

**Mechanisms Regulating the Dopamine Transporter
and Their Impact on Behavior**

A Dissertation Presented

By

CAROLYN GRAY SWEENEY

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

FEBRUARY 26, 2018

NEUROSCIENCE

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This work was undertaken in the Graduate School of Biomedical Sciences

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February 26, 2018

I dedicate this dissertation to Calvin. You loved working on your “thesis” beside me through the writing process, and you are a constant source of joy to everyone around you. I love you little man.

Acknowledgements

First and foremost, I would like to thank Haley Melikian. I came to her lab in the Fall of 2012. It was my first rotation as a graduate student, and she made an incredible impact even then. During my first lab meeting, she asked me, as she asks everyone, “what is the biological question?” I find myself constantly going back to this statement every time I talk about my research. Further, I think “what is the biological question?” every time I read a paper or go to a talk/seminar. I’m sure I will always think of that first lab meeting for the rest of my scientific career. Over the years, Haley has helped develop me into the scientist I am today. Every week, we have one-on-one meetings, and these sessions were critical for honing hypotheses and developing new ideas. We almost always went over our 45 minute meeting time (sorry Rita, Sijia, and Patrick). However, our long meetings were constantly full of back and forth discussion, and I looked forward to this time each week. Of course, not all of these meetings were positive, but over the course of six years, I look back on them as paramount in producing my thesis work. It should be noted that we didn’t spend all of these morning meetings talking science. Haley was always there to share a delicious vegetarian recipe or advice on how to juggle a graduate student career as a new mother.

I would like to thank my TRAC/DEC members. Andrew Tapper, Mary Munson, Daryl Bosco, Reid Gilmore, and Gilles Martin. Thank you for the help and support

over the years. Randy Blakely, thank you for traveling to serve as my external reviewer.

Each story presented within this thesis would not be possible without support:

Chapter II: Thank you to Brad Tremblay for helping to complete all of the dose response curves. I had just returned from maternity leave, and this support was critical so that I could focus on other projects. Harald Sitte has been a source of support for this chimera project since before I began working it myself. In fact, this paper might not have happened at all if it were not for his advice, suggesting that there might be something interesting behind the differential substrate affinities I observed while characterizing my mutants. Thomas Stockner provided a lot of advice for thinking about how terminal domains can impact substrate binding pockets.

Chapter III: This story was a source of a lot of headaches, and it felt like there were more dead-ends than scientific discoveries. However, I am proud of the work that eventually came from it, and I am excited to see where this story goes. Brian Alejandro worked hard to test the link between Rin and Ack1 inactivation. Further, he helped to re-characterize Rin in all of our lab's cell lines. Rita, I remember the day we sat down and said "is Rin even in these cells?" It was a scary possibility to think about with respect to each of our projects. We worked together to go back through the data, through our notebooks, and through

previous lab members' notebooks to find all the evidence. It was like we were detectives.

Chapter IV: This story would not have been possible without the support of so many from the BNRI community. Rubing and Susanna taught me behavioral assays, and tips and tricks for stereotaxic surgeries. Patrick, thanks for performing SO MANY slice biotinylations. You're very talented, and it is greatly appreciated. Valeria, thank you for all of the imaging. Gilles and Jenya, thank you for all of the electrophysiological data and analysis.

To my lab-mates, you guys are everything! Over the years, this group has changed in size and in personalities. Sijia, thank you for always answering my questions, and for all of the hilarious conversations. The lab isn't the same without you, but I'm sure you're doing amazing things at Biogen. Rita, my bay-mate and scientific travel buddy, thank you for always being there to talk to. We always joke about how the lab is really quiet when you're not around, but it's true. You are the energy force that keeps the lab going. Patrick, thank you for working so hard on my projects, and for your insight on all Starbucks beverages. I would never know what a Unicorn Frappuccino looks like in real life without you.

I would like to acknowledge the table and chairs outside the Melikian lab in the BNRI. I know this may seem odd, but this table was very important. Upon first sight, it seemed like a place to put coffee and snacks so that we could sneak out

quickly during an incubation and rehydrate or eat. However, we also sat there and talked constantly about our lives and our experiments. This table was the site of countless therapy sessions, and it was also the site of really great scientific ideas. I remember cleaning out the drawers while we packed up the BNRI and laughing with Patrick, Rita, Lauren, and Valeria as we talked about the “old days” in the lab.

Thank you to my husband, Seth Campbell. He has been incredibly supportive since the day I said, “I think I want to go back to school.” Graduate school has its ups and downs, and he has been there for all of them. It should be noted that he suggested that I spend more time writing my acknowledgements to him than writing about my research within this thesis. Needless to say, I didn’t take his advice.

To Calvin, thank you for always making me laugh. The times you toddled around the lab while I finished an experiment were always an adventure. I promise I didn’t let you touch anything toxic or radioactive.

To the rest of my family and friends, thank you for being an amazing support network. Mom, Dad, Ted, Andrew, Rachel, Brooke, Peter, the rest of the Sweeney family, the Gray family, and the Campbell family – thank you!

Abstract

Dopamine (DA) is central to movement, reward, learning, sleep, and anxiety. The dopamine transporter (DAT) spatially and temporally controls extracellular dopamine levels by taking DA back up into the presynaptic neuron following evoked release. Multiple lines of evidence from studies using pharmacological DAT blockade or genetic DAT deletion demonstrate that DAT availability at the plasma membrane is required for maintenance of homeostatic DA levels. Therefore, intrinsic mechanisms that regulate the transporter's availability at the plasma membrane may directly impact downstream DA signaling cascades and DA-dependent behaviors. Acute, regulated DAT internalization in response to protein kinase C (PKC) activation has been well documented, however the physiological importance of this mechanism remains untested. Due to DAT's critical role in regulating DA levels, it is essential to understand mechanisms that acutely regulate DAT function and surface expression, and further, how these mechanisms contribute to DA related behaviors.

DAT has intracellular amino and carboxy termini, which contain domains for transporter phosphorylation, recruitment to and from the plasma membrane, and sites for protein-protein interactions. To test whether these domains work synergistically for DAT function and regulated endocytosis I made DAT/SERT chimeras, in which I switched DAT's amino, carboxy, or both termini with that of SERT, a homologous transporter with highly divergent intracellular domains. I

demonstrated that DAT's amino and carboxy termini synergistically contribute to substrate and select competitive inhibitor affinities. Additionally, I demonstrated that the amino terminus is required for PKC-stimulated DAT endocytosis, and that both N- and C-termini are required for downstream Ack1-dependent regulation of DAT endocytosis.

To test the physiological importance of PKC-stimulated DAT endocytosis *in vivo*, I knocked down Rin, a GTPase required for PKC-stimulated DAT trafficking. This study was the first to achieve AAV-mediated, conditional, and inducible gene silencing in neurons. Using this AAV approach, I demonstrated a critical role for Rin GTPase signaling and DAT trafficking in both anxiety and locomotor response to cocaine.

Taken together, this thesis 1) adds to the understanding of DAT functional and endocytic mechanisms and 2) is the first to report the physiological impact of Rin signaling and DAT endocytosis in DA behavior.

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Chapter II of this dissertation appeared as a separate publication:

Sweeney CG, Tremblay BP, Stockner T, Sitte HH, Melikian HE (2017) Dopamine Transporter Amino and Carboxyl Termini Synergistically Contribute to Substrate and Inhibitor Affinities. J Biol Chem 292:1302-1309.

Symbols and Abbreviations

5HT	Serotonin (5-hydroxytryptamine)
AAV	Adeno-associated virus
Ack1	Activated CDC42 kinase 1
ADE	anomalous dopamine efflux
ADHD	attention deficit hyperactive disorder
Akt	Protein Kinase B
AMPH	Amphetamine
ASD	Autism Spectrum Disorder
β-CFT	(–)-2-β-Carbomethoxy-3-β-(4-fluorophenyl)tropane (WIN 35,428)
β-CIT	(–)-2β-Carbomethoxy-3β-(4-iodophenyl)tropane; RTI-55
BBS	Bungarotoxin Binding Site
BPD	Bipolar Disorder
BTX	Bungarotoxin
CaM	Calmodulin
CaMKII	Calmodulin-dependent protein kinase II
co-IP	Co-immunoprecipitation
CPP	Conditioned Place Preference
CTxB	Cholera toxin B-subunit
DA	Dopamine
DAG	Diacylglycerol
DAT	Dopamine Transporter
DAT/C-SERT	DAT(1-583)/SERT(601-630)

DAT/SERT/DAT	DAT(1-59)/SERT(79-600)/DAT(584-620)
dDAT	<i>Drosophila</i> DAT
Dox	Doxycycline
DS	Dorsal Striatum
DTDS	Dopamine transporter deficiency syndrome
EGF	Epidermal growth factor
EL2	Extracellular Loop 2
Flot-1	Flotillin-1
FSCV	Fast-scan cyclic voltammetry
GABA	Gamma-Amino Butyric Acid
GAP	GTPase activating protein
GDNF	glial-derived neurotrophic factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
GST	glutathione S-transferase
GTP	Guanosine triphosphate
GWAS	Genome wide association study
hDAT	Human dopamine transporter
IHC	Immunohistochemistry
IL4	Intracellular loop 4
IRES	Internal ribosome entry site
KD	Knockdown
KI	Knock in
KO	Knockout
KOR	Kappa opioid receptor

L-DOPA	L-3,4-dihydroxyphenylalanine
Lepr	Leptin receptor
LeuT	Leucine Transporter
LMS	Locomotor sensitization
mDAT	mouse DAT
MDMA	3,4-Methylenedioxymethamphetamine
METH	Methamphetamine
MPH	Methylphenidate
MSN	Medium spiny neuron
N-DAT/SERT	DAT(1-59)/SERT(79-620)
N-SERT/DAT	SERT(1-78)/DAT(60-620)
NAc	Nucleus accumbens
ND	Not determined
NDRI	Norepinephrine dopamine reuptake inhibitor
NE	Norepinephrine
NET	Norepinephrine transporter
NGF	Nerve growth factor
NK1R	Substance P receptor, neurokinin 1 receptor
NMDAR	N-methyl-D-aspartate receptor
PACAP38	Pituitary adenylate cyclase-activating polypeptide 38
PD	Parkinson's Disease
PICK1	Protein interacting with C kinase 1
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein Kinase C

