays a critical role in D206L/H547A-increased DA uptake. To determine whether the increased DA uptake exhibited by H547A is due to transporter conformational alterations, a functional assay called the substituted cysteine accessibility method was utilized. This assay is specific for the measurement of the reactivity
of a mutation of hDAT at I159 (isoleucine to cysteine, I159C) to MTSET ([2-(trimethylammonium) ethyl] methane thiosulfonate), which inhibits DA uptake by impeding DA translocation (Loland et al. 2004). As shown in Figure 3.8A, a pilot study was designed to determine the optimal MTSET concentration that significantly decreases DA uptake in MTSET-sensitive E2C-I159C construct, while the MTSET-insensitive E2C-hDAT construct remains unchanged. The addition of MTSET to E2C-I159C produced a 36.96% and 55.10% decrease in DA uptake at 0.5 mM and 1.0 mM, respectively, compared to WT hDAT, which is consistent with previous reports (Loland et al. 2004, 2008). Based on the pilot study, the effects of MTSET on the combination of E2C/H547A, E2C-I159C/H547A or E2C-I159A/H547A was further determined (Figure 3.8B). Compared to their respective controls (100%), the addition of MTSET did not affect DA transport in E2C and E2C/H547A; however, DA uptake was decreased by 54.02 % in E2C-I159C ($t_{(8)} = 6.34, p<0.001$), 69.60 % in E2C-I159C/H547A ($t_{(8)} = 5.24, p<0.001$), and 41.46% in E2C-I159A/H547A ($t_{(8)} = 2.14, p<0.05$). Comparing the difference among the MTSET-treated groups, two-way ANOVA analysis reveals significant main effects of genotype ($F_{(4,40)}=139.67, p<0.001$) and treatment ($F_{(1,40)}=40.03, p<0.001$), and genotype × treatment interaction ($F_{(4,40)}=2.62, p<0.05$). Post hoc analyses with single comparison show that compared to E2C, DA uptake was significantly decreased by 37.25% in E2C-I159C ($t_{(8)} = 4.66, p<0.001$). The combination of E2C-I159C with H547A further resulted a 17.57% reduction of DA uptake in E2C-I159C/H547A ($t_{(8)} = 2.17, p<0.05$), suggesting that the outward-open state in H547A produces greater
accessibility to the engineered MTSET binding site, I159C. Interestingly, the elimination of MTSET-sensitive binding in E2C-I159A/H547A (33 ± 4.1%) reversed the E2C-I159C-induced decrease in DA uptake relative to E2C-I159C/H547A (58.54 ± 7.7, t(8) = 2.92, p<0.05). Greater DA uptake inhibition in the presence of MTSET suggests that the H547A mutant causes the transporter to favor a more outward-facing conformation, which may explain its dramatic increase (196%) in DA uptake.

It has been previously demonstrated that the H547A-induced increase in V\textsubscript{max} is mediated by alterations in basal PKC activity (Quizon et al. 2016). Recent studies reported that DAT kinetics is also regulated by a reciprocal phosphorylation and palmitoylation mechanism, where increased phosphorylation results in decreased DAT V\textsubscript{max} and palmitoylation, and vice-versa (Moritz et al. 2015; Rastedt et al. 2016; Foster and Vaughan 2017). To determine whether the H547A-induced increase in DA uptake is a result of alteration of basal palmitoylation levels, the V\textsubscript{max} of DA uptake in WT hDAT and H547A was determined in the presence or absence of palmitoylation inhibitor 2-BP (2-bromopalmitate). As shown in Figure 3.8C and 3.8D, a pilot study was designed to determine the time-dependent inhibitory effect of 2-BP on DA uptake. At the zero time point, 2-BP had no inhibitory effect on DA uptake in WT hDAT and H547A (Figure 3.8C). At two-hour point, two-way ANOVA reveals a significant main effect of treatment (F(1,5)=81.71, p<0.001) and a trend significance of genotype × treatment (F(1,5)=5.88, p=0.06). Post hoc analysis shows that compared to their respective controls, 2-BP significantly produced a
38% decrease in WT hDAT ($t_{(6)} = 3.45, p<0.05$) and 62% in H547A ($t_{(6)} = 2.97, p<0.05$). Compared to WT hDAT, 2-BP further produced a 25% decrease in DA uptake in H547A ($t_{(6)} = 2.60, p<0.05$). This suggests that H547A alters basal palmitoylation, resulting in enhanced DA uptake. Taken together, these results suggest that the “DA enhancer” effect observed in H547A may be a result of both conformational alterations as well as changes in basal phosphorylation and palmitoylation.

### 3.4 DISCUSSION

The current study evaluated the effects of single (D206L, D381L), double (D206L/H547A), and triple (Y88F/D206L/H547A) mutants on basal DA uptake, Tat-induced inhibition of DA uptake, and transporter conformational transitions. These studies also evaluated whether a mutation on Tat protein would disrupt Tat-DAT interaction. First, introducing a mutation on recombinant Tat1-86 at position 19 (K19A, which directly interacts with Y88 on hDAT) resulted in the attenuation of Tat-induced inhibition of DA uptake in WT hDAT. A second major finding reveals that D206L and D206L/H547A preserved normal DA uptake, while D381L and Y88F/D206L/H547A resulted in decreased DA uptake activity. The inhibitory effect of Tat on DA uptake and binding as observed in WT hDAT was attenuated by D206L, D206L/H547A, and Y88F/D206L/H547A. It was also confirmed that the dramatic increase in DA uptake that was observed in H547A (Quizon et al. 2016) is due to the transporter’s shift to a more outward-facing conformation, as well as a palmitoylation-dependent mechanism that increases DAT $V_{\text{max}}$. Taken together, these results provide mechanistic insights into
identifying targets on DAT for Tat binding which may aid in drug discovery efforts for correcting dysfunctional dopaminergic neurotransmission in patients afflicted with HAND.

Ideally, the mutant constructs should preserve normal DA uptake while impeding Tat-DAT binding by counteracting the inhibitory effects of Tat on DA uptake. The computational model of hDAT and the Tat-DAT complex reveals that D-D206 and D-D381 of hDAT match these ideal characteristics (the prefix T- indicates Tat and D- indicates DAT hereafter). First, D-D206 and D-D381 are solvent exposed, which indicates that they are not involved in direct interaction with other parts of hDAT. Therefore, this model predicts that mutating these two residues may not disturb the hDAT transport process. Second, residues D-D206 and D-D381 are far away from the dopamine binding site, which indicates that the mutation of these two residues is not likely to influence substrate (i.e., dopamine) binding. Third, residues D-D206 and D-D381 of hDAT form two hydrogen bonds with T-R56 and one hydrogen bond with T-R57 of Tat, respectively, which indicates that the mutation of D-D206 and D-D381 are capable of interfering with hDAT and Tat binding. On the other hand, residues D-H547, D-D206, and D-Y88 could form hydrogen bonds with T-R49, T-R57, and T-K19, respectively. According to previously published computational and experimental validation data, the single hDAT mutants H547A, D206L, or Y88F could significantly attenuate the binding between DAT and Tat. It could be observed that D-H547, D-D206, and D-Y88 independently interact with Tat (Figure 3.1A, 3.1B), which suggests the possible additive effect of the double or
triple mutation on impeding Tat-DAT binding. Two hydrogen bonds in the Tat-DAT interface were eliminated by double mutation D206L/H547A on hDAT (Fig. 3.1C), and three hydrogen bonds in the Tat-DAT interface were eliminated by triple mutation Y88F/D206L/H547A on hDAT (Figure 3.1D). Therefore, it could be expected that double mutation D206L/H547A and triple mutation Y88F/D206L/H547A would be more effective in inhibiting Tat-DAT binding than single mutation H547A, D206L, or Y88F. Results of [3H]DA uptake assays for mutant D206L, D381L, D206L/H547A, and Y88F/D206L/H547A show that: (1) except for Y88F/D206L/H547A, these mutations do not significantly alter the $K_m$ of dopamine uptake (Table 3.1 and Table 3.3); (2) both D206L and D206L/H547A preserve normal hDAT function, while D381L and Y88F/D206L/H547A decrease the $V_{max}$ of dopamine uptake (Table 3.1 and Table 3.3); (3) all tested mutants, except for D381L, attenuate the Tat-induced inhibition of DA uptake (Figure 3.5). Out of all the mutants, only D381L did not match the computational predictions via the pharmacological validation studies. This may be explained by the fact that compared to D206L which is capable of binding to both T-R56 (with two hydrogen bonds at 2.1 Å and 2.2 Å distance) and T-R57 (with one hydrogen bond at 2.0 Å) of Tat, D381L has been only observed to bind to T-R57, with a relatively larger distance (one hydrogen bond at 2.4 Å, data not shown). Despite this one discrepancy, these overall results are consistent with the computational predictions, which confirm the computational model of the Tat-DAT complex.
To further validate the Tat-DAT complex model, it was necessary to confirm whether mutating key recognition binding sites on Tat protein would also result in the disruption of the interaction between Tat and hDAT. This computational model has predicted that Y88 of DAT directly interacts with K19 of Tat, which has been confirmed in the current study through co-immunoprecipitation with mouse striatal synaptosomes as well as DA uptake and WIN35,428 binding studies with PC12 cells expressing WT hDAT. These results have shown that mutating K19 to an alanine (K19A) is able to disrupt Tat-DAT binding as evidenced by decreased optical density on the co-immunoprecipitation experiment as well as the attenuation of the inhibitory effects of wild-type recombinant Tat\textsubscript{1-86} \textit{in vitro} (Figure 3.2). Although the disruption is not as profound as mutating C22 of Tat into a glycine (C22G) which causes Tat to lose all functionality (Zhu et al. 2009; Aksenov et al. 2009; Midde et al. 2012), K19A exhibits a marked difference compared to WT recombinant Tat\textsubscript{1-86}. These findings support the site-directed mutagenesis studies and further confirm the computational predictions. Future studies should focus on delving further into Tat recognition residues and conducting site-directed mutagenesis experiments on rTat\textsubscript{1-86} to support the previous studies on DAT.