PMA	12-myristate 13-acetate
Rack1	Receptor of activated protein C kinase 1
RFP	Red fluorescent protein
Ric	Ras related protein which interacted with calmodulin
Rin	Ras-like in neurons
Rit	Ras-like in tissue
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SERT	Serotonin transporter
SERT/C-DAT	SERT(1-600)/DAT(584-620)
SERT/DAT/SERT	SERT(1-78)/DAT(60- 583)/SERT(601-630)
shRNA	short hairpin ribonucleic acid
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SSRI	Selective serotonin reuptake inhibitor
Syn1A	Syntaxin 1A
TET	Tetracycline
TfR	Transferrin receptor
TH	tyrosine hydroxylase
TIRF	total internal reflection fluorescence
TM6	Transmembrane 6
TRE	Tetracycline responsive element
tTA	Tetracycline transactivator
VMAT	Vesicular monoamine transporter

VTA	Ventral tegmental area
WT	Wildtype
y2h	Yeast 2-hybrid

Chapter I

Introduction

I.A Dopaminergic Signaling

Dopamine (DA) is a catecholamine neurotransmitter required for movement, reward, learning, motivation, and anxiety (Wise, 2004; Schultz, 2007; Zweifel et al., 2011; Russo and Nestler, 2013). DA dysregulation is associated with Parkinson's disease (PD), attention deficit hyperactivity disorder (ADHD), and addictive disorders (Iversen and Iversen, 2007; Sulzer, 2011). Therefore, it is necessary to understand mechanisms that regulate DA and its associated signaling cascades. DA is synthesized from its precursor, tyrosine, through an enzymatic pathway that was initially described by Hermann Blaschko in 1939. In the first, rate-limiting step, tyrosine hydroxylase (TH) converts tyrosine to L-DOPA. Next, aromatic L-amino acid decarboxylase converts L-DOPA to dopamine (Blaschko, 1952; Molinoff and Axelrod, 1971). DA was originally hypothesized to serve solely as a precursor to norepinephrine (NE). However, Arvid Carlsson and colleagues discovered that DA is in fact a neurotransmitter (Carlsson et al., 1957; Carlsson et al., 1958). They rescued reserpine-induced akinesia in mice and rabbits with a L-DOPA treatment. This resulted in an increase of DA without concomitant NE increase, demonstrating that DA is in fact a neurotransmitter. Carlsson also described DA's profile throughout the brain and showed that it is concentrated in the basal ganglia (Carlsson, 1993).

Basal ganglia brain structures include the striatum, pallidum, subthalamic nucleus, substantia nigra, and ventral tegmental area (VTA) (Groenewegen, 2003). The striatum is the largest basal ganglia nucleus, and it is subdivided into dorsal (caudate nucleus and putamen) and ventral (nucleus accumbens, NAc) regions. The dorsal striatum (DS) primarily receives DAergic innervation from the substantia nigra pars compacta (SNc). The SNc to DS pathway (nigrostriatal pathway) is commonly associated with motor planning and voluntary movement. NAc receives DAergic innervation from the VTA (mesolimbic pathway), and this pathway is commonly associated with reward-related learning and motivated behavior (Burke et al., 2017).

The striatum is a heterogeneous hub of connections. In addition to DAergic inputs from VTA and SNc, it receives glutamatergic inputs from cortical, thalamic, and limbic regions (Burke et al., 2017). The principle striatal neurons are the medium spiny neurons (MSNs), which make up approximately 95% of total striatal neurons. The remaining striatal neurons are GABAergic and cholinergic interneurons. The MSNs are classically subdivided into two groups based upon the DA receptors they express (D1R vs. D2R) and structures to which they project. The D1-like receptors are $G_{s/olf}$ coupled receptors and include D1 and D5, whereas the D2-like receptors are $G_{i/o}$ coupled and include D2, D3, and D4 (Gerfen and Surmeier, 2011). In a simplified model of MSN signaling, D1R-

expressing MSNs project through the basal ganglia's "direct pathway" to disinhibit thalamic neurons to promote behavior. In contrast, the D2R expressing MSNs use the "indirect pathway" to inhibit basal ganglia output nuclei, and reduce behavioral output (Graybiel, 2000; Gerfen and Surmeier, 2011).

Dopamine impacts both direct and indirect pathways by modulating MSN excitability and synaptic plasticity at glutamatergic terminals. MSNs are quiescent without glutamatergic input. DA receptor activation alters voltage-dependent ion channel properties in the MSN (Kreitzer and Malenka, 2008; Gerfen and Surmeier, 2011). This modulation is slow (order of seconds) compared to fast excitatory glutamatergic signaling. Additionally, DA acts upon afferent glutamatergic terminals that project to the striatum. D1R activity in glutamatergic terminals suppresses excitatory signaling onto the MSN (Nicola et al., 2000). Taken together, DA signaling within the striatum is critical to shape excitatory input onto MSNs, which signal through both direct and indirect pathways to regulate behaviors such as locomotor activity and reward. Therefore, mechanisms that alter DA signaling within the synapse can shape excitatory signaling at both pre- and post-synaptic sites. DAergic terminals express the cocaine- and amphetamine-sensitive DA transporter (DAT), which regulates extracellular DA levels through presynaptic reuptake. Here, I provide evidence suggesting that DAT is required for DAergic homeostasis.

I.B Dopamine transporter (DAT)

Julius Axelrod first discovered catecholamine reuptake, by observing radiolabeled epinephrine and norepinephrine accumulation in rodent tissues (Axelrod et al., 1959; Whitby et al., 1961; Iversen, 2000). Snyder and Coyle demonstrated that distinct transporters exist for DA (DAT) and NE (NET) (Coyle and Snyder, 1969). However, the monoamine transporter field began to accelerate greatly when clones were isolated for DAT, NET, and SERT (5HT transporter) (Blakely et al., 1991; Giros et al., 1991; Kilty et al., 1991; Pacholczyk et al., 1991). DAT, NET, and SERT belong to the Solute Carrier 6 (SLC6) gene family, which also includes other Na⁺/Cl⁻-coupled transporters for GABA and glycine, among others (Kristensen et al., 2011). These transporters use Na⁺ and Cl⁻ co-transport down their concentration gradients to drive substrate transport. DAT transports DA into the presynaptic neuron using an alternating access mechanism, in which DAT cycles between outward facing to an inward facing states. DA binds to DAT in the outward facing state, and DAT undergoes conformational changes that expose DA to the intracellular milieu where it is released. (Forrest and Rudnick, 2009).

SLC6 structural elements include twelve transmembrane spanning domains, a large, glycosylated extracellular loop (EL2), and intracellular N- and C-termini. While these elements were predicted topologically (Blakely et al., 1991; Kilty et al., 1991; Pacholczyk et al., 1991; Chen and Reith, 2000), structures for the

bacterial homolog leucine transporter (LeuT) (Yamashita et al., 2005) and *Drosophila melanogaster* DAT (dDAT) (Penmatsa et al., 2013) confirmed these findings. Moreover, dDAT structure revealed elements that are specific the eukaryotic monoamine transporter, such as a kink in the DAT C-terminus, next to the plasma membrane. This kink causes a C-terminal helical structure to create a “gate” along the intracellular face. Little is known about the N-terminal structure and the distal C-terminal structure due to their highly disordered nature. However, it is known that the intracellular N- and C-termini serve as sites for multiple posttranslational modifications and protein binding sites, and their importance in DAT regulation will be discussed in Chapters II and III.

DAT Proteome

As stated previously, DAT's N- and C-termini serve as scaffolds for DAT-associated proteins. These DAT-associated complexes are critical for regulating DAT function. While numerous studies have identified DAT-associated proteins, the functional implication resulting from each protein's association with DAT has been characterized to varying extent. Table I-1 summarizes known DAT-associated proteins, their DAT binding domains, and functional implications for the DAT-association. Taken as a whole, it demonstrates that DAT-associated molecules are required for membrane targeting, trafficking, and forward/reverse DA transport. This suggests that DAT function is tightly regulated through a DAT-associated complex. Some DAT-associated molecules have been and/or will be

discussed further within this introduction including D2R (Bolan et al., 2007; Lee et al., 2007), Rin (Navaroli et al., 2011), CaMKII α (Fog et al., 2006b; Rickhag et al., 2013a), KOR (Kivell et al., 2014), G $\beta\gamma$ (Garcia-Olivares et al., 2013; Garcia-Olivares et al., 2017), PIP₂ (Hamilton et al., 2014; Khelashvili et al., 2015a; Khelashvili et al., 2015b; Khelashvili and Weinstein, 2015), Syn1A (Lee et al., 2004; Binda et al., 2008; Cartier et al., 2015), and Flot-1 (Cremona et al., 2011; Sorkina et al., 2013). Given that DAT N- and C-termini serve as sites for multiple protein interactions, it raises the hypothesis that these two terminal domains collectively create a DAT-binding scaffold. In Chapter III, I report tools for investigating DAT-associated proteins to further test how the N- and C-termini synergistically create a DAT associated regulatory complex.

Table I-1
DAT-associated proteins/lipids

Protein	N-Terminus		C-terminus		Means of discovery	Functional Implication	Reference
	Association	Residues	Association	Residues			
α - synuclein	No	-	Yes	598-620	Y2H, Co-IP, FRET	Increased V_{max} , Increased AMPH-stimulated efflux	Lee 2001, Butler 2015
14-3-3 ζ	N.D.	-	N.D.	-	Co-IP	N.D., 14-3-3 ζ mice have less DAT protein	Ramshaw 2013
CaMKII α	No*	-	Yes	612-617	Y2H, Co-IP, GST-pulldown	Required for AMPH-stimulated DA efflux, *N-terminus is CaMKII α substrate	Fog 2006, Rickhag 2013
Carboxypeptidase E	No	-	Yes	583-620	Y2H, Co-IP, GST-pulldown	Increased V_{max}	Zhang 2009
Ctr9	N.D.	-	Yes	577-579	Y2H, Co-IP, GST-pulldown	Increased V_{max}	De Gois 2015
D2R	Yes ¹ /No ²	1-15 ¹ /- ²	No	-	Co-IP, GST-pulldown ¹ /Flag Δ 1-55 DAT, co-IP ²	Promote DAT PM insertion and increased DA uptake	Lee 2007 ¹ / Bolan 2007 ²
DJ-1	No	-	No	-	Co-IP, GST-pulldown	Associates with DAT IL4, Increased DA uptake	Luk 2015
Epsin/Eps5	N.D.	-	N.D.	-	siRNA screen, Co-IP	Required for PKC-stimulated internalization	Sorkina 2006
Flot-1	N.D.	-	N.D.	-	Co-IP	Required for PKC-stimulated internalization and AMPH stimulated efflux ³ /Not required for PKC stimulated internalization ⁴	Cremona 2011 ³ / Sorkin 2013 ⁴
GPR37	N.D.	-	N.D.	-	Co-IP	reduced V_{max}	Marazziti 2007
G β γ	No	-	Yes	582-596	Co-IP, GST-pulldown, Far Western	Reduced V_{max} , Promotes efflux	Garcia-Olivares 2013, 2017
Hic5	No	-	Yes	561-590	Y2H, Co-IP, GST-pulldown	Reduced V_{max}	Carneiro 2002
KOR	N.D.	-	N.D.	-	Co-IP, FRET	Increased V_{max} , increased surface expression	Kivell 2014
NEDD4-2	N.D.	-	N.D.	-	siRNA screen, Co-IP	Required for PKC-stimulated internalization	Sorkina 2006
Parkin	N.D.	-	Yes	-	Co-IP/Co-IP, GST-pulldown	Increased V_{max} and surface expression ⁵ /Reduced V_{max} , competes with α - synuclein for binding ⁶	Jiang 2004 ⁵ / Moszczynska 2007 ⁶
PICK1	No	-	Yes	617-620	Y2H, Co-IP	Increased V_{max} , Required for PM insertion	Torres 2001, Madsen 2012, Wu 2017