The study of multiple mutants has traditionally been utilized to uncover key mechanistic insights underlying monoamine transporter pharmacokinetics and function (Penado et al. 1998; Itokawa et al. 2000). Furthermore, because Tat protein interacts with more than one recognition residue at a time, combined mutant constructs were generated to evaluate the effects of these key binding
targets on DAT functionality as well as their ability to attenuate the inhibitory effects of Tat protein. As mentioned in the previous section, all mutants attenuated the inhibitory effects of Tat protein on DA uptake and WIN35,428 binding, except for D381L (Figure 3.5). While the single mutants D206L and D381L preserved and decreased DA uptake respectively, the double mutant D206L/H547A and triple mutant Y88F/D206L/H547A resulted in a respective increase and decrease in uptake (Table 3.3, Figure 3.3). In order to evaluate whether the kinetic differences in uptake and binding were due to alterations in cell surface expression, biotinylation studies were conducted. Both D206L and D206L/H547A preserved normal DAT surface expression while Y88F/D206L/H547A showed a significant decrease (Figure 3.3). Since DAT surface expression is an indicator of DA uptake efficiency (Zhu and Reith 2008), these results correspond with the kinetic data from Table 3.3 showing a decreased $V_{\text{max}}$ and $K_m$ for Y88F/D206L/H547A. It is interesting to note that the combination of three mutants resulted in a marked decrease in DA uptake, despite the fact that single mutants Y88F and D206L preserve uptake (Midde et al. 2015) and H547A dramatically enhances it (Quizon et al. 2016). The current trend that was observed so far regarding the combinations of key mutants residues based on the Tat-DAT complex model is that double mutants (e.g., Y88F/H547A) increase $[^3\text{H}]\text{DA} \ V_{\text{max}}$ while triple mutants (e.g., Y88F/K92M/H547A) show a dramatic decrease (Sun et al. 2019). While most double and triple mutants in monoamine transporters have been reported to result in decreased DA uptake (Itokawa et al., 2000; Kitayama et al., 1992;
Penado et al., 1998), it is interesting to observe that a mutating another key Tat residue (i.e., Y88F or D206L) alongside H547A has so far resulted in an increase in DA uptake $V_{\text{max}}$, a relatively rare occurrence in published literature. This suggests that H547 plays a key role in the DA translocation process. Elucidating the mechanisms underlying enhanced DA uptake $V_{\text{max}}$ through these mutants will pave the way for a novel therapeutic target for the treatment of DAT-mediated dysregulation of dopaminergic function.

The current study has found that combining the D206L mutation with H547A (D206L/H547A) resulted in an increase in DA uptake which is consistent with a previously generated double mutant, Y88F/H547A (Sun et al. 2019). This suggests that H547A plays a critical role in the DA uptake process. In order to investigate whether DAT undergoes possible conformation changes with the H547A-hDAT mutant, the substituted cysteine accessibility method (SCAM) was utilized, an assay that allows the functional evaluation of the transporter's conformational state (Torres et al. 2003). Previous reports indicate that the accessibility of a cysteine inserted into position 159 in DAT (Ile159Cys), the norepinephrine transporter (NET, Ile155Cys), and serotonin transporter (SERT, Ile179Cys) is highly dependent on whether the transporter is outward or inward facing (Chen and Rudnick 2000; Loland et al. 2004). If the extracellular gate is open (outward-facing), the inserted cysteine (Ile159Cys in DAT) is accessible to the extracellular environment and inaccessible when the extracellular gate is closed (Loland et al. 2004). The addition of sulfhydryl-reactive, cell-impermeable, and positively-charged compound [2-(trimethylammonium) ethyl]methylene
thiosulfonate (MTSET) to a transporter construct with the Ile159Cys mutation inactivates the transporter, resulting in a 60% inhibition of DAT activity, allowing the use of specific DA uptake as a functional measure for I159C reactivity to MTSET (Loland et al. 2004, 2008). Using these principles, inserting a cysteine in position 159 alongside the H547A mutation will allow the evaluation of the accessibility of this engineered cysteine: greater DA uptake inhibition in the combined E2C-I159C/H547A mutant indicates greater accessibility of I159C to the extracellular space, suggesting that the transporter has been rendered to a more outward-facing conformation, which was demonstrated in Figure 3.8. This suggests that DA translocation more readily occurs due to a structural change induced by the H547A mutation, allowing the transporter to exist more favorably in an outward facing state, which facilitates enhanced DAT uptake. Further MTSET assays on double mutants D206L/H547A and Y88F/H547A must be conducted in order to confirm whether a similar conformation change is responsible for the elevated DA uptake observed in these mutants.

Finally, this study investigated whether the increased DA uptake in H547A is due to alterations in basal palmitoylation activity. Palmitoylation is a posttranslational modification wherein a cysteine receives palmitate, a C16-saturated palmitic acid, through a thioester linkage (Resh 2006; Foster and Vaughan 2011). Phosphorylation and palmitoylation in DAT have been found to work in a reciprocal manner, with an increase in phosphorylation coinciding with a decrease in palmitoylation (Moritz et al. 2015). Phosphorylation (usually mediated by PKC) reduces DA uptake through increased DAT endocytic
trafficking, while palmitoylation has been found to increase DA uptake (Moritz et al. 2015; Rastedt et al. 2016; Foster and Vaughan 2017). It was previously reported that there is an increased sensitivity to PMA-induced phosphorylation in H547A compared to WT hDAT (Quizon et al. 2016). To determine whether this corresponds to altered basal palmitoylation in H547A, \( V_{\text{max}} \) values in the presence or absence of palmitoylation inhibitor, 2-BP, as measured. H547A displayed a greater sensitivity to 2-BP-induced downregulation of DA uptake compared to WT hDAT, which suggests that the H547A-induced increase in \( V_{\text{max}} \) is highly dependent on palmitoylation activity. Future studies should address whether the increased DA uptake by other H547A double mutants(Y88F/H547A and D206L/H547A) is due to alterations in basal palmitoylation.

In summary, the functional influence of single hDAT mutants D206L and D381L as well as double and triple mutants D206L/H547A and Y88F/D206L/H547A in basal DA transport and Tat-induced inhibition of DA transport were investigated. Along with functional residues identified from previous studies, this characterization of the Tat-DAT binding complex will aid in the development of novel compounds that would block Tat while preserving normal DAT activity. This study also revealed that H547A enhances DA uptake through a conformational alteration, opening up the possibilities for creating therapeutic compounds for correcting disrupted dopaminergic neurotransmission. Future studies should explore the creation of small molecule compounds that would interact with these critical recognition binding residues to aid in the
development of prophylactics for HIV-positive patients in the early stages of disease.
Table 3.1. Summary of kinetic properties and inhibitory activities in [³H]DA uptake in WT hDAT and mutants

<table>
<thead>
<tr>
<th></th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (pmol/min/10&lt;sup&gt;5&lt;/sup&gt; cells)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DA</td>
</tr>
<tr>
<td>WT hDAT</td>
<td>12.43 ± 2.50</td>
<td>1.39 ± 0.36</td>
<td>1810 ± 490</td>
</tr>
<tr>
<td>D206L</td>
<td>13.09 ± 3.55</td>
<td>1.66 ± 0.78</td>
<td>1970 ± 520</td>
</tr>
<tr>
<td>D381L</td>
<td>4.54 ± 0.95*</td>
<td>1.04 ± 0.34</td>
<td>340 ± 40*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. values from five to seven independent experiments performed in duplicate. * p < 0.05, unpaired student's t test compared to WT hDAT.
Table 3.2. Summary of kinetic properties and inhibitory activities in $[^{3}\text{H}]$WIN35,428 binding in WT hDAT and mutants

<table>
<thead>
<tr>
<th></th>
<th>$B_{\text{max}}$ (pmol/10$^5$ cells)</th>
<th>$K_d$ (µM)</th>
<th>IC$_{50}$ (nM)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DA</td>
<td>Cocaine</td>
<td>GBR12909</td>
</tr>
<tr>
<td>WT</td>
<td>3.23 ± 0.58</td>
<td>5.98 ± 0.73</td>
<td>1350 ± 382</td>
<td>308 ± 55</td>
<td>770 ± 193</td>
<td></td>
</tr>
<tr>
<td>D206L</td>
<td>2.87 ± 0.96</td>
<td>6.37 ± 1.42</td>
<td>1882 ± 409</td>
<td>566 ± 116</td>
<td>1618 ± 456</td>
<td></td>
</tr>
<tr>
<td>D381L</td>
<td>2.00 ± 0.63</td>
<td>17.52 ± 6.57*</td>
<td>670 ± 292</td>
<td>1480 ± 276***</td>
<td>2620 ± 404**</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. values from five to seven independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ unpaired student’s $t$ test compared to WT hDAT.
### Table 3.3. Summary of kinetic properties and inhibitory activities in [³H]DA uptake in WT hDAT and mutants

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ (pmol/min/10⁵ cells)</th>
<th>$K_m$ (µM)</th>
<th>IC₅₀ (nM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DA</td>
</tr>
<tr>
<td>WT hDAT</td>
<td>26.62 ± 3.86</td>
<td>0.45 ± 0.21</td>
<td>467 ± 85</td>
</tr>
<tr>
<td>D206L/H547A</td>
<td>41.82 ± 2.96*</td>
<td>0.69 ± 0.10</td>
<td>695 ± 37</td>
</tr>
<tr>
<td>Y88F/D206L/</td>
<td>10.78 ± 0.98*</td>
<td>1.92 ± 0.37*</td>
<td>1830 ± 587*</td>
</tr>
<tr>
<td>H547A</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. values from five to seven independent experiments performed in duplicate. * $p < 0.05$, unpaired student's t test compared to WT hDAT.
Figure 3.1 Key residues D-H547, D-Y88 and D-D206 involved in the HIV-1 Tat-DAT binding. (A) Typical Tat-DAT binding complex from MD trajectory. Tat and DAT are represented as gold and cyan ribbons, respectively. The dashed box indicates the binding surface between Tat and DAT. (B) Residues T-K19, T-R49 and T-R57 of HIV-1 Tat are represented as ball-stick style and colored in yellow. Residues D-H547, D-Y88 and D-D206 are represented as ball-stick style and colored in green. Dashed lines represent inter-molecular hydrogen bonds with labeled distances. (The prefix T- and D- indicates residues of Tat and DAT, respectively). (C) Double mutation D-D206L/D-H547A on TAT-hDAT structure. D-H547A mutation eliminates one hydrogen bond with T-R49, and D-D206L mutation eliminates the hydrogen bond with T-R57. (D) Double mutation D-Y88F/D-D206L/D-H547A on TAT-hDAT structure. Addition to the mutational effects of D-Y88F, D-D206L, and D-H547A also eliminate the hydrogen bond with T-K19.
Figure 3.2 Disruption of the direct interaction between Tat and DAT by mutated Tat1-86.