PIP ₂	Yes	3,5,19,27,35,51	N.D.	-	PIP2 sensor, Co-IP, GST-pulldown, Molecular dynamic simulation	Promotes AMPH-stimulated DA efflux, Reduced AMPH-stimulated locomotor activity in <i>Drosophila</i>	Hamilton 2014, Khelashvili 2015
PKC β	N.D.	-	N.D.	-	Co-IP	Increased AMPH-stimulated efflux, Increased V _{max}	Johnson 2005, Chen 2009
Rack1	Yes	-	N.D.	-	Y2H	-	Lee 2004, Franekova 2008
Rin	N.D.	-	Yes	587-596	Y2H, FRET	Required for PKC-stimulated internalization	Navaroli 2011
Syn1A	Yes	1-33	N.D.	-	Y2H, Co-IP, GST-pulldown	Reduced V _{max} , Increased AMPH stimulated efflux	Binda 2008, Lee 2004, Cervinski 2010
Synaptogyrin-3	Yes	-	No	-	mbSUS, Co-IP, FRET, GST-pulldown	Increased V _{max}	Egana 2009

^{1,2}Discrepancy regarding D2R. ¹Finding from Lee et al., 2007. ²Finding from Bolan et al., 2007.

^{3,4}Discrepancy regarding Flotillin. ³Finding from Cremona et al., 2011. ⁴Finding from Sorkina et al., 2013.

^{5,6}Discrepancy regarding Parkin. ⁵Finding from Jiang et al., 2004. ⁶Finding from Moszczynska et al., 2007.

DAT and psychostimulants

DAT is the molecular target for psychostimulants and their analogs, therapeutic agents, and neurotoxins. Select DAT inhibitors and their respective K_i values are listed in Table II-1 (Sweeney et al., 2017). Cocaine was originally described as a DA reuptake inhibitor using radioligand binding assays (Ritz et al., 1987). Cocaine binds the monoamine transporters, DAT, NET, and SERT, with equimolar affinity, and at high doses, cocaine inhibits voltage gated sodium channels (Reith et al., 1986). Multiple cocaine analogs have been developed with enhanced affinity for specific monoamine transporters. For example, WIN35,428 (β -CFT) binds DAT with approximately 15x higher potency than cocaine, and it displays slight selectivity for DAT and NET over SERT (K_i = 27, 33, and 133nM respectively) (Eshleman et al., 1999; Kristensen et al., 2011; Sweeney et al., 2017).

AMPH is a psychostimulant that disrupts DAT function at numerous levels (Sulzer et al., 2005b). First, AMPH is a competitive DAT substrate (Sitte et al., 1998). It competes with extracellular DA for DAT binding sites, and is then translocated in the cytosol. AMPH is also a vesicular monoamine transporter (VMAT) substrate and, once in the cytosol, AMPH blocks DA repackaging into vesicles. Further, once translocated into vesicles, AMPH serves as a weak base, therefore disrupting the proton gradient necessary for DA vesicular reuptake (Sulzer and Rayport, 1990; Sulzer et al., 1993; Freyberg et al., 2016). Both direct

VMAT blockade and proton gradient disruption result in increased cytosolic DA accumulation, which promotes nonvesicular DA release, or efflux, through DAT. AMPH transitions DAT from an “efflux reluctant” state to an “efflux willing” state without interfering with forward transport mechanisms (Khoshbouei et al., 2004). The “willing” state requires AMPH-induced increases in intracellular Na^+ and Ca^{2+} (Khoshbouei et al., 2003; Gnegy et al., 2004). Additionally, N-terminal DAT phosphorylation is required for AMPH-stimulated efflux (Khoshbouei et al., 2004). AMPH promotes active $\text{CaMKII}\alpha$ binding to DAT’s C-terminus, which in turn results in phosphorylated N-terminal serines (Fog et al., 2006a; Rickhag et al., 2013a). Additionally, AMPH-stimulated efflux requires $\text{PKC}\beta$ activity as demonstrated with $\text{PKC}\beta$ inhibitors and $\text{PKC}\beta^{-/-}$ mice. (Johnson et al., 2005; Zestos et al., 2016). AMPH also elicits DAT internalization in a PKC-independent mechanism (Saunders et al., 2000; Boudanova et al., 2008a). The Gnegy group reported that upon initial exposure to AMPH (<2min), DAT inserts into the plasma membrane; however, following longer treatments with AMPH, (10-30 min), DAT sequesters from the plasma membrane through rapid endocytosis (Chen et al., 2009). Below, I will discuss AMPH-stimulated efflux mechanism in detail in addition to regulated DAT endocytic mechanisms.

I.C Dopamine Transporter Endocytic Trafficking

Membrane trafficking is a central mechanism that exerts significant control over synaptic transmission in the CNS. It is required for signal transduction (Sorkin and Von Zastrow, 2002), AMPA receptor turnover and synaptic plasticity (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Kennedy and Ehlers, 2006), and synaptic vesicle biogenesis (Hannah et al., 1999; Buckley et al., 2000). Much like receptors involved in synaptic transmission, DAT undergoes regulated trafficking. While we know much about regulated DAT trafficking mechanisms, the physiological importance of regulated DAT trafficking remains unknown. Here I present what is known about regulated DAT trafficking, and in Chapter IV, I will test the physiological importance behind a known trafficking mechanism.

Constitutive DAT internalization

At steady state, DAT internalizes and recycles to the plasma membrane. DAT expression in intracellular compartments at steady state was initially observed using electron microscopy in SN, VTA, striatum, and NAc (Nirenberg et al., 1996; Hersch et al., 1997; Nirenberg et al., 1997b; Nirenberg et al., 1997a). Further evidence for constitutive internalization came from Melikian and Buckley, using cellular fractionation techniques. They observed that, at steady state, DAT localizes to TfR-+ endosomes (Melikian and Buckley, 1999). To test whether significant basal DAT trafficking occurs, Loder and Melikian used a reversible

biotinylation assay or “internalization assay” to directly measure DAT internalization rates following both temperature-dependent recycling blockade and pharmacological recycling inhibition (Loder and Melikian, 2003). Basal DAT trafficking has since been demonstrated by many other studies in heterologous expression systems (Loder and Melikian, 2003; Li et al., 2004; Holton et al., 2005; Sorkina et al., 2005; Boudanova et al., 2008b; Sorkina et al., 2009; Navaroli et al., 2011; Sakrikar et al., 2012; Chen et al., 2013; Gabriel et al., 2013; Hong and Amara, 2013; Wu et al., 2015; Vuorenpaa et al., 2016; Wu et al., 2017), and in *ex vivo* preparations (Eriksen et al., 2009; Gabriel et al., 2013; Hong and Amara, 2013; Block et al., 2015). Hong and Amara, using an antibody feeding assay in rat midbrain cultures transduced with DAT, found that DAT constitutively internalized and recycled to the plasma membrane (Hong and Amara, 2013). However, this study relied upon DAT overexpression, rather than endogenously expressed DAT. The Gether group demonstrated constitutive endocytosis of endogenously expressed DAT in ventral midbrain cultures, using a fluorescent cocaine analog (JHC 1-64) (Eriksen et al., 2009). Additionally, the Sorkin group, using a knock-in mouse expressing HA-tagged DAT, reported intracellular HA-DAT in ventral midbrain slices (Block et al., 2015), consistent with findings from the Gether laboratory (Eriksen et al., 2009). However, little is known about constitutive DAT trafficking in striatal nerve terminals. Block et al. saw no intracellular DAT populations in striatal slices from HA-DAT knock-in mouse (Block et al., 2015). In contrast with these findings, Gabriel et al. reported

that recycling blockade and dynamin inhibition in *ex vivo* striatal slices reduced surface DAT populations, showing that DAT internalizes from and recycles to the plasma membrane under steady state conditions (Gabriel et al., 2013).

Dopamine Transporter Endocytic Signals

Canonical endocytic motifs include a dileucine motif ([D/E]XXXLI or DXXLL, where D/E is an acidic amino acid, X is any amino acid, L is leucine, and I is isoleucine) or a tyrosine-based motif (NPXY where N is asparagine, P is proline, X is any amino acid, and Y is tyrosine or YXX ϕ , where ϕ is a bulky hydrophobic residue) (Bonifacino and Traub, 2003). DAT has five dileucine-like motifs and two tyrosine based motifs; however, these sequences are not required for DAT endocytosis (Loland et al., 2002; Granas et al., 2003; Holton et al., 2005). Holton et al. used a gain-of-function approach to test whether DAT N- or C-terminus was sufficient to drive internalization of endocytic defective reporter proteins. They determined that DAT's C-terminus, and more specifically, a ten amino acid motif, FREKLAYAIA (587-596), was necessary and sufficient for both constitutive and PKC-stimulated endocytosis. Within the FREKLAYAIA sequence there are overlapping sequences that are required for constitutive internalization (LAYAIA) and PKC-stimulated internalization (FREK, discussed below). Interestingly, when the FREK (587-590) sequence was mutated to alanines, DAT underwent enhanced basal endocytosis (Boudanova et al., 2008b). These data suggested that DAT endocytic rates are negatively regulated, and that the C-terminus is

required for the negative regulatory mechanism. Additional support for a C-terminal role in negatively regulating DAT endocytosis came from an ADHD patient with a C-terminal point mutation distal to the FREKLAYAIA sequence (R615C) (Sakrikar et al., 2012). This mutation results in enhanced constitutive endocytic rates, consistent with negative regulation at the DAT C-terminus (Sakrikar et al., 2012; Wu et al., 2015). The Sorkin laboratory demonstrated that DAT's N-terminus is also required for negatively regulating DAT endocytosis (Sorkina et al., 2009). They truncated DAT's entire N-terminus, and replaced this sequence with YFP-tag (Δ 1-65 DAT). Using an antibody-feeding assay, they observed that Δ 1-65 DAT underwent enhanced constitutive endocytosis. Further, they determined that the most membrane proximal residues (60-65) mediate N-terminal negative regulation. Whether these N- and C-terminal mechanisms function independently from one another or are synergistically required to maintain negative endocytic regulation for DAT remains an unanswered question in the field, and I will test this question in Chapter III.