(A) Co-IP of wild-type recombinant Tat1-86 (rTat1-86) and its mutants with DAT was performed by immunoprecipitation (IP) with anti-DAT antibody as a bait and immunoblot (IB) with anti-Tat antibody. Mouse striatal synaptosomes were preincubated with rTat1-86 (+, lane 2 from left) and Tat mutants (C22G, K19A, lane 3 and 4 from left) or without (−, lane 1 from left). Wild-type rTat1-86 bound to agarose beads was able to immunoprecipitate DAT (lane 2), which was diminished in C22G and K19A mutants.

(B) Mutation of rTat1-86 at Cys22 or Lys19 attenuates Tat-induced inhibition of DA uptake. PC12 cells transiently transfected with WT hDAT were preincubated with or without 140 nM rTat1-86 and Tat mutants at room temperature for 20 min followed by addition of a single mixed concentration (5 nM) of [3H]DA. Specific DA uptake was determined in the presence of 10 µM nomifensine. Heated rTat1-86 was used as a negative control.

(C) Mutation of rTat1-86 at Cys22 or Lys19 attenuates Tat-induced inhibition of DAT binding site. PC12 cells transiently transfected with WT hDAT were preincubated with 5 nM [3H]WIN35,428 on ice for 2 h in the presence or absence of 140 nM rTat1-86 and Tat mutants. Specific [3H]WIN35,428 binding was determined in the presence of 30 µM cocaine. Heated rTat1-86 was used as a negative control. Data are expressed as means from four to five independent experiments ± S.E.M. ** p<0.01, compared to control (in the absence of Tat).
Figure 3. Kinetic analysis of [3H]DA uptake and DAT surface expression in WT hDAT and mutants. DA uptake was determined in intact PC12 cells expressing WT hDAT (WT), D206L, D381L (A), D206L/H547A, or Y88F/D206L/H547A (B) using six concentrations of DA ranging from 0 μM to 5 μM mixed with a fixed concentration of [3H]DA (500,000 dpm/well, specific activity: 21.2 Ci/mmol). In parallel, nonspecific uptake of each concentration of [3H]DA (in the presence of 10 μM nomifensine, final concentration) was subtracted from total uptake to calculate the specific DAT-mediated uptake. The $V_{\text{max}}$ and $K_m$ values were calculated by fitting the data to the Michaelis-Menten equation and represent the means from four to five independent experiments ± S.E.M. * $p < 0.05$ compared to the respective WT hDAT (unpaired Student’s $t$ test). (C-D) Biotinylation assays were used to analyze DAT cell surface expression in PC12 cells expressing WT hDAT or its mutants. Representative immunoblots (see Appendix B for supplementary data) and quantification are shown as means ± S.E.M (n=4-5). *$p$<0.05, **$p$<0.01 compared to WT.
Figure 3.4. Saturation binding of $[^3]$H]WIN35,428 in intact PC12 cells transfected with WT hDAT and mutants. PC12 Cells expressing WT hDAT (WT), D206L, or D381L (A) or WT, D206L/H547A, or Y88F/D206L/H547A (B) were incubated with indicated concentrations of $[^3]$H]WIN35,428 (0.5 – 30 nM) on ice for 2 h while in parallel, nonspecific wells were incubated with both 30 μM cocaine (final concentration) and $[^3]$H]WIN35,428. The $B_{\text{max}}$ and $K_d$ values were generated via a nonlinear one-site binding curve fit and represent the means from four independent experiments (mean ± S.E.M., n=4). *$p$<0.05 compared to WT (unpaired Student’s t test).
Figure 3.5. Inhibitory effects of Tat on $[^3]$H]DA uptake and $[^3]$H]WIN35,428 binding in WT hDAT and mutants. (A-B) PC12 cells expressing WT hDAT (WT) or mutant were preincubated with or without recombinant Tat$_{1-86}$ (rTat$_{1-86}$, 140 nM, final concentration) at room temperature for 20 min followed by the addition of $[^3]$H]DA. In parallel, nonspecific uptake (in the presence of 10 μM nomifensine, final concentration) was subtracted from total uptake to calculate DAT-mediated uptake. Data are presented as a percentage of untreated control per group, expressed as means ± S.E.M (n=3-4). Values in DPM for WT vs D206L and D381L: WT (control, 825.63 ± 46 vs Tat, 592.63 ± 55.04), D206L (control, 789.75 ± 163.55 vs Tat, 735.75 ± 177.90), D381L (control, 652.90 ± 90.04 vs Tat, 433.50 ± 94.74). Values in DPM for WT vs D206L/H547A: WT (control, 1524.88 ± 124.23 vs Tat, 913.00 ± 110.79), D206L/H547A (control, 997.63 ± 123.89 vs Tat, 961.88 ± 108.27). (C) PC12 cells expressing WT or mutant were incubated with or without recombinant Tat$_{1-86}$ (rTat$_{1-86}$, 140 nM, final concentration) and $[^3]$H]WIN35,428 on ice for 2 h. In parallel, nonspecific binding (in the presence of 30 μM cocaine, final concentration) was subtracted from total binding to calculate specific binding. Values in DPM: WT (control, 7118.25 ± 397.41 vs Tat, 5312.50 ± 481.03), D206L/H547A (control, 7978.00 ± 655.94 vs Tat, 8522.00 ± 1115.47). *$p< 0.05$ compared to the percentage of control (in the absence of Tat).
Figure 3.6. Effects of hDAT mutants on transporter conformational transitions. Mutants D381L and D206L do not affect zinc regulation of [³H]DA uptake (A) and [³H]WIN35,428 binding (B). Double mutant D206L/H547A and triple mutant Y88F/D206L/H547A further enhanced zinc-induced decrease in DA uptake (C) while attenuating zinc’s effect on WIN binding (D). PC12 cells transiently expressing WT hDAT (WT) or mutants were incubated with buffer alone (control) or three concentrations of ZnCl₂ (1, 10, or 100 μM, final concentration) followed by [³H]DA uptake or [³H]WIN35,428 binding (n = 5-6). The graphs illustrate specific [³H]DA uptake and [³H]WIN35,428 binding expressed as mean ± S.E.M. of the respective controls set to 100% for mutants. *p < 0.05, ***p < 0.001 compared to control.
Figure 3.7. Effects of hDAT mutants on functional efflux of basal DA and MPP⁺. Functional DA efflux of D206L (A) and D381L (B), and MPP⁺ efflux of D206L and D381L (C), and D206L/H547A-hDAT and Y88F/D206L/H547A (D) with their respective WT hDAT (WT) controls. PC12 cells transfected with WT or mutants were preincubated with KRH buffer containing [³H]DA (0.05 µM, final concentration) or [³H]MPP⁺ (0.005 µM, final concentration) at room temperature for 20 min. After incubation, cells were washed and incubated with fresh buffer at indicated time points. Subsequently, the buffer was removed from cells, and radioactivity in the buffer and residual radioactivity in the cells was counted. Each fractional efflux of [³H]DA or [³H]MPP⁺ in WT or mutants was expressed as a percentage of total [³H]DA or [³H]MPP⁺ in the cells at the start of the experiment. Fractional [³H]DA or [³H]MPP⁺ efflux levels at 1, 10, 20, 30, 40 and 50 min are expressed as a percentage of total [³H]DA with preloading with 0.05 µM or total [³H]MPP⁺ with preloading with 0.005 µM introduced to the cells at the start of the experiment (n = 4-5). *p<0.05, **p<0.01 compared to WT (Bonferroni t-test).
Figure 3.8. H547A renders an inserted cysteine more reactive to MTSET inactivation and alters basal palmitoylation compared to WT hDAT. HEK293 cells transiently expressing WT hDAT (WT) or E2C hDAT constructs were treated with a range of concentrations (0.1, 0.5, 1.0 mM) of MTSET for 10 mins followed by the addition of 5 nM $^3$H]DA for 8 mins. (A) the concentration-dependent effects of MTSET on DA uptake (*$p<0.05$, **$p<0.001$; n=3). (B) Effects of 1 mM MTSET on DA uptake in E2C, E2C-I159C, E2C/H547A, E2C-I159C/H547A, and E2C-I159A/H547A (*$p<0.05$, **$p<0.01$ compared to the respective controls). #$p<0.001$, compared to E2C; ×$p<0.05$ compared to E2C-I159C; ××$p<0.05$ compared to E2C-I159C/H547A. (C) Effects of 2-BP on DA uptake at zero time point in WT hDAT and H547A. Raw $V_{\text{max}}$ values at 0h: WT hDAT (control, 4.57 ± 0.59 vs 2-BP, 5.36 ± 0.74), H547A (control, 5.63 ± 1.24 vs 2-BP, 6.41 ± 1.45). (D) Effects of 2-BP on DA uptake at two-hour time point in WT hDAT and H547A. *$p<0.05$ compared to control; #$p<0.05$ compared to 2-BP treated WT. Raw $V_{\text{max}}$ values at 2h: WT hDAT (control, 8.80 ± 0.92 vs 2-BP, 5.55 ± 0.92), H547A (control, 16.33 ± 0.99 vs 2-BP, 5.66 ± 1.93).
CHAPTER 4

PROOF-OF-CONCEPT STUDIES USING NOVEL ALLOSTERIC MODULATORS TO ESTABLISH THEIR POTENTIAL FOR THERAPEUTIC APPLICATION IN HAND

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ABSTRACT: Cocaine abuse has been shown to increase the incidence of HIV-1 associated neurocognitive disorders. It has been demonstrated that HIV-1 Tat allosterically modulates dopamine (DA) reuptake via the human DA transporter (hDAT). This study determined whether a novel allosteric modulator, SRI-32743, pharmacologically blocks Tat binding to hDAT and alleviates Tat-potentiated cocaine rewarding effects in inducible HIV-1 Tat transgenic (iTat-tg) mice. SRI-32743 inhibited \[^3\text{H}\]DA uptake (IC\(_{50}\), 9.9 µM) with a 17-fold greater inhibition than the potency of \[^3\text{H}\]WIN35,428 binding (IC\(_{50}\), 168 µM) with 68.4% and 71.4% of its \(E_{\text{max}}\), respectively. Tat (140 nM) induced a 28% reduction in \[^3\text{H}\]DA uptake which was attenuated by SRI-32743 and SRI-31596. SRI-32743 alone and SRI-31596 alone did not alter DAT uptake. Both SRI-32743 and indatraline, a competitive DAT inhibitor, increased the cocaine IC\(_{50}\) values of \[^3\text{H}\]DA uptake by 45% and 172%, respectively. The cocaine (10 µM)-induced dissociation rate (\(k_\text{-1} = 0.147 \pm 0.022 \text{ min}^{-1}\)) of \[^3\text{H}\]WIN35,428 binding was slowed by the addition of 50 nM SRI-32743 in combination with cocaine (\(k_\text{-1} = 0.086 \pm 0.014 \text{ min}^{-1}\)) and by 50 nM SRI-32743 alone (\(k_\text{-1} = 0.069 \pm 0.010 \text{ min}^{-1}\)). Following a 14 day-doxycycline treatment to induce Tat protein expression, the iTat-tg mice exhibited a 2-fold potentiation of cocaine-CPP which was dose-dependently ameliorated by pretreatment of SRI-32743 (1 or 10 mg/kg/day, i.p.) prior to CPP. These results demonstrate that developing allosteric modulatory molecules which attenuate cocaine and Tat binding to DAT will aid drug discovery efforts in the search for therapeutic interventions for HIV-infected patients who are concurrent abusers of cocaine.
4.1 INTRODUCTION

The introduction of antiretroviral therapy (ART) in the mid-nineties has transformed HIV-1 infection from a terminal diagnosis into a manageable chronic disease. Currently, there is an estimated thirty-seven million people worldwide living with HIV (UNAIDS 2016). This is a direct consequence of improved access to ART leading to increased life expectancy and dramatic reduction in HIV- or AIDS-related deaths (Simioni et al. 2010; Bonnet et al. 2013). Because most ART cannot efficiently cross the blood-brain barrier (BBB), the brain serves as a viral reservoir that continues to infect susceptible cells in the brain. As a result, up to 70% of HIV-1 positive individuals suffer from neurological complications that are collectively known as HIV-associated neurocognitive disorders (HAND). There are no known pharmacological therapeutics for the treatment of HAND. Since neurodegeneration is irreversible, an early intervention strategy is key.

Dysregulation of dopaminergic neurotransmission has been implicated in the persistence of HIV infection as well as the development of HAND (Nath et al. 2000; Purohit et al. 2011; Gaskill et al. 2017; Zhu et al. 2018). Clinical evidence and animal studies have found dopaminergic neuron damage and disruptions in DA metabolism in HIV-positive patients (Koutsilieri et al. 2002b; Silvers et al. 2006; Scheller et al. 2010; Horn et al. 2013; Meulendyke et al. 2014). The dopamine transporter (DAT), the main regulator of DA homeostasis in the brain via dopamine (DA) reuptake, is implicated in HIV infection as well. For instance, clinical imaging studies reveal decreased levels of DAT in the brains of HIV-positive patients with dementia (Wang et al. 2004). Drugs of abuse such as
cocaine, which inhibit DAT uptake and cause extracellular DA overflow, have been found to exacerbate viral replication and accelerate HAND neuropathogenesis (Peterson et al. 1990; Bagasra and Pomerantz 1993; Roth et al. 2002; Steele et al. 2003; Dahal et al. 2015). In HIV-positive patients with a history of cocaine abuse, DAT availability in the brain is further decreased compared to patients with HIV-associated dementia, which correlated with diminished performance in neurocognitive tests (Chang et al. 2008). Consequently, drug-induced increases in DA levels were found to promote HIV viral replication and infection of macrophages and lymphocytes (Scheller et al. 2000; Gaskill et al. 2009, 2013). Furthermore, as infected cells continue to accumulate in the brain's viral reservoirs, neurotoxic viral proteins are released. These viral proteins have been associated with the widespread neuropathology in the brain as well as the development of HAND (Nath and Geiger 1998).

Among the viral proteins, Tat (trans-activator of transcription), has been found to play a critical role in HIV-1 infection, viral replication, and the development of neurocognitive deficits that lead to HAND (Mocchetti et al. 2012; Gaskill et al. 2017; Zhu et al. 2018).

Tat directly inhibits DAT through an allosteric mechanism, where it does not directly compete with the transporter’s central substrate binding site (Zhu et al. 2011; Midde et al. 2013; Yuan et al. 2015; Sun et al. 2017). DAT inhibition leads to increased synaptic DA levels and continued Tat release, thereby exacerbating viral infection and disruptions in dopaminergic signaling (Nath and Geiger 1998; Purohit et al. 2011). Allosteric modulation is a novel approach to
disrupt Tat-DAT binding by developing compounds to attenuate Tat binding to DAT with minimal influence on basal DA transport. A novel series of quinazoline structure-based compounds (the SRI compounds) have been developed, which function as allosteric modulators of monoamine transporters and act as partial antagonists of DA uptake without the full inhibitory profile that is typical of classic competitors of DAT (Pariser et al. 2008; Rothman et al. 2015). It has been recently found that these SRI compounds attenuate the inhibitory effects of Tat as well as cocaine on DA uptake and binding (Zhu et al. 2011; Sun et al. 2017). The computational model of the Tat-hDAT complex reveals that SRI-30827 interferes with the interaction of Y470 and Y88 with the EL6 region of hDAT, which most likely interferes with Tat binding on hDAT via an allosteric mechanism (Sun et al. 2017). It is therefore of great clinical and scientific interest to utilize pharmacological probes that could block Tat binding to hDAT, especially in HIV-positive patients who are concurrent abusers of cocaine.

In this study, novel allosteric DAT modulators were screened using a heterologous cell line expressing wild-type human DAT (WT hDAT). After selecting the most potent compounds, allosteric properties and ability to attenuate the inhibitory effects of recombinant Tat_{1-86} (rTat_{1-86}) protein in vitro was characterized. Finally, it was determined whether the SRI compound would alleviate Tat-potentiated cocaine rewarding effects in an inducible Tat transgenic mouse model (iTat-tg). These proof-of-concept studies utilizing SRI compounds as pharmacological probes will aid in characterizing the allosteric sites of DAT,
paving the way for new therapeutic avenues for treating both cocaine addiction and cocaine-potentiated HAND in HIV-positive individuals.

4.2 METHODS

4.2.1 Materials

Rat pheochromocytoma (PC12) cells and CHO cells were obtained from ATCC (Manassas, VA). 3,4-[7-3H]DA (28 Ci/mmol) and [N-methyl-3H]WIN 35,428 (82.62 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). The novel DAT allosteric modulators (SRI compounds), were synthesized at Southern Research Institute (Birmingham, AL). Cocaine hydrochloride and other fine chemicals/reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

4.2.2 Molecular modeling

Molecular Docking techniques were used to identify the binding poses of the hDAT homology model and SRI-32743 using Autodock Vina (Trott and Olson 2010). The homology model for hDAT was constructed using methods described previously (Yuan et al. 2015). The 3D geometrical structure of each SRI compound was built with GaussView 6 (Dennington et al. 2016). This 3D structure, of each SRI compound was then used to generate a conformational ensemble of 20 different compounds with varying rotatable bonds, which were generated using OMEGA from the OpenEye Scientific Software package (Hawkins et al. 2010). The atomic charges for the SRI compound were determined as restrained electrostatic potential (RESP) fitted charges based on the first-principles electronic structure calculations at the B3LYP/6-31 G* level.
with geometry optimization by using the Gaussian09 program (Bayly et al. 1993; Frisch et al. 2009). The ligand-binding site of the hDAT homology model was discovered by selecting a search space that included the extracellular end of the transmembrane domain of hDAT in close proximity to the Tat protein. The best protein-ligand binding conformation for the SRI compound was selected according to the best binding score given by AutoDock Vina. For each SRI compound, the selected binding conformation was combined with the constructed hDAT-Tat structure with tLeap module of the AMBER16 Software package (Case et al. 2005). Then the SRI-hDAT-Tat complex were optimized with 4,000 steps of the deepest decent energy-minimization and 4,000 steps of the conjugate gradient energy-minimization by using the Sander module of the AMBER 16 package. This series of optimized minimization steps were carried out by applying a harmonic constraint only on the protein and gradually reducing the force constant from 300, 200, 100, 75, 50, and 25 kcal/mol/Å (Yuan et al. 2015). A final minimization step was carried out without applying any harmonic constraint. Docking models and figures were generated using Pymol (Schrödinger 2015).