Protein Kinase C (PKC) stimulated trafficking

Initial studies reporting DAT sequestration to intracellular compartments, following protein kinase C (PKC) activation, began in the late 1990's using radioligand binding assays (Zhu et al., 1997; Pristupa et al., 1998) and immunofluorescently-tagged DAT (Pristupa et al., 1998). These findings, in conjunction with reports that both SERT (Qian et al., 1997) and NET

(Apparsundaram et al., 1998) downregulate in response to PKC in a trafficking dependent manner, raised the hypothesis that DAT undergoes enhanced internalization following PKC activation. Two independent reports in 1999 demonstrated that DAT rapidly internalizes following PKC activation (Daniels and Amara, 1999; Melikian and Buckley, 1999). Melikian and Buckley used a biotinylation assay to demonstrate that PKC activation, via treatment with the phorbol ester PMA, reduced DAT surface expression (Melikian and Buckley, 1999). Daniels and Amara also used PMA to activate PKC in cells overexpressing GFP-DAT and used immunocytochemistry to identify intracellular DAT populations (Daniels and Amara, 1999). Since these original findings, investigators have sought out the mechanisms underlying PKC-stimulated DAT endocytosis.

The specific PKC isoforms required for PKC-stimulated DAT downregulation remains a critical unanswered question in the field. Protein kinase C is a cellular kinase that phosphorylates serines and threonines on target proteins. The ten PKC isoforms are subdivided into three classes, conventional PKC (cPKC), novel PKC (nPKC), and atypical (aPKC) (Steinberg, 2008). PKC α , β_I , β_{II} , and γ make up the cPKC family. They require Ca^{2+} and diacylglycerol (DAG) for activation. nPKCs (δ , ϵ , η , θ , and μ) are Ca^{2+} -independent and require only DAG for activation. aPKC (ζ , ι , and λ) require neither Ca^{2+} nor DAG for activation. It

should be noted that the phorbol ester, PMA, activates only cPKC and nPKC isoforms.

Doolen and Zahniser reported that DAT downregulation required only cPKC based upon pharmacological inhibition (Doolen and Zahniser, 2002). However, this study was conducted *Xenopus* oocytes, which, presumably do not have the molecular factors contained in an intact DA nerve terminal. Fog et al., reported that PKC α used the DAT N-terminus as a substrate for phosphorylation (Fog et al., 2006b). It is known that PKC activation stimulates DAT phosphorylation; (Huff et al., 1997; Vaughan et al., 1997; Foster et al., 2002; Granas et al., 2003; Fog et al., 2006a); however, this phosphorylation is not required for PKC-stimulated endocytosis. Chen et al. mutated three putative PKC phosphorylation sites on DAT (S262, S586, and T613), and this mutant maintained PKC-stimulated downregulation (Chang et al., 2001). In addition to those three PKC consensus sites, DAT's distal N-terminus contains five serines (S2, S4, S7, S12, S13) that are phosphorylated following PKC activation (Foster et al., 2002). However, the Gether group truncated the first 22 DAT residues, and showed that this domain is not required for PKC-stimulated DAT internalization either by PMA or activation of the G_q-coupled substance P receptor, NK1R (Granas et al., 2003). This finding demonstrated that the first 22 DAT residues, including these distal N-terminal serines, are not required for PKC stimulated DAT internalization. The Fleckenstein group identified PKC β I, β II, and γ as DAT-associated in a co-IP from

rat striatal synaptosomes, however, they did not follow up on these studies with functional data (Hadlock et al., 2011). Work from the Gnegy group demonstrated that PKC β is required for DAT plasma membrane insertion rather than internalization (Chen et al., 2009; Chen et al., 2013). PKC β is activated by PMA, however, it is hypothesized that other PKC isoforms, activated by PMA, drive DAT internalization to a greater effect than PKC β DAT insertion, such that the net effect is the observed downregulation (Bermingham and Blakely, 2016). Taken as a whole, the PKC isoforms required for DAT internalization in terminals remains unknown, and in the absence of effective isoform-specific PKC activators and inhibitors, this question remains unanswered.

PKC activation causes additional DAT posttranslational modifications. DAT is palmitoylated at its membrane-proximal C-terminus (Foster and Vaughan, 2011). PKC activation reduces palmitoylated DAT; however, PKC-induced palmitoylation reductions do not affect PKC-stimulated DAT endocytosis (Foster and Vaughan, 2011; Moritz et al., 2015). Ubiquitination is another PKC-induced DAT posttranslational modification. Miranda et al. first reported that PKC activation results in DAT ubiquitination at lysines 19, 35, and 599 (Miranda et al., 2005). However, mutating these three ubiquitination sites did not block PKC-stimulated DAT endocytosis, suggesting that these three residues, and the resulting ubiquitination at these sites, are not required for regulated endocytosis (Miranda et al., 2007). However, PKC-mediated endocytosis was blocked when

two of these sites, K19 and K35, were mutated in conjunction with an additional, putatively non-ubiquitinated lysine, K27 (Miranda et al., 2007). This DAT triple mutant (N3K) does not internalize in response to PKC activation. This finding correlates DAT ubiquitination with PKC-stimulated endocytosis. However, more directly, it demonstrates that lysines 19, 27, and 35 are required for PKC-stimulated DAT endocytosis.

In addition to three N-terminal lysines, DAT also has C-terminal domains required for PKC-stimulated DAT endocytosis. As discussed previously in this chapter, the Melikian laboratory showed a non-canonical endocytic motif, FREKLAYAIA, is both necessary and sufficient for PKC-stimulated DAT endocytosis (Holton et al., 2005; Boudanova et al., 2008a). To identify putative DAT interacting proteins involved in regulated DAT endocytosis, a yeast 2-hybrid screen was performed using FREKLAYAIA as bait. This screen identified the small, neuronal Ras-like GTPase, Rin as a DAT binding protein (Navaroli et al., 2011). FRET microscopy studies demonstrate that Rin binds directly to DAT, and internalization assays in PC12 cells showed that Rin activity is required for PKC-stimulated endocytosis.

Even though Rin binds DAT, and its activity is required for PKC-stimulated endocytosis, it is unknown how Rin drives DAT endocytosis. GTP-bound Rin is found in complex with Par6 and Cdc42 (Hoshino et al., 2005), which prompted the hypothesis that Cdc42 activity is required for regulated DAT endocytosis. Wu

et al. showed that Cdc42 negatively regulates DAT endocytic rates (Wu et al., 2015). This finding is consistent with the negative regulatory mechanism or “endocytic brake” that is associated with DAT’s N- or C-terminus (Boudanova et al., 2008b; Sorkina et al., 2009). Cdc42 activates the nonreceptor tyrosine kinase, Ack1, which Wu et al. demonstrated negatively regulates DAT endocytosis downstream of PKC and Cdc42 activation (Wu et al., 2015). Whether Rin sequesters Cdc42 such that it cannot activate Ack1, and therefore, cannot serve as a DAT endocytic brake remains untested. Ack1 contains a PPXY motif that is required for binding to the E3 ubiquitin ligase, Nedd4-2, and Nedd4-2 activity downregulates Ack1 expression (Chan et al., 2009). Interestingly, Nedd4-2, was identified in an siRNA screen as a required molecule for PKC-stimulated DAT endocytosis (Sorkina et al., 2006; Vina-Vilaseca and Sorkin, 2010). Whether Nedd4-2 mediated Ack1 downregulation is required to drive Ack1-dependent DAT endocytosis down stream of PKC activation remains unknown.

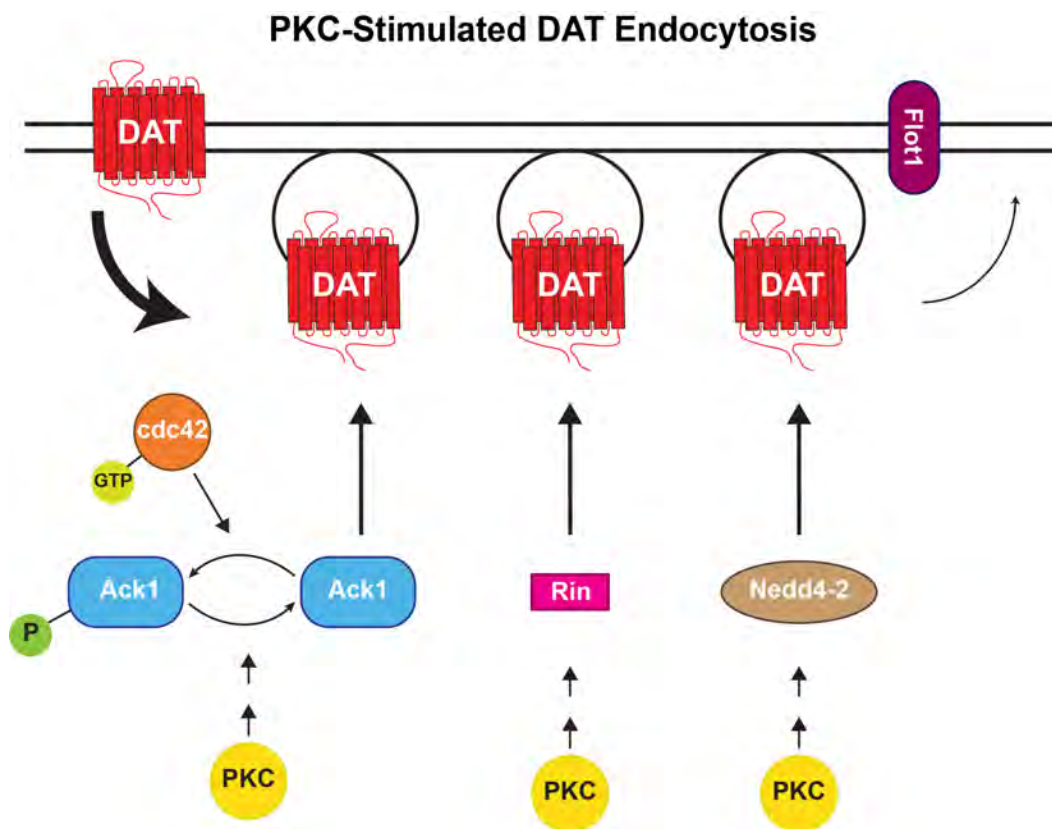


Figure I-1. PKC stimulated DAT endocytosis. DAT rapidly internalizes and displays reduced insertion rates following protein kinase C activation. PKC-stimulated DAT endocytosis requires Ack1 inactivation, Rin activity, Nedd4-2, and Flot1. Future studies are required to test whether these mechanisms are distinct or part of a coordinated signaling cascade (Adapted from Wu et al., 2015).