4.2.3 Construction of plasmids

Synthetic cDNA encoding the WT hDAT sequence (NCBI, cDNA clone MGC: 164608 IMAGE: 40146999) subcloned into pcDNA3.1+ (provided by Dr. Haley E Melikian, University of Massachusetts) was used to propagate plasmid DNA. Plasmid DNA were propagated and purified using the Qiagen Hi-speed maxi prep plasmid DNA isolation kit (Qiagen, Valencia, CA, USA).
4.2.4 Cell culture

PC12 cells (CRL-1721, ATCC, Manassas, VA) were maintained at 37°C (5% CO₂) with RPMI-1640 medium (ATCC, Manassas, VA) supplemented with horse serum (10%), fetal bovine serum (5%), and penicillin-streptomycin (100 U/ml). Chinese hamster ovary cells (CHO cells, ATCC, CRL-61) were maintained in F12 medium with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). For transfection, cells were seeded into poly-D-lysine-coated 24 well plates at a density of 1×10⁵ cells/well. After 24h, cells were transfected with WT or mutant DAT plasmids using Lipofectamine 2000 (Life Tech, Carlsbad, CA) based on the manufacturer’s instruction. Cells were used for the experiments after 24 h of transfection.

4.2.5 [³H]DA uptake assay

To determine IC₅₀ values of the SRI compounds or cocaine via [³H] DA uptake, assays in PC12 cells transfected with WT hDAT was carried out according to our previous studies (Quizon et al. 2016; Sun et al. 2017). Briefly, assays were performed in duplicate in a final volume of 250 µl. Cells were washed twice with Krebs-Ringer-HEPES (KRH) buffer and incubated with a range of SRI concentrations (0.1 nM to 100 μM), cocaine concentrations (1nM-1mM) or a fixed concentration of SRI-32743 (50 nM) or indatraline (10 nM) at room temperature for 10 min. Next, cells were incubated for 8 min with a fixed concentration of mixed [³H]DA (0.05 μM, 250,000 DPM per well). In parallel, the non-specific [³H]DA uptake was determined in the presence of nomifensine (10 µM). Cells were lysed in 1% SDS for an hour and lysates were transferred into
scintillation vials containing cocktail. After overnight incubation, samples were read on a liquid scintillation counter (model Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences, Waltham, MA). The IC$_{50}$ values were determined using Prism 8.0 (GraphPad Software Inc., San Diego, CA).

To determine the effects of SRI compounds on Tat-induced inhibition of DA uptake, [$^3$H]DA uptake assays were performed on PC12 cells transiently expressing WT hDAT, according to our previously reported methods (Quizon et al. 2016). In brief, cells were harvested and resuspended in 1X KRH buffer and incubated for 20 min at room temperature with or without 140 nM of recombinant Tat$_{1-86}$ (ImmunoDX, Woburn, MA). The samples were then incubated with 0.05 µM [$^3$H]DA for 8 min then filtered through Whatman GF/B glass filters (presoaked with 1XKRH buffer containing 1 mM pyrocatechol for at least 3 h). Filters were washed three times with 3 ml of ice-cold 1X KRH buffer containing pyrocatechol using a Brandel cell harvester (model M-48; Brandel Inc., Gaithersburg, MD). Radioactivity was determined as described above.

4.2.6 [$^3$H]WIN35,428 binding assay

To determine the IC$_{50}$ values of SRI compounds via [$^3$H]WIN35,428 binding, competitive inhibition assays were carried out in duplicate in a final volume of 250 µl using previously published methods (Sun et al. 2017). In brief, PC12 cells transiently expressing WT hDAT were washed once with sucrose-phosphate buffer and were then incubated with a range of concentrations of SRI compounds (0.1 nM to 100 µM) in the presence of [$^3$H]WIN 35,428 (5 nM) on ice for 2 h. Non-specific binding was determined in the presence of 30 µM cocaine.
After incubation, the intact cells in each well were washed twice with ice-cold assay buffer, lysed by 1% SDS for 1 h and subjected to liquid scintillation counting.

For the binding dissociation assay, experiments were conducted in a final volume of 500 µl according to our previous publications (Zhu et al. 2011; Sun et al. 2017). Intact CHO cells transiently expressing WT hDAT on 24-well poly-D-lysine coated plates were incubated with a fixed concentration of [³H]WIN 35,428 (5 nM) on ice for 2 h (steady state) followed by 2 washes of ice-cold assay buffer. Non-specific [³H]WIN 35,428 binding was determined by addition of β-CFT naphthalenedisulfonate monohydrate (10 µM). Buffer was added to all wells before the initiation of the dissociation experiments and 50 µl of 10 µM cocaine, and/or 50 nM SRI-32743 were added to assigned wells at indicated time points. In condition 1 (10 µM cocaine only), 50 µl of cocaine was added to the 60 min time point; 10 min later, cocaine was added to the 50 min time point, and so on. At the 0 min time point, cocaine was added and immediately washed twice alongside all the other wells. For condition 2 (10 µM cocaine + 50 nM SRI-32743), 50 µl of cocaine and 50 µl SRI-32743 were added to the 60 min time point; 10 min later, cocaine and SRI-32743 were added to the 50 min time point, and so on. At the 0 min time point, only cocaine was added and immediately washed twice alongside all the other wells. The cells were lysed with 1% SDS for 1 h and ran through a liquid scintillation counter the next day. For data analyses, the 0 min timepoint where cocaine added but immediately washed was set as 100% to normalize samples at each time point in condition 1 and 2.
4.2.7 Animals

Inducible Tat transgenic mice (iTat-tg) mice were generated at the University of Florida where all animal studies were performed. The iTat mouse line genetically expresses a tetracycline-on (TETON) system, which is integrated into the regulator for the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter. The TETON system is coupled to the Tat1-86 gene, and with doxycycline (Dox) administration, induces Tat1-86 expression in the mouse brain. In the present study, 8 to 14-week-old male iTat mice were administered either saline (control) or Dox for 14 days to determine whether the Tat-induced potentiation of cocaine-CPP is reversed by allosteric modulator SRI-32743 (0.1, 1, or 10 mg/kg/day). C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME), the background strain of iTat-tg mice, were used as non-transgenic control. Mice were housed in 12 h light/dark cycle and had access to food and water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Florida and conform to National Research Council guidelines.

4.2.8 Cocaine Conditioned Place Preference (cocaine-CPP)

Mice were conditioned via a cocaine conditioned place preference (cocaine-CPP) paradigm with the apparatus and automated measurement system (San Diego Instruments, San Diego, CA) as previously described (Carey et al. 2007; Paris et al. 2014). iTat-tg mice were tested for initial place preference followed by 14-day administration of vehicle (0.9% saline) or Dox as described above. On days 15 and 16, mice were administered (i.p.) vehicle (100% DMSO)
or SRI-32743 (0.1, 1, 10 mg/kg/day) 60 min prior to cocaine or saline place conditioning for 30 min. Preconditioning preference was determined by measuring the amount of time individual mice spent in each chamber during a 30-min testing period. After administration of cocaine (10 mg/kg s.c.), mice were immediately confined for 30 min in the initially non-preferred chamber. Conditioning with vehicle (0.9% saline, s.c.) followed 4 h later in a similar manner, but paired to the initially preferred chamber. This cocaine-saline conditioning ‘cycle’ was repeated for two cycles, one cycle per day, for a total of 2 days. Mice were tested for preference 24 h after the completion of conditioning by allowing the mice free access to the apparatus and measuring the time they spent in each chamber over a 30-min testing period. On day 17, all mice were given a final place preference test.

4.2.8 Data analysis and statistics

Descriptive statistics and graphical analyses were used as appropriate. Results are presented as mean ± SEM, and n represents the number of independent experiments for each experiment group. IC_{50} values for cocaine inhibition of [{^3}H]DA uptake and [{^3}H]WIN35,428 binding were calculated from inhibition curves by nonlinear regression analysis with a one-site model of variable slope. E_{max} for [{^3}H]DA uptake and [{^3}H]WIN35,428 binding were derived from IC_{50} values and were calculated based on a previously reported equation (Rothman et al. 2015). For the dissociation of [{^3}H]WIN35,428 binding induced by cocaine, the dissociation rate (K_{-1}) was determined by the specific [{^3}H]WIN35,428 binding through non-nonlinear regression analysis using a single component
dissociation model. Both kinetic parameters (cocaine IC\textsubscript{50} and k\textsubscript{-1}) were calculated by GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA). For experiments involving comparisons between unpaired samples, separate ANOVAs followed by appropriate post hoc tests (Bonferroni’s or Student’s unpaired t test) were used. All statistical analyses were performed using IBM SPSS Statistics version 24. Statistical significance was determined by a minimum value of \( p<0.05 \).