DAT mobility in membrane microdomains

Multiple reports indicate that DAT localizes to distinct membrane microdomains (Adkins et al., 2007; Foster et al., 2008; Hong and Amara, 2010; Cremona et al., 2011; Navaroli et al., 2011; Rahbek-Clemmensen et al., 2017). Membrane microdomains are small, heterogeneous, dynamic domains that can facilitate cellular functions by compartmentalizing cellular factors (Simons and Ikonen, 1997; Pike, 2006). Flotillin (Flot-1) is an integral membrane protein involved in signaling, endocytosis, and cytoskeletal interactions (Otto and Nichols, 2011). Cremona et al. reported that Flot-1 is required for DAT localization to cholesterol-rich domains, and that Flot-1 is required for PKC-stimulated DAT internalization (Cremona et al., 2011). However, the Sorkin group showed that while Flot-1 is required for DAT lateral mobility in the membrane; it is not required for PKC-stimulated DAT endocytosis (Sorkina et al., 2013). While follow up studies are necessary to test whether Flot-1 is in fact required for PKC-stimulated endocytosis, Cremona et al.'s findings raise an interesting hypothesis that regulated DAT endocytosis requires DAT localization in discrete microdomains. In support of this hypothesis, the DAT/Rin interaction is enriched in membrane rafts as measured with CTxB labeling (Navaroli et al., 2011). Given that Rin is required for DAT internalization following PKC activation, these findings correlate DAT membrane localization and PKC-stimulated DAT endocytosis. Further, the ADHD associated DAT mutant, R615C, displays altered microdomain localization (Sakrikar et al., 2012). R615C is an endocytic gain-of-function mutant, as

demonstrated by increased basal internalization rates. Taken together, these data correlate R615C's enhanced basal endocytosis with disrupted plasma membrane localization, further supporting the hypothesis that microdomain localization is required for regulated DAT endocytosis.

Voltage-dependent DAT internalization

Consistent with reports that DAT localizes to discrete microdomains, the Gether group recently used super-resolution microscopy to demonstrate that DAT localizes to cholesterol-rich domains in CAD cells and *ex vivo* preparations (Rahbek-Clemmensen et al., 2017). Further, they showed that DAT microdomain localization decreased following NMDA receptor activation, suggesting that DAT undergoes activity-dependent lateral diffusion. While this finding does not speak to whether DAT internalizes or recycles back to the plasma membrane following depolarization, reports from the Khoshbouei group support voltage dependent DAT internalization (Richardson et al., 2016). They used TIRF microscopy and surface biotinylation approaches to monitor fluorescently-tagged DAT during depolarization and hyperpolarization in HEK cells and cultured neurons. They observed DAT surface losses in response to depolarization, and enhanced surface expression following hyperpolarization. However, it should be noted that the only means of membrane depolarization used in this paper was KCl treatment. Whether this mechanism holds true under more physiological depolarization conditions remains untested.

Substrate-Mediated DAT Trafficking

In addition to constitutive, PKC-stimulated, and voltage-dependent trafficking mechanisms, DAT undergoes rapid internalization following substrate translocation. As discussed previously, amphetamines are a potent, competitive DAT substrate. They increase extracellular levels by competing with DA for uptake into the presynaptic terminal, by promoting an “efflux willing” state that allows for reverse DA transport through DAT, and they cause rapid DAT endocytosis which results in less DAT at the plasma membrane for reuptake (Sulzer et al., 2005a). Initial reports, using Flag-DAT in EM4 cells showed that AMPH treatment increased intracellular DAT populations (Saunders et al., 2000). A surface biotinylation assay confirmed that AMPH-stimulated DAT sequestration was due to a loss of surface DAT populations (Kahlig et al., 2004). AMPH-stimulated DAT internalization is inhibited by pretreatment with cocaine, suggesting that this process requires AMPH translocation through DAT to the cytosol intracellular AMPH. Consistent with this idea, introducing AMPH via patch pipette was sufficient to elicit enhanced DAT endocytosis (Kahlig et al., 2006). The Melikian group reported that AMPH-stimulated DAT trafficking uses pathways distinct from PKC-stimulated DAT internalization, given that the C-terminal endocytic motif FREKLAYAIA is not required for AMPH-stimulated DAT internalization, and pharmacological PKC blockade did not affect AMPH-stimulated DAT internalization in PC12 cells (Boudanova et al., 2008a).

As stated earlier, AMPH activates CaMKII, which results in CaMKII binding to DAT's C-terminus (Fog et al., 2006a). Additionally, following AMPH exposure, CaMKII inactivates Akt, and Akt inactivation is required for DAT internalization (Garcia et al., 2005; Wei et al., 2007). These data demonstrate that following AMPH treatment, CaMKII's association with DAT is required for two important DAT mechanisms – endocytosis and efflux. Further, these data demonstrate that Akt negatively regulates DAT surface expression, and following Akt inactivation, DAT rapidly internalizes. The notion of negatively regulating DAT surface expression is a recurrent theme within the DAT regulatory field (Boudanova et al., 2008b; Sorkina et al., 2009; Wu et al., 2015), suggesting that multiple factors stabilize DAT surface populations, and following a stimulus (PKC activation, AMPH treatment), an “endocytic brake” is released, allowing for rapid internalization. In addition to Akt-dependent AMPH-stimulated endocytosis, the Amara group showed that AMPH activates Rho GTPase, and Rho activity is required for AMPH-stimulated DAT internalization (Wheeler et al., 2015). However, how this Rho-dependent mechanism relates to the Akt-dependent mechanism remains unknown.

In addition to AMPH, dopamine, the endogenous DAT substrate, causes DAT internalization. Chi and Reith reported dose-dependent DAT surface loss following pretreatment with DA. While others report DA-stimulated DAT

internalization (Saunders et al., 2000) with 100 μ M DA, Daniels and Amara saw no effect on DAT plasma membrane levels with DA treatment with 10 μ M DA (Daniels and Amara, 1999). The difference in their findings may be due to different cellular expression systems, or rather, a reflection of the dose-dependence of DA-stimulated DAT internalization.

Other DAT Trafficking Mechanisms

Neurons have other mechanisms for acutely regulating DAT surface expression. The autoreceptor, D2R is expressed on DA nerve terminals. Multiple lines of evidence suggest that following D2R activation, DAT surface expression increases (Bolan et al., 2007; Lee et al., 2007; Chen et al., 2013). Lee et al, used Co-IP and GST-tagged N-terminal peptides to show that DAT associates with D2R at its N-terminus (Lee et al., 2007). Following D2R activation with quinperole, DAT functionally upregulates as measured by increased V_{max} , which the authors attribute to increased surface expression. It should be noted that Bolan et al. also report that D2R activation increases DAT surface expression; however, they report that the N-terminus is not required for either DAT/D2R complex or for DAT upregulation (Bolan et al., 2007). Bolan et al., further demonstrated that D2R-dependent DAT upregulation requires ERK1/2. The Gnegy group provided additional support for increased DAT insertion following D2R activation (Chen et al., 2013). They treated striatal synaptosomes with quinperole, and measured increased DAT surface expression with a surface

biotinylation assay (Chen et al., 2013). Chen et al. further demonstrated that D2R-mediated DAT surface upregulation requires PKC β , for *PKC β ^{-/-}* mice did not display quinperole-induced DAT plasma membrane insertion. D2R is a G α_i coupled receptor, which raises the question as to whether this mechanism is specific to the DAT/D2R interaction or more broadly attributed to G α_i signaling cascades. Kivell et al. used Co-IP and FRET to show that DAT exists in complex with the kappa opioid receptor (KOR) (Kivell et al., 2014). KOR is expressed in DAT+ varicosities in the NAc (Svingos et al., 2001), suggesting that KOR activation and signaling via G α_i cascades, could regulate DAT function and surface expression. Like D2R, KOR activation increased DAT function as a result of increased DAT surface expression (Kivell et al., 2014). They further reported that this increase requires ERK1/2 signaling downstream of KOR activation, consistent with the D2R-mediated mechanism reported by Bolan et al.

Zhu et al. reported a novel DAT trafficking mechanism that is GDNF and Vav2 dependent (Zhu et al., 2015). GDNF signals through the receptor tyrosine kinase, Ret. Downstream of Ret activation, Vav2 negatively regulates DAT surface expression. Further, they showed that both *Ret^{+/-}* and *Vav2^{-/-}* mice have increased DAT surface expression, and increased DAT transporter activity. Interestingly, this mechanism is specific to the NAc, as *Ret^{+/-}* and *Vav2^{-/-}* mice show no changes in DAT surface expression or transporter activity in the DS. Ret/Vav2 dependent DAT trafficking does not require PKC activity, suggesting a

novel mechanism for negatively regulating DAT surface expression. Cocaine increases DAT surface expression (Daws et al., 2002; Little et al., 2002); however, in the NAc of *Vav2*^{-/-} mice, cocaine decreased DAT surface expression. *Vav2*^{-/-} mice are not hyperactive; however, they do not sensitize to the locomotor effects of cocaine. This finding is the first to link a DAergic behavior to an acute DAT trafficking mechanism. It is known that DAT maintains DAergic homeostasis, and DAT trafficking mechanisms regulate DAT availability. However, the physiological outcome of DAT trafficking events remains poorly understood. This finding in *Vav2*^{-/-} mice correlates DAT surface expression regulation with locomotor sensitization to cocaine, suggesting that other acute DAT endocytic mechanisms can have profound consequences on DAergic behavior. I will be testing the physiological impact of PKC-stimulated DAT endocytosis in Chapter IV.