**4.3 RESULTS**

4.3.1 **SRI-32743 directly blocks Tat binding sites on hDAT**

Docking studies were carried out with the insight that the SRI compounds inhibit the binding of the Tat protein to the hDAT, leaving dopamine binding unaffected (Sun et al. 2017). 3D models in Figure 4.1A show the Tat protein (green) bound to the hDAT homology model (cyan) with bound DA (purple) and the ligand binding site for the SRI compounds. DA is bound to the central cavity while SRI-32743 (yellow) and Tat are allosterically bound to the extracellular region of the transmembrane domain where the Tat protein interfaces with hDAT. The identified ligand-binding site is one of three pockets where the Tat protein forms close contact with the hDAT. Figure 4.1B shows a closer view of the ligand-binding site with SRI-32743’s geometry complementing the groove of the pocket. Figure 4.1C shows a global view of SRI-32743 and Tat binding to one of the three known Tat binding pockets on the surface of hDAT. Figure 4.1D shows a magnified surface view of Tat protein and SRI-32743 bound to the Tat binding pocket on hDAT.
4.3.2 SRI-32743 has the highest potency for inhibiting $[^3]$H]DA uptake among all compounds screened

All 14 SRI compounds (Figure 4.2) were screened for the ability to inhibit $[^3]$H]DA uptake and $[^3]$H]WIN35,428 binding in PC12 cells transiently expressing WT hDAT. As shown in Table 4.1, SRI-32743 displays the highest potency in inhibiting $[^3]$H]DA uptake (IC$_{50}$: $9.86 \pm 1.47$ µM; E$_{max}$: $68.4 \pm 8.56\%$) and an E$_{max}$ value below 70%, indicative of partial efficacy (Rothman et al. 2015). On the other hand, SRI-35280-2 (IC$_{50}$: $678.00 \pm 72.79$ µM; E$_{max}$: $83.6 \pm 8.46\%$) and SRI-36997 (IC$_{50}$: $348.37 \pm 54.56$ µM; E$_{max}$: $83.2 \pm 8.40\%$) have elevated IC$_{50}$ and E$_{max}$ values compared to other compounds. For $[^3]$H]WIN35,428 binding, SRI-32743 (168.49 ± 63.94 µM) has the least potency for binding to the DA binding site compared to other SRI compounds. Because SRI-32743 is a chiral drug, the individual IC$_{50}$ values of its R- and S- enantiomers for $[^3]$H]DA uptake and $[^3]$H]WIN35,428 binding to hDAT were screened. For $[^3]$H]DA uptake, R-SRI-32743 (IC$_{50}$: $16.02 \pm 2.0$ µM, $t(7) = 2.37, p<0.05$; E$_{max}$: $76.4 \pm 9.09\%$) displayed an IC$_{50}$ value that was higher than that of the original racemate SRI-32743, while IC$_{50}$ and E$_{max}$ values of S-SRI-32743 (IC$_{50}$: $7.01 \pm 2.0$ µM; E$_{max}$: $70.7 \pm 9.99\%$) remain unchanged. On the other hand, $[^3]$H]WIN35,428 binding shows that R-SRI-32743 (IC$_{50}$: $10.76 \pm 1.7$ µM, $t(7) = 2.81, p<0.05$; E$_{max}$: $54.3 \pm 8.69\%$) and S-SRI-32743 (IC$_{50}$: $4.69 \pm 1.5$ µM, $t(7) = 2.92, p<0.05$; E$_{max}$: $47.1 \pm 8.97\%$) displayed IC$_{50}$ and E$_{max}$ values that are significantly lower than that of SRI-32743.
4.3.3 SRI-32743 increased the IC\textsubscript{50} of cocaine

Like cocaine, indatraline is a potent direct competitor of \[^3\text{H}\]DA uptake (Pariser et al. 2008; Zhu et al. 2011; Sun et al. 2017). For these experiments, indatraline was used as a positive control to assess whether SRI-32743 is a direct competitor for DAT binding sites by determining its ability to alter cocaine’s inhibitory effects on \[^3\text{H}\]DA uptake. As reported in Table 4.2, compared to the cocaine-only control (IC\textsubscript{50}: 196 \pm 14 nM), the addition of 10 nM Indatraline significantly increased cocaine’s IC\textsubscript{50} by 172% (IC\textsubscript{50}: 533 \pm 114 nM, \(t_{(6)} = 2.97, \ p<0.05\)). On the other hand, the addition of 50 nM SRI-32743 only resulted in a 45% increase in cocaine’s IC\textsubscript{50} (IC\textsubscript{50}: 285 \pm 30 nM, \(t_{(6)} = 2.68, \ p<0.05\)). This suggests that SRI-32743 is not a direct competitor for DA binding sites.

4.3.4 SRI-32743 slowed the dissociation rate of \[^3\text{H}\]WIN35,428

To further confirm the allosteric properties of SRI-32743, dissociation assay, which has been traditionally used to identify allosteric modulation of ligands, was performed (Nandi et al. 2004; Pariser et al. 2008; Schmitt et al. 2013). It has been previously reported that SRI-20040, SRI-20041, and SRI-30827 significantly slowed the \[^3\text{H}\]WIN35,428 binding dissociation rate (k\textsubscript{\text{-1}}) in WT hDAT (Zhu et al. 2011; Sun et al. 2017). As shown in Table 4.3, the dissociation of \[^3\text{H}\]WIN35,428 initiated by the addition of cocaine (10 \(\mu\)M) proceeded in a monotonic manner which was well described by a single component dissociation model (k\textsubscript{\text{-1}} = 0.147 \pm 0.022 \text{ min}^{-1}; Condition 1). In Condition 3 (10 \(\mu\)M Cocaine + 50 nM SRI-32743), the addition of 50 nM SRI-32743 after cocaine significantly decreased cocaine-induced \[^3\text{H}\]WIN35,428
dissociation in WT hDAT (k<sub>-1</sub> = 0.086 ± 0.014 min<sup>-1</sup>, t<sub>(7)</sub> = 2.419, p<0.05) compared with cocaine alone. For Condition 2, 50 nM of SRI-32743 significantly slowed down [<sup>3</sup>H]WIN35,428 binding dissociation (k<sub>-1</sub> = 0.069 ± 0.010 min<sup>-1</sup>, t<sub>(6)</sub> = 3.196, p<0.02) compared to Condition 1. These results indicate that SRI-32743 does not compete with the DAT-selective inhibitor, [<sup>3</sup>H]WIN35,428 and demonstrates properties consistent with known allosteric modulators (Rothman et al. 2002; Zhu et al. 2011; Sun et al. 2017).

4.3.5 Both SRI-32743 and SRI-31596 attenuated the inhibitory effects of Tat on DAT uptake

It has been previously demonstrated that Tat protein induces a 20-30% decrease in specific [<sup>3</sup>H]DA uptake in cells expressing WT hDAT (Midde et al. 2013, 2015; Quizon et al. 2016; Sun et al. 2019). In the present study, SRI-32743 and SRI-31596 were used as pharmacological probes to determine whether Tat protein inhibited DAT by acting on allosteric binding sites. The addition of 140 nM recombinant Tat<sub>1-86</sub> resulted in a 28% decrease in specific [<sup>3</sup>H]DA uptake in WT hDAT (72.2 ± 4.47%, t<sub>(7)</sub> = 2.098, p<0.05). On the other hand, the addition of either SRI-32743 alone or SRI-31596 alone did not alter specific [<sup>3</sup>H]DA uptake compared to untreated control (SRI-32743 DPM: 2704 ± 453; SRI-31596 DPM: 2618 ± 480; Untreated Control DPM: 2714 ± 447). The inhibitory effects of 140 nM recombinant Tat<sub>1-86</sub> in WT hDAT cells without SRI treatment was attenuated by the addition of 50 nM SRI-32743 (120.9 ± 10.82%, t<sub>(8)</sub> = 4.16, p<0.01) and 50 nM SRI-31596 (145.4 ± 5.78%, t<sub>(8)</sub> = 10.01, p<0.001). These results show that
SRI-32743 and SRI-31596 block Tat’s inhibitory effect on DAT on allosteric binding sites.

4.3.6 Systemic in vivo administration of SRI-32743 alleviates Tat-potentiatiated cocaine reward in iTat-tg mice

iTat-tg mice were tested for initial place preference followed by 14-day administration of saline or Dox. On days 15 and 16, mice were administered (i.p.) vehicle (100%DMSO) or SRI-32743 (0.1, 1, 10 mg/kg/day) 60 min prior to cocaine or saline place conditioning for 30 min. On day 17, all mice were given a final place preference test. As shown in Figure 4.4.A, SRI-32743 administration dose-dependently alleviates potentiation of cocaine-CPP in iTat-tg mice treated with Dox. Figure 4.4.B shows that a selected dose (1 mg/kg) of SRI-32743 attenuates potentiated cocaine-CPP in iTat-tg mice following 14-day Dox. Two-way ANOVA analysis reveal a significant main effect of place conditioning ($F_{(1,274)} =10.43$, $p=0.0014$) and treatment ($F_{(1,274)} =2.31$, $p=0.044$), and significant interaction of treatment and conditioning ($F_{(5,274)} =2.945$, $p=0.013$). Whereas treatment with Dox for 14 d significantly potentiated cocaine-CPP over saline-treated littermates (†; $p=0.025$), indicating that iTat potentiates cocaine-CPP, treatment with SRI-32743 ameliorates the Tat-induced potentiation (‡; $p=0.004$).

4.4 DISCUSSION

As a follow-up to previous work (Sun et al. 2017), the current study tested the feasibility of utilizing novel quinazoline-based allosteric modulators to attenuate the effects of Tat inhibition on DAT function as well as Tat-potentiatiated cocaine rewarding in the inducible HIV-1 Tat transgenic mouse model. There are
six major findings in this study. First, preliminary results from the computational model predicts that SRI-32743 blocks Tat at the known Tat binding sites on hDAT. Second, among the SRI compounds screened, SRI-32743 has the highest potency for inhibiting $[^3H]DA$ uptake while leaving $[^3H]WIN35,428$ binding unaffected as evidenced by its lowest binding potency. Third, both SRI-32743 and indatraline increased the cocaine IC$_{50}$ values of $[^3H]DA$ uptake. However, SRI-32743 did this to a lesser degree compared to indatraline. This suggests that unlike indatraline, SRI-32743 is not a direct competitor for DA binding sites on DAT. Fourth, SRI-32743 alone or in combination with cocaine slowed the dissociation rate of $[^3H]WIN35,428$ compared to cocaine alone, demonstrating that SRI-32743 does not compete with the putative DA or cocaine binding sites on DAT. Fifth, both SRI-32743 and SRI-31596 attenuated the inhibitory effects of Tat protein on DA uptake, thereby confirming computational predictions regarding the SRI compounds’ ability to block Tat binding on DAT. Finally, systemic pre-treatment with SRI-32743 ameliorated Tat-potentiated cocaine-CPP in the iTat-tg mouse model. These results demonstrate that utilizing allosteric modulatory compounds that disrupt Tat and DAT binding raise the exciting possibility for potential therapeutics for the prevention of HAND especially in HIV-infected patients with concurrent cocaine abuse.