I.D Animal Models of Dopamine Transporter

Acute DAT trafficking mechanisms regulate DAT availability at the plasma membrane. It is hypothesized that acutely altering DAT availability will impact extracellular DA levels, and therefore impact DA signaling cascades; however, this hypothesis remains untested. DAT animal models support the hypothesis that altered DAT activity will have profound consequences on DAergic pathways and behaviors. *DAT*^{-/-} mice demonstrate that DAT is required for maintaining homeostatic DA signaling. *DAT*^{-/-} mice have increased extracellular DA; however,

reduced total DA stores and reduced DA release upon single pulse stimulation (Gainetdinov et al., 1998; Jones et al., 1998b; Benoit-Marand et al., 2000). *DAT*^{-/-} mice have less striatal TH, but overall TH mRNA is not reduced. Further, TH activity is increased in *DAT*^{-/-} mice, as indicated by increased L-DOPA levels and unchanged L-amino acid decarboxylase levels (Jones et al., 1998a). Additionally, D1R and D2R are downregulated in *DAT*^{-/-} mice (Giros et al., 1996). Taken as a whole, these data demonstrate that DAT is required for maintaining DA homeostasis at both pre- and postsynaptic neurons. Behaviorally, the broad disruption in DA homeostasis results in hyperactivity. However, *DAT*^{-/-} mice still display conditioned place preference (CPP) to cocaine and self-administer cocaine (Rocha et al., 1998; Sora et al., 1998). These surprising results suggested that either DAT is not required for cocaine induced reward and motivation, or that *DAT*^{-/-} mice have profound molecular compensation within their reward circuitry. A cocaine insensitive DAT KI (DAT-CI) mouse was critical in demonstrating that DAT is indeed required for cocaine's rewarding properties (Chen et al., 2006). The Gu group used a chimeric protein approach, swapping out mouse DAT (mDAT) sequences with dDAT sequences because dDAT has 10-fold lower affinity for cocaine than mDAT. This approach revealed three residues (L104V, F105C, A109V) required for DAT/cocaine binding (Wu and Gu, 2003; Chen et al., 2005). DAT-CI mice do not display cocaine-induced hyperlocomotion nor CPP to cocaine (Chen et al., 2006). The Gu laboratory used the DAT-CI mice to test the minimal DAergic circuitry sufficient for cocaine's

rewarding and locomotor effects (O'Neill et al., 2014; Wu et al., 2014). AAV-mediated WT DAT delivery to DAT-CI mice in the rostromedial striatum rescued cocaine-induced locomotion, whereas WT DAT expression in the DS and medial NAc did not restore cocaine-induced locomotion. Moreover, WT DAT expression in each of these brain regions failed to rescue CPP to cocaine (O'Neill et al., 2014). However, WT DAT injection into ventral midbrain (VTA and SNc), rescued cocaine CPP (Wu et al., 2014). Taken together, these data demonstrate not only that cocaine's rewarding properties require DAT, but also that DAT expression in both DS and NAc is required for cocaine reward.

I.E DAT mutants in disease states

As previously stated, DAT functions to clear synaptic DA, and therefore, regulates descending DA signaling cascades. DAergic dysfunction is implicated in numerous disease states including Parkinson's Disease (PD), attention deficit hyperactivity disorder (ADHD), bipolar disorder (BPD), autism spectrum disorder (ASD), schizophrenia, and drug abuse. Therefore, dysfunctional DAT can alter synaptic homeostasis, and possibly lead to DA-related disease states. Here, I provide description of DAT coding variants associated with multiple DAergic disease states, to support the hypothesis that altered DAT function may contribute to pathophysiology of DAergic disease states.

Dopamine Transporter Deficiency Syndrome

Dopamine transporter deficiency syndrome (DTDS) is an autosomal recessive disorder characterized by infantile/juvenile-onset parkinsonism dystonia (Kurian et al., 2009; Kurian et al., 2011; Ng et al., 2014). Patients express DAT point mutations (Leu368Gln, Pro395Leu, Val158Phe, Pro554Leu, Gly327Arg, Gln439X, Pro529Leu, Leu224Pro, Arg521Trp) that abolish DAT function due to reduced plasma membrane expression. Cellular studies revealed that loss of function arises from DAT mutant retention in the ER due to misfolding (Chiba et al., 2014; Beerepoot et al., 2016; Asjad et al., 2017). Recent studies using pharmacochaperoning provide insight as to possible therapeutics for these children. Pharmacochaperoning requires reversible, small molecule binding to folding intermediates, as a means to lower the free energy necessary to achieve the trajectory of a fully folded protein (Chiba et al., 2014). One such pharmacochaperone is noribogaine, which binds DAT in an inward facing conformation. In flies, expressing DTDS mutants causes reduced sleep; however, noribogaine treatment rescues this phenotype. These data suggest that molecular pharmacochaperoning approaches could serve as possible therapeutics for these patients (Beerepoot et al., 2016; Asjad et al., 2017).

Adult Parkinsonism

A patient, presenting with both early onset PD and ADHD, was identified as heterozygous for two distinct DAT missense alleles (I132F and D412N) (Hansen

et al., 2014). The Gether group performed kinetic analysis of both mutant transporters. I132F showed significantly reduced DA uptake compared to WT DAT, and D421N showed no measurable V_{\max} . The complete absence of DA uptake in D421N cannot be attributed to surface expression loss. Rather, D421N expresses significantly more protein compared to WT DAT controls. The authors attribute the lack of DA uptake to deficient Na^+ binding. Indeed, the mutated aspartate is predicted to be critical for the Na^+ binding site (Penmatsa et al., 2013). Further, D421N mutant cells undergo anomalous dopamine efflux (ADE), meaning they display an outward DA current at steady state. Cellular studies describing ADE show that this DA current occurs through DAT and is blocked with cocaine and methylphenidate (Mazei-Robison et al., 2008; Bowton et al., 2010; Bowton et al., 2014). Hansen et al. show that D421E ADE can be rescued with AMPH treatment. The I321F mutation occurs in DAT's sixth transmembrane region (TM6). While TM6 is critical for substrate binding and translocation, the mutated isoleucine is not predicted as required for substrate binding. Consistent with this prediction, I312F DAT has equal affinity for the DAT inhibitor β -CFT. Rather, the authors predict that substituting isoleucine for phenylalanine may impact substrate translocation mechanisms, resulting in the observed reduction in V_{\max} .

A rare DAT coding variant (V24M) was discovered in a PD patient in a screen for genetic variants possessing altered blood metabolite levels (Long et al., 2017).

The authors note that this individual had increased blood levels of dopamine sulfate; however, they speculate whether altered dopamine sulfate levels are a consequence of the mutation or due to L-DOPA treatment for PD. This mutation occurs at DAT's N-terminal domain, which is required for protein interactions, posttranslational modifications, DA reverse transport, and substrate translocation (Vaughan and Foster, 2013).

Attention deficit hyperactivity disorder (ADHD)

ADHD is characterized by hyperactivity, disorganization, inattentiveness, and/or impulsivity (American Psychiatric Association, 2013b). Common therapies for ADHD include methylphenidate (Ritalin), a competitive DAT antagonist, and amphetamine (Adderall), a competitive DAT substrate (Pliszka, 2007). Polymorphisms in the DAT gene were found in ADHD patients (Cook et al., 1995; Yang et al., 2007). Further, *DAT*^{-/-} mice display a hyperactive phenotype (Giros et al., 1996). Taken together, these observations provide evidence DAT is a risk allele for ADHD, and disrupted DAT function may contribute to ADHD pathology. Several functional DAT coding variants were identified in ADHD patients including A559V, R615C, V24M, and L164F; however, the latter two mutants have not been characterized (Mergy et al., 2014a). Interestingly, as previously noted, the V24M mutant was identified in an adult PD patient (Long et al., 2017).

A559V

A559V mutation was found in patients with ADHD, bipolar disorder (BD), and autism spectrum disorder (Grunhage et al., 2000; Mazei-Robison and Blakely, 2005; Bowton et al., 2014). This mutant undergoes ADE. A559V DAT's ADE is attributed to enhanced sensitivity to intracellular Na^+ levels, which results in a voltage dependent DA leak (Mazei-Robison et al., 2008). Further, data suggests that mechanistically, ADE may also arise from increased D2R activity. Cells, overexpressing A559V DAT and endogenously expressing D2R, demonstrate reduced ADE following treatment with the D2R antagonist, raclopride (Bowton et al., 2010). Downstream of D2R, cells expressing A559V have increased CaMKII activity, PKC β activity, and DAT N-terminal phosphorylation (Bowton et al., 2010; Bowton et al., 2014). These findings are consistent with reports that DAT-mediated DA efflux requires PKC β , CaMKII, and N-terminal phosphorylation (Kantor and Gnegy, 1998; Khoshbouei et al., 2004; Johnson et al., 2005; Fog et al., 2006a; Chen et al., 2013; Zestos et al., 2016). Interestingly, AMPH treatment, which promotes DAT-mediated DA efflux in WT DAT, rescues A559V DAT's ADE. The authors propose this finding as a possible mechanism to explain the paradox of using psychostimulants to alleviate hyperactivity in ADHD patients (Mazei-Robison et al., 2008).

Above, describes cellular experiments for the A559V mutant; however, they do not explain how this point mutation affects DAergic phenotypes *in vivo*. To test

this, the Blakely group generated an A559V DAT KI mouse (Mergy et al., 2014a; Mergy et al., 2014b; Davis et al., 2018). This mouse has increased extracellular DA levels, which the authors attribute to previously reported ADE. Increased extracellular DA result in tonically active D2R activity, which inhibits vesicular DA release at steady state. Further, increased extracellular DA increases the duration of D2R-mediated sIPSCs (Mergy et al., 2014b). Taken as a whole, these data demonstrate that this DAT point mutation alters DAergic signaling.

Interestingly, A559V DAT mice are not hyperactive, despite having increased extracellular DA levels. However, their locomotor response to AMHP and MPH is blunted (Mergy et al., 2014b). In a recent report with A559V DAT mice, Davis et al. demonstrate that this mutation results in enhanced motivation for sucrose reward, and increased impulsivity, which the authors attribute to their enhanced desire for reward (Davis et al., 2018). Therefore, altered DA regulation resulting from a DAT point mutation results in DAergic phenotypes.

R615C

R615C is a rare DAT coding variant, originally discovered in a cohort of Irish patients with ADHD (Bellgrove et al., 2009; Sakrikar et al., 2012). The mutated arginine is located at DAT's distal C-terminus. This mutant displays a gain-of-function endocytic phenotype in cells. It internalizes significantly faster than WT DAT at steady state levels. Further, PKC activation and AMPH treatment

stimulate WT DAT endocytosis; however, they have no effect on R615C endocytic rates suggestive of a ceiling effect in transporter endocytic rates (Sakrikar et al., 2012).

Additionally, this mutant displays increased CaMKII association and consistent with this finding, R615C has increased N-terminal phosphorylation. Further, this mutant shows less association with Flot-1. Consistent with disrupted Flot-1 association, R615C DAT localizes to different membrane compartments than WT DAT (Sakrikar et al., 2012).

DAT plasma membrane expression is negatively regulated. A negative endocytic regulatory mechanism or endocytic brake stabilizes DAT at the plasma membrane. As stated previously, Ack1 inactivation releases the endocytic brake, and results in enhanced DAT endocytosis (Wu et al., 2015). Given the gain-of-function endocytic trafficking phenotype displayed by R615C DAT, Wu et al. hypothesized that imposing negative endocytic regulation upon this mutant would rescue the endocytic phenotype. Using a constitutively active Ack1 (S445P), they rescued enhanced R615C internalization rates back to WT DAT rates. This finding raised the possibility that regulating DAT endocytic rates could have implications for ADHD treatment. All mechanistic characterization of R615C mutant has been in cellular expression systems; however, they suggest that dysregulated DAT trafficking could have profound phenotypic consequences. An

in vivo model of this mutant is necessary to test how this mutation, and downstream disrupted regulation of DAT and DA signaling, contributes to ADHD-like symptoms.

Autism Spectrum Disorder

Autism spectrum disorder (ASD) is characterized by deficits in social interaction or communication, and restrictive and/or repetitive behavioral patterns (American Psychiatric Association, 2013a). Altered DA signaling is reported in ASD-like behavior (Ernst et al., 1997; Gunaydin and Deisseroth, 2014; Lee et al., 2017; Paval, 2017). Consistent with this idea, rare genetic variants for DAT have been identified in probands with ASD, suggesting that DA clearance deficits can contribute to ASD presentation. T356M, R51W, and A559V point mutations have been identified in ASD patients. As described previously, A559V mutation was originally identified in patients with ADHD and BPD, and whole exome sequencing identified this rare allele in two unrelated males diagnosed with ASD (Bowton et al., 2014).

T356M

T356M is a *de novo* DAT coding variant found in an ASD patient (Neale et al., 2012; Hamilton et al., 2013). T356 is located in DAT's seventh transmembrane domain, and is conserved across species including mammals, *Drosophila*, and *C.*

elegans. Hamilton et al. showed that the T356M mutation did not affect DA affinity, but significantly reduced V_{\max} , which is not due to loss of surface T356M DAT. They attribute V_{\max} reductions, in part, to the mutant's ADE. Additionally, this mutant displayed reduced AMPH-stimulated DA efflux. Taken together with diminished uptake, these findings suggest that T356M has reduced capacity for transport in both forward and reverse directions. Additionally, Hamilton et al., showed that reduced uptake rates and blunted AMPH-stimulated efflux could be partially rescued with zinc (Hamilton et al., 2015). Zinc coordinates sidechains of extracellular DAT residues H193, H375, and E396, and it promotes an outward facing DAT conformation (Norregaard et al., 1998; Loland et al., 1999; Pifl et al., 2009; Li et al., 2015).

To assess the physiological impact of this mutation *in vivo*, they used the UAS/GAL4 system in *Drosophila* to express T356M DAT over a dDAT null background. Compared to flies overexpressing WT hDAT, the T356M flies are hyperactive, and they do not demonstrate increased locomotor activity following AMPH treatment. These data suggest that ADE results in a hyperactive state due to increased extracellular DA. Interestingly, A559V also displays ADE; however, in the KI mouse model, this mutant is not hyperactive (Mergy et al., 2014b). Taken together, these data suggest that increased extracellular DA resulting from ADE alone does not contribute to hyperactivity.

R51W

Whole exome sequencing identified the rare DAT variant, R51W (Cartier et al., 2015). The mutated arginine is located at the DAT N-terminus, which is a site for multiple protein-protein interactions. Syntaxin1a (Syn1A) associates with DAT's N-terminus, and this interaction is required for AMPH-stimulated DA reverse transport (Binda et al., 2008). R51W reduces DAT/Syn1A interaction, and consistent with this finding, displays reduced AMPH-stimulated DA efflux. Cartier et al. used the UAS/GAL4 system in flies to express R51W in DA neurons over a dDAT null background. This fly shows normal baseline locomotor activity compared to flies expressing WT DAT; however, AMPH-stimulated increases in locomotor activity are blunted in R51W flies compared to hDAT controls (Cartier et al., 2015). This finding supports the hypothesis that AMPH-stimulated efflux is critical for AMPH-stimulated increases in locomotor activity, however, it does not point to the importance of this mutation in ASD-related behaviors.

Taken as a whole, DAT mutants in DTDS, PD, ADHD, ASD, and BPD demonstrate DAT's critical role in regulating DA signaling. While many of these mutations do not affect total DAT or surface DAT populations, the diminished DAT function and regulation significantly impact DA signaling and DAergic behavior. These mutants support the hypothesis that DAT regulation is required to maintain homeostatic DA signaling.

I.F Rin (Rit2)

Our laboratory demonstrated that the small, neuronal Ras-like GTPase, Rin, binds directly to DAT and is required for PKC-stimulated DAT internalization (Navaroli et al., 2011). However, the following questions remain unanswered: 1) what is the physiological impact of PKC-stimulated DAT internalization? and 2) what is the significance of Rin signaling *in vivo*? In Chapter IV, I will test both of these questions. Here, I present what is known about Rin GTPase biology.

Rin signaling

The Ras GTPase superfamily is composed of small (~25 kD) proteins subdivided into families: Ras, Rho, Arf, Rab, Sar, and Ran (Colicelli, 2004). GTPases act as molecular switches that cycle between GTP-bound (active) and GDP-bound (inactive) states. In the GTP-bound state, they display high affinity for effector molecules. Following GTP hydrolysis, catalyzed by GTPase activating proteins (GAPs), they dissociate from effector molecules. GTPases are inactive in the GDP-bound state. The exchange of GDP for GTP is the rate limiting step in GTPase signaling cycle. A guanine nucleotide exchange factor (GEF) catalyzes the release of GDP, which allows for GTP loading, and downstream effector binding (Colicelli, 2004). Ras-like GTPases exert multiple functions in neurons, including axonal initiation, growth, guidance, and branching (Hall and Lalli, 2010).

Rin (Rit2), and its homolog, Rit, were initially identified in two independent screens. Lee et al., using degenerate primers for Ras-GTPases, screened retinal cDNA libraries for novel Ras-like GTPases (Lee et al., 1996). From this screen, they identified two novel GTPases, Rin and Rit. Rin (Ras-like in neurons) is expressed exclusively in brain and retina, whereas Rit (Ras-like in tissues) is expressed ubiquitously in all tissues. In an independent study, Wes et al. screened for calmodulin binding proteins in *Drosophila* retina (Wes et al., 1996). Their screen identified a novel GTPase, Ric (Ras-related protein which interacted with calmodulin), as a ubiquitously expressed Ras-like GTPase, which they identified as homologous to Rin and Rit.

Rin, Rit, and Ric all lack the classical CAAX domain (where C is a cysteine, A is an aliphatic amino acid, and X is any amino acid) necessary for lipid modification (prenylation, myristoylation, and palmitoylation) and plasma membrane association. These three GTPases all contain a polybasic domain at their C-termini which is believed to mediate plasma membrane association in the absence of lipid modification (Lee et al., 1996; Wes et al., 1996; Heo et al., 2006).

Both Lee et al. and Wes et al. identified Rin as a calmodulin (CaM) binding protein, and this finding was later confirmed in an *in vitro* binding assay performed by the Hoshino group (Lee et al., 1996; Wes et al., 1996; Hoshino and

Nakamura, 2003). CaM binds Ca^{2+} , serving as an intermediate protein to mediate Ca^{2+} signaling in the cell. It is abundantly expressed in the neuron and is reported to bind over 100 proteins in the brain (Xia and Storm, 2005). The physiological consequence of Rin-calmodulin interaction remains untested.

Nerve growth factor (NGF) activates Rin (Spencer et al., 2002; Shi et al., 2005a). The Andres group showed that H-Ras is required for Rin activation downstream of NGF and EGF signaling. Further, they show that NGF-stimulated Rin activation occurs in neuronal expression systems (PC6 cells, a subline of the pheochromocytoma PC12 cells, and SK-N-MC-IXC, a subline of the parental SK-N-MC cell) and not in non-neuronal systems (HEK and Vero cells) (Spencer et al., 2002). This finding suggests that neuronal specific factors are required for Rin activation, consistent with Rin localization limited to neurons.

The neurotrophic peptide, pituitary adenylate cyclase (AC) activated polypeptide 38 (PACAP38), activates Rin (Shi et al., 2008). PACAP induces neurite outgrowth in PC12 cells (Somogyvari-Vigh and Reglodi, 2004; Vaudry et al., 2009). This neuropeptide activates downstream signaling cascades required for survival, proliferation, neurite outgrowth, and trophic factor induction (Waschek, 2002). Shi et al. demonstrated $\text{G}\alpha_i$ -mediated PACAP signaling through Src kinase to activate Rin (Shi et al., 2008).

It should be noted that many studies investigating Rin activity relied heavily upon constitutively active and dominant negative Rin, Rit, and Ras mutants. Given the extensive homology in effector domains among these proteins, and among the superfamily of Ras-like GTPases, one cannot distinguish as to whether the results seen are indeed due to activated Rin-like proteins, or rather, due to sequestration of effectors proteins for other Ras-like GTPases necessary for queried signaling cascades. The specific GAPs and GEFs required for Rin and Rit GTPase activity remain unknown. The Hoshino group report that Rin binds to mSOS (GEF); however, they did not test whether mSOS exerts GEF activity on Rin either *in vitro* or *in vivo* (Hoshino and Nakamura, 2002). Gene silencing studies provide a more Rin-specific approach to ask questions regarding Rin-related biology. The Andres group used Rin knockdown in PC6 cells to characterize the PACAP38-Src-Rin signaling cascade. Further, some Rin signaling studies were performed in non-neuronal heterologous expression systems. Spencer et al. reported NGF-induced Rin activation in neuronal expression systems but not in non-neuronal expression systems (Spencer et al., 2002), necessitating the importance of using *in vivo* systems to study endogenous Rin activity *in situ*. In unpublished data from our laboratory, I demonstrate that Rin-targeted shRNAs are efficacious for reducing Rin expression in heterologous expression systems and in mouse brain (Chapter IV).

Rin in vivo

The gene that encodes Rin, *RIT2*, has been identified as a risk allele in numerous GWAS studies. *RIT2* mutations or deletions have been identified in patients with Parkinson's disease (Latourelle et al., 2012; Pankratz et al., 2012; Emamalizadeh et al., 2014; Labbe and Ross, 2014; Liu et al., 2015; Lu et al., 2015; Nie et al., 2015; Wang et al., 2015a; Zhang et al., 2015; Emamalizadeh et al., 2017), schizophrenia (Glessner et al., 2010; Emamalizadeh et al., 2017), autism spectrum disorder (Liu et al., 2016; Emamalizadeh et al., 2017), bipolar disorder (Emamalizadeh et al., 2017), essential tremor (Emamalizadeh et al., 2017), and speech delay (Bouquillon et al., 2011). As previously stated, Rin is required downstream of PACAP signaling in PC12 cells to induce neurite outgrowth, and reports show that that PACAP signaling is required for increased cell survival of midbrain DAergic neurons (Takei et al., 1998; Reglodi et al., 2004). Whether Rin/*RIT2* mediates this effect, and further, whether this pathway is disrupted in Rin-related neurodegenerative pathologies remains unknown.

Rit2 was identified by the Nestler laboratory as having increased Δ FosB binding to its promotor in NAc from mice receiving chronic cocaine treatment (Renthal et al., 2009). Gene expression changes are a proposed mechanism for long-term changes in reward circuitry following chronic drug abuse (Hyman et al., 2006; Nestler, 2008). Δ FosB is a stable transcription factor that is upregulated following cocaine exposure, and its long lasting expression is hypothesized to mediate long-lasting effects of drugs of abuse (Nestler, 2008). Whether Rin is indeed

transcriptionally regulated through Δ FosB, and further, whether Rin activity is altered following chronic cocaine treatment, must be tested further. However, this study is the first to propose a link between Rin expression and drug abuse.

Prior to unpublished work described in this thesis (Chapter IV), very little was known about Rin's role *in vivo*. A published abstract by the Andres group reports that in *Rin*^{-/-} mice, brain Akt signaling is increased, p38 MAPK signaling decreased, and these mice have improved outcomes in response to traumatic brain injury (Pannell et al., 2015). However, with the absence of published data to go on, we cannot ascertain what importance, if any, Rin plays in neurobiology. Additionally, a germline knockout is sensitive to compensatory mechanisms throughout development. Furthermore, multiple lines of evidence support that Rin activity may be critical in DAergic neurons, and a conditional Rin KD or KO is critical for investigating these DA-specific mechanisms. In Chapter IV I will test, for the first time, the physiological importance of Rin activity in DA neurons.

Summary

DA signaling cascades are tightly regulated to maintain homeostatic DAergic function. DAT regulates descending DA systems through DA reuptake, thereby spatially and temporally limiting DA signaling events. Small alterations in DAT function can critically impact DAergic systems. Single point mutations in DAT are associated with PD, ADHD, ASD, and BPD. DAT point mutations often do not

affect DA uptake rates (V_{\max}) or surface expression. However, having dysregulated function is sufficient to disrupt the DA network. DAergic neurons intrinsically regulate DAT availability through regulated trafficking events. However, the physiological consequences resulting from acute DAT endocytosis remains unknown. I hypothesize that disrupting regulated DAT endocytosis will impair DAT's critical and delicate control on DA signaling events; therefore, disrupting DAergic homeostasis. Within this thesis, I will discuss DAT domains required for functional DA uptake (Chapter II) and regulated trafficking events (Chapter III). Further, I will test the hypothesis that regulated DAT trafficking is required for DAergic behavior (Chapter IV).

Preface to Chapter II

This chapter was previously published separately in:

Sweeney, C. G., Tremblay, B. P., Stockner, T., Sitte, H. H., and Melikian, H. E.
(2017) Dopamine Transporter Amino and Carboxyl Termini Synergistically
Contribute to Substrate and Inhibitor Affinities. *J Biol Chem* **292**, 1302-1309

Author contributions:

Sweeney CG, Sitte HH, and Melikian HE designed research

Sweeney CG, Tremblay BP performed research

Sweeney CG and Melikian HE analyzed research

Sweeney CG, Stockner T, Sitte HH, and Melikian HE interpreted data

Sweeney CG, Stockner T, Sitte HH, and Melikian HE wrote the manuscript

Chapter II

Dopamine Transporter Amino and Carboxy Termini Synergistically Contribute to Substrate and Inhibitor Affinities

Authors: Sweeney CG, Tremblay BP, Stockner T, Sitte HH, and Melikian HE

II.A Summary

Extracellular dopamine and serotonin concentrations are determined by the presynaptic dopamine (DAT) and serotonin (SERT) transporters, respectively. Numerous studies have investigated the DAT and SERT structural elements contributing to inhibitor and substrate binding. To date, crystallographic studies have focused on conserved transmembrane domains, where multiple substrate binding and translocation features are conserved. However, it is unknown what, if any, role the highly divergent intracellular amino and carboxy termini contribute to these processes. Here, we used chimeric proteins to test whether DAT and SERT amino and carboxy termini contribute to transporter substrate and inhibitor affinities. Replacing the DAT amino terminus with that of SERT had no effect on DA transport V_{\max} , but significantly decreased DAT substrate affinities for DA and amphetamine. Similar losses in uptake inhibition were observed for small DAT inhibitors, whereas substituting the DAT carboxy terminus with that of SERT affected neither substrate nor inhibitor affinities. In contrast, the N-terminal

substitution was completely tolerated by the larger DAT inhibitors, which exhibited no loss in apparent affinity. Remarkably, all affinity losses were rescued in DAT chimeras encoding both SERT amino and carboxy termini. The sensitivity to amino terminal substitution was specific for DAT, as replacing the SERT amino and/or carboxy termini affected neither substrate nor inhibitor affinities. Taken together, these findings provide compelling experimental evidence that DAT amino and carboxy termini synergistically contribute to substrate and inhibitor affinities.

II.B Introduction

Psychostimulant drugs include therapeutics for multiple psychiatric disorders, such as depression and ADHD, as well as drugs of abuse (Torres et al., 2003a; Kristensen et al., 2011; Vaughan and Foster, 2013; German et al., 2015). Determining how these drugs interact with their molecular targets is essential for understanding psychostimulant addiction as well as psychiatric treatment. The monoamine transporters DAT and SERT are the primary targets for a variety of psychostimulants, such as cocaine and amphetamines (AMPH, METH, MDMA), as well as the SSRI and NDRI classes of antidepressants (Kristensen et al., 2011; Vaughan and Foster, 2013; Gaffaney et al., 2014; German et al., 2015). Cocaine and SSRI/NDRI inhibit these transporters by directly blocking DA and 5HT reuptake, whereas amphetamine and its congeners are competitive DAT

and SERT substrates. Once in the cytosol, amphetamines mediate monoamine release from vesicles in a VMAT-dependent manner (Freyberg et al., 2016) and drive their efflux via their respective plasma membrane transporters (Sitte and Freissmuth, 2015).

DAT and SERT belong to the SLC6 family of Na⁺- and Cl⁻-dependent solute carriers, which are topologically similar, having twelve transmembrane domains and intracellular N- and C-termini (Torres et al., 2003a; Kristensen et al., 2011; Vaughan and Foster, 2013; German et al., 2015). Crystal structures of the SLC6 bacterial homologs LeuT (Yamashita et al., 2005), *Drosophila* DAT (dDAT) (Penmatsa et al., 2013), and more recently human SERT (Coleman et al., 2016) have confirmed the predicted topologies of SLC6 transporters, shed light on the mechanism of substrate translocation, and better mapped inhibitor/substrate binding sites. While critical in understanding transporter structure and function, these transporter crystallographic studies lack structural insights into the N-terminus and much of the C-terminus, due to their highly disordered properties. Mutagenesis studies have revealed that DAT and SERT intracellular N- and C-terminal domains contribute to transporter regulated internalization (Holton et al., 2005; Miranda et al., 2007; Boudanova et al., 2008b; Sorkina et al., 2009; Rickhag et al., 2013b; Seimandi et al., 2013; Bermingham and Blakely, 2016), post-endocytic trafficking (Bermingham and Blakely, 2016; Vuorenmaa et al., 2016), biosynthetic trafficking (Sitte et al., 2004; Farhan et al., 2007; Sucic et al.,

2011; Sucic et al., 2013; Bermingham and Blakely, 2016), and amphetamine-stimulated substrate efflux (Khoshbouei et al., 2004; Guptaroy et al., 2009; Sitte and Freissmuth, 2010; Sucic et al., 2010; Rickhag et al., 2013a). Moreover, DAT and SERT N- and C- terminal coding variants have been identified in ADHD and autism spectrum disorder patients (Prasad et al., 2005; Prasad et al., 2009; Sakrikar et al., 2012; Cartier et al., 2015; Wu et al., 2015), further implicating these regions as important for transporter function, and necessitating the need for greater understanding into how they may contribute to transporter mechanisms of action. Computational modeling of the DAT N-terminus (Khelashvili et al., 2015a; Khelashvili et al., 2015b) and both SERT N- and C-termini (Fenollar-Ferrer et al., 2014) has shed light onto their secondary structure. Moreover, these studies have raised the possibility that these domains may interact with the plasma membrane and, potentially, each other. However, there remains a dearth of experimental data testing whether or not these domains contribute to substrate and inhibitor affinities. In the current study, we asked whether DAT and SERT N- and C-termini influenced substrate and inhibitor affinities. To address this question, we generated DAT/SERT chimeras in which the N-, C-, or both termini are exchanged between their respective DAT or SERT core proteins. DAT and SERT transmembrane cores share 52% sequence identity; however