It has been previously demonstrated that Tat inhibits DA function by binding to allosteric sites on DAT at specific recognition residues validated through a combination of computational modeling predictions and pharmacological screening (Midde et al. 2013, 2015; Yuan et al. 2016b; Quizon
et al. 2016; Sun et al. 2019). These residues do not lie on the central binding site of hDAT, but may share the allosteric binding sites for both the SRI compounds and Tat protein (Sun et al. 2017). Interestingly, preliminary results from the computational modeling study appears to support this hypothesis. The three binding pockets on hDAT that most favorably binds to Tat based on the outward-open model of hDAT have been identified. The molecular dynamics simulations, with the addition of SRI-32743 to the Tat-hDAT model, show that SRI-32743 favorably binds to one of the three Tat binding pockets on hDAT. Upon closer inspection, these preliminary results show that SRI-32743 directly blocks Tat protein from fully binding to its binding pocket on hDAT (Figure 4.1). This prediction was validated experimentally in cells expressing WT hDAT. Both SRI-32743 and SRI-31596 attenuated Tat-induced inhibition of DA uptake compared to the non SRI-treated Tat-exposed control (Figure 4.3). Previous studies have reported that SRI-30827 and SRI-20041 interact with key Tat recognition residues Y470 and Y88 on hDAT (Midde et al. 2013, 2015; Sun et al. 2017). Further studies are warranted in order to identify the specific residues on hDAT as well as Tat that are involved in the ability of SRI-32743 as well as SRI-31596 for blocking Tat binding to hDAT.

Among the SRI compounds screened, SRI-32743 has the highest potency for inhibiting [3H]DA uptake while leaving [3H]WIN35,428 binding unaffected as evidenced by its lowest binding potency (Table 4.1). These results are interesting because an ideal allosteric compound should have minimal or partial inhibitory action on DAT with no effect on DA binding (Ananthan et al. 2002; Rothman et al.
2002, 2015; Pariser et al. 2008). That is, the compound must be able to modulate transporter function (i.e., [³H]DA uptake) without competing for the central substrate binding site (i.e., as represented by [³H]WIN35,428 binding, which has the highest binding selectivity for DAT). This condition would only be true if the compound acts on sites that are distinct from the active binding site—i.e., in an allosteric manner (Hasenhuetl et al. 2019). This finding agrees with previous screenings conducted on rat synaptosomes where SRI-31142 was found to have a 1000-fold weaker potency to block [³H]WIN35,428 binding compared to its higher potency for inhibiting [³H]DA uptake (Rothman et al. 2015; Moerke et al. 2018). The current study also found that SRI-32743 and indatraline, a competitive DAT inhibitor, increased the cocaine IC₅₀ values of [³H]DA uptake. However, SRI-32743 increased cocaine IC₅₀ values of [³H]DA uptake to a lower degree compared to indatraline, which is consistent with previous findings with SRI-20040 (Zhu et al. 2011). This suggests that unlike indatraline, SRI-32743 is not a direct competitor for DA binding sites on DAT, effectively demonstrating its allosteric characteristics.

Finally, as an initial test to determine the feasibility of utilizing allosteric modulators like the SRI compounds to disrupt the potentiating effects of Tat on cocaine-CPP, we utilized the iTat-tg mouse model. It has been previously found that Tat protein potentiates cocaine-CPP in this mouse model due to Tat-induced neurotoxicity and damage to dopaminergic areas of the brain (Paris et al. 2014). This mouse model recapitulates many aspects of the neuropathological phenotypes observed in HAND patients (Kim et al. 2003) and has been widely
used to test the *in vivo* effects of Tat protein. In this study, SRI-32743 was administered via i.p. injection, because of its excellent BBB penetration (data not shown). Similar to the *in vitro* results shown in Figure 4.3, SRI-32743 blocked the potentiating effects of Tat on cocaine-CPP in a dose-dependent manner (Figure 4.4). These results demonstrate the potential for using allosteric DAT modulators like the SRI compounds to block the effects of Tat *in vivo*. Future studies should determine whether the SRI compounds could ameliorate already established cocaine-CPP in these mice. More studies regarding the ability of SRI compounds to improve learning and memory in this mouse model would also be beneficial, due to the cognitive deficits experienced by patients with HAND.

Overall, these results demonstrate that developing allosteric modulatory molecules which attenuate cocaine and Tat binding to DAT offer the potential for preventing HAND especially in HIV-infected patients who are concomitant abusers of cocaine.
Table 4.1. Summary of competitive inhibition studies on SRI compounds.

<table>
<thead>
<tr>
<th>Drug</th>
<th>DAT Uptake $IC_{50}$ (µM)</th>
<th>DAT Uptake $E_{max}$ %</th>
<th>DAT Binding $IC_{50}$ (µM)</th>
<th>DAT Binding $E_{max}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRI-29213</td>
<td>21.53 ± 3.50</td>
<td>69.4 ± 8.06</td>
<td>43.68 ± 14.62</td>
<td>69.4 ± 8.36</td>
</tr>
<tr>
<td>SRI-29786</td>
<td>21.34 ± 4.71</td>
<td>70.6 ± 8.45</td>
<td>35.44 ± 14.02</td>
<td>71.1 ± 7.64</td>
</tr>
<tr>
<td>SRI-30827</td>
<td>18.96 ± 4.81</td>
<td>74.2 ± 8.38</td>
<td>9.82 ± 1.70</td>
<td>74.8 ± 9.09</td>
</tr>
<tr>
<td>SRI-31142</td>
<td>24.68 ± 8.70</td>
<td>72.6 ± 8.19</td>
<td>7.15 ± 1.03</td>
<td>68.4 ± 9.35</td>
</tr>
<tr>
<td>SRI-31596</td>
<td>10.38 ± 0.59</td>
<td>70.5 ± 8.48</td>
<td>7.94 ± 1.30</td>
<td>68.4 ± 9.46</td>
</tr>
<tr>
<td>SRI-32743</td>
<td>9.86 ± 1.47</td>
<td>68.4 ± 8.56</td>
<td>168.49 ± 63.94</td>
<td>71.4 ± 7.40</td>
</tr>
<tr>
<td>SRI-35280-2</td>
<td>678.00 ± 72.79</td>
<td>83.6 ± 8.46</td>
<td>9.73 ± 2.26</td>
<td>73.3 ± 9.11</td>
</tr>
<tr>
<td>SRI-35282-2</td>
<td>83.00 ± 24.96</td>
<td>78.7 ± 8.16</td>
<td>13.19 ± 0.76</td>
<td>74.1 ± 8.40</td>
</tr>
<tr>
<td>SRI-36997</td>
<td>348.37 ± 54.56</td>
<td>83.2 ± 8.40</td>
<td>20.58 ± 1.00</td>
<td>71.3 ± 8.07</td>
</tr>
<tr>
<td>SRI-36998</td>
<td>12.09 ± 1.31</td>
<td>73.7 ± 9.04</td>
<td>5.32 ± 0.50</td>
<td>67.0 ± 9.85</td>
</tr>
<tr>
<td>SRI-39040</td>
<td>14.97 ± 0.47</td>
<td>75.8 ± 8.96</td>
<td>7.86 ± 1.04</td>
<td>69.7 ± 9.14</td>
</tr>
<tr>
<td>SRI-39041</td>
<td>12.61 ± 1.02</td>
<td>76.9 ± 8.95</td>
<td>6.41 ± 0.61</td>
<td>73.8 ± 9.91</td>
</tr>
<tr>
<td>SRI-32743 (R)</td>
<td>16.02 ± 2.0</td>
<td>76.4 ± 9.09</td>
<td>10.76 ± 1.7</td>
<td>54.3 ± 8.69</td>
</tr>
<tr>
<td>SRI-32743 (S)</td>
<td>7.01 ± 2.0</td>
<td>70.7 ± 9.99</td>
<td>4.69 ± 1.5</td>
<td>47.1 ± 8.97</td>
</tr>
</tbody>
</table>

Note: The $E_{max}$ of cocaine is 100%
Table 4.2. Effects of SRI-32743 on cocaine-induced inhibition of [³H]DA uptake in WT hDAT.

<table>
<thead>
<tr>
<th></th>
<th>Condition 1 (Cocaine)</th>
<th>Condition 2 (Cocaine + Indatraline)</th>
<th>Condition 3 (Cocaine + SRI-32743)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (nM)</td>
<td>196 ± 14</td>
<td>533 ± 114*</td>
<td>285 ± 30*</td>
</tr>
</tbody>
</table>

*P<0.05 compared to Condition 1. Data are presented as means ± SEM (n=4).
Table 4.3. Effects of SRI-32743 on cocaine-mediated dissociation of [³H]WIN35,428 binding in WT hDAT.

<table>
<thead>
<tr>
<th>Condition 1 (Cocaine)</th>
<th>Condition 2 (SRI-32743)</th>
<th>Condition 3 (Cocaine + SRI-32743)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.147 ± 0.022</td>
<td>0.069 ± 0.010*</td>
<td>0.086 ± 0.014*</td>
</tr>
</tbody>
</table>

*P<0.05 compared to Condition 1. Data are presented as means ± SEM (n=4).
Figure 4.1 Preliminary molecular modeling of the Tat binding interface of the hDAT homology model. DAT is shown in cyan, DA in purple, SRI-32743 in yellow, and Tat protein in green. (A) The global view of the binding of Tat (green ribbon) and SRI-32743 (yellow stick) to hDAT. Notice that DA (purple sphere) is bound to the central cavity while SRI-32743 and Tat are allostERICALLY bound to the extracellular vestibule. (B) A closer view of SRI-32743 bound to the external surface of hDAT highlighting how SRI-32743’s geometry compliments the shape of the surface. (C) A global view of SRI-32743 and Tat binding to one of the three known Tat binding pockets on the surface of hDAT. (D) A magnified surface view of Tat protein and SRI-32743 competing for the Tat binding pocket on hDAT.
Figure 4.2 Novel series of quinazoline structure-based compounds screened. All of these compounds possess a common 4-quinazolinamine core structure. SRI-29786 possess a monocyclic substituent and all others possess the fused bicyclic 6-(7-methylimidazo(1,2-a)pyridyl system at the 2-position of the quinazoline ring. Compounds SRI-31142, SRI-31596, and SRI-32743 are capable of crossing the BBB and are water-soluble.
Figure 4.3 SRI-32743 attenuates Tat-induced inhibition of DA uptake in WT hDAT. Specific [\(^{3}H\)]DA uptake in intact PC12 cells expressing WT hDAT was performed in the presence or absence of Tat alone or SRI compounds, or Tat + SRI compounds. Data are presented as a percent of untreated control per group, expressed as mean ± S.E.M. (*p<0.01 compared to control with no Tat, # p<0.01 compared to WT hDAT within Tat exposure group, n = 4-6).
Figure 4.4 Systemic administration of SRI-32743 alleviates Tat-potentiated cocaine reward in iTat-tg mice. (A) SRI-32743 administration dose-dependently alleviates potentiation of cocaine-CPP in iTat-tg mice treated with Dox. (B) Selected dose (1 mg/kg) of SRI-32743 attenuates potentiated cocaine-CPP in iTat-tg mice following 14-day Dox. (†; p=0.025; ‡; p=0.004). N=22-26 mice/bar as listed.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

The widespread availability of ART will increase the number of people living with HIV worldwide. It has been estimated that by the year 2030, the number of HIV-positive patients with HAND will grow by 5- to 10-fold (Cysique et al. 2011). Mounting evidence from clinical and basic science research areas have demonstrated the link between HIV viral protein Tat, dopaminergic neurotransmission, and the dopamine transporter in the pathogenesis of HAND. Tat protein blocks DAT leading to extracellular DA overflow, neurotoxicity, and impaired dopaminergic neurotransmission that is further potentiated in the presence of drugs of abuse such as cocaine. There are no targeted therapeutics for the treatment of HAND, and due to the irreversible nature of neurodegenerative diseases, a preventive early intervention strategy is critical. Drug discovery efforts should focus on developing prophylactic neuroprotective agents for HIV-positive patients to be taken with ART that would block Tat without impeding normal DAT function.

This dissertation explored DAT as a potential target for disrupting Tat-DAT binding through integrated computational modeling studies coupled with in vitro pharmacological validation. Additional critical residues on hDAT for Tat were identified, which includes H547 and D206. This work was the first to uncover
H547A, an hDAT mutant that dramatically enhanced DAT function through alterations in structure and sensitivity to posttranslational modifications like phosphorylation and palmitoylation. The combination of DAT mutations were also characterized, Y88F/D206L and Y88F/D206L/H547A, providing insights into developing compounds that would block Tat binding on hDAT, presumably through allosteric binding sites. An initial in vivo proof-of-concept study showed the feasibility of utilizing allosteric modulator SRI-32743 to block Tat-potentiated cocaine-CPP in iTat-tg mice.

The findings from this dissertation will contribute to improved understanding of the dopamine transporter, which has been implicated not only in the development of HAND, but in various diseases that stem from dopaminergic dysregulation. Information from these studies will help contribute to developing novel therapeutics for the treatment of such dopaminergic imbalances as well as serve as the blueprint for creating an early intervention strategy for the prevention of the development of HAND in HIV-positive patients.

5.2 FUTURE DIRECTIONS

The impact of HIV infection and the Tat protein on dopaminergic dysregulation has been well studied (Purohit et al. 2011; Gaskill et al. 2017; Zhu et al. 2018). HIV infection has been found to cause significant neuronal loss in brain areas such as the PFC and the basal ganglia, which is reflected in neurocognitive decline observed in patients with HAND (Küper et al. 2011). The PFC, via both DA and norepinephrine (NE), is involved in complex cognitive processing and organization of higher-order thinking and goal-directed behaviors
Elevated levels of NE during the course of HIV infection has also been found to increase viral replication and disease progression (Cole et al. 1998; Ironson et al. 2008; Dever et al. 2016). Due to its high sequence homology to DAT (80%) (Andersen et al. 2015), NET actively participates in the reuptake of both NE and DA (Morón et al. 2002). The similarities between DAT and NET necessitate further studies looking into the interaction between Tat protein and NET to determine additional therapeutic targets for preventing HAND in HIV-infected individuals.

The work presented in this dissertation has laid the foundations for characterizing the Tat-DAT binding complex. However, these studies were carried out in vitro, which may not accurately reflect what occurs in vivo. The iTat-tg mouse is a valid model that is widely used to study the biological effects of Tat protein in vivo (Kim et al. 2003). Compared to cell-based studies that rely on a fixed concentration of recombinant Tat1-86, the iTat-tg mouse endogenously expresses Tat protein after induction with doxycycline. Common problems with recombinant Tat1-86 such as instability, oxidation, and degradation (Gaskill et al. 2017) are avoided when the protein is endogenously expressed in a closed biological system such as a transgenic mouse.

Further in vitro characterization studies of the Tat-DAT and Tat-NET binding pocket can identify amino acid substitutions on DAT or NET that block the effects of Tat while preserving normal transporter function. To determine whether these findings hold true in vivo, knock-in mouse models bearing the ideal DAT and/or NET mutations determined from in vitro studies should be
generated and crossed with the iTat-tg mice and characterized pharmacologically and behaviorally. The SRI compounds should continue to be tested on various behavioral paradigms utilizing the iTat-tg mice. In this manner, Tat-DAT and Tat-NET disruption is tackled from both genetic and pharmacologic approaches, paving the way for the ongoing development and further refinement of small allosteric modulatory compounds that block the inhibitory effects of Tat while preserving basal DAT and NET function.
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APPENDIX A
CHAPTER 2 SUPPLEMENTARY INFORMATION

Table A.1. Summary of inhibitory activities in [³H]WIN 38,428 binding in WT-hDAT and mutated hDAT

<table>
<thead>
<tr>
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<th>IC₅₀ (nM)</th>
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<tbody>
<tr>
<td></td>
<td>DA</td>
</tr>
<tr>
<td>WT hDAT</td>
<td>1350 ± 382</td>
</tr>
<tr>
<td>H547A-hDAT</td>
<td>2416 ± 652</td>
</tr>
</tbody>
</table>

* p<0.05 compared with WT hDAT (unpaired Student’s t test)
Figure A.1 Supplementary data for Chapter 2.

**Top left.** Surface (biotinylated) and intracellular DAT expression. Samples were loaded onto the gel and run under same experimental condition. Lanes indicate biotinylated protein (b, d, f, h) and intracellular protein (a, c, e, g). Lanes d and f were cropped and represented as surface DAT immunoblots for WT hDAT and H547A mutant, respectively, in Fig 2B (top panel).

**Lower left.** Total DAT expression. Samples were duplicated and loaded onto the gel and run under same experimental condition for WT hDAT (a, b), H547A (c, d), H547P (e, f), and D206L (g, f, unpublished data). Lanes b and d were cropped and represented as total DAT immunoblots for WT hDAT and H547A mutant, respectively, in Figure 2B (top panel).

**Blots on right.** The same blots shown in left were reprobed with β-tubulin antibody, which indicate an equal sample protein was loaded onto each lane.
Figure B.1. Representative immunoblots of total and biotinylated WT hDAT (a, b) and D381L (c, d).

**Top-left:** Total DAT expression in PC12 cells expressing WT hDAT or mutant. The total fraction was loaded in duplicate onto the gel under the same experimental conditions. Lanes a and c were cropped to represent Total DAT for WT and D381L, respectively, in Figure 3C.

**Bottom-left:** Biotinylated (surface) and intracellular DAT expression in PC12 cells expressing WT hDAT or mutant. The biotinylated and intracellular fractions were loaded in duplicate onto the gel under the same experimental conditions. Lanes a and c were cropped to represent Biotinylated DAT for WT and D381L, respectively, in Figure 3C.
Blots on right: β-tubulin antibody was used to re-probe the corresponding blots on the left in order to show that equal amounts of protein were loaded on each sample lane.
Figure B.2. Representative immunoblots of total and biotinylated WT hDAT and D206L.

Top-left: Total DAT expression in PC12 cells expressing WT hDAT or mutant. The total fraction was loaded in duplicate onto the gel under the same experimental conditions. Lanes a and g were cropped to represent Total DAT for WT and D206L, respectively, in Figure 3C.

Bottom-left: Biotinylated (surface) and intracellular DAT expression in PC12 cells expressing WT hDAT or mutant. The biotinylated and intracellular fractions were loaded in duplicate onto the gel under the same experimental conditions. Lanes a and g were cropped to represent Biotinylated DAT for WT and D206L, respectively, in Figure 3C.

Blots on right: β-tubulin antibody was used to re-probe the corresponding blots on the left in order to show that equal amounts of protein were loaded on each sample lane.
Figure B.3. Representative immunoblots of total and biotinylated WT hDAT, D206L/H547A, and Y88F/D206L/H547A.

**Top-left:** Total DAT expression in PC12 cells expressing WT hDAT or mutant. The total fraction was loaded in duplicate onto the gel under the same experimental conditions. Lanes d, e, and f were cropped to represent Total DAT for WT, D206L/H547A, and Y88F/D206L/H547A, respectively, in Figure 3C.

**Bottom-left:** Biotinylated (surface) and intracellular DAT expression in PC12 cells expressing WT hDAT or mutant. The biotinylated and intracellular fractions were loaded in duplicate onto the gel under the same experimental conditions. The WT, D206L/H547A, and Y88F/D206L/H547A lanes were cropped to represent Biotinylated DAT in Figure 3C.

**Blots on right:** β-tubulin antibody was used to re-probe the corresponding blots on the left in order to show that equal amounts of protein were loaded on each sample lane.
APPENDIX C

CHAPTER 2 COPYRIGHT PERMISSION

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About this article

Publication history

Received
27 June 2016
Accepted
17 November 2016
Published
14 December 2016

DOI
https://doi.org/10.1038/srep39048

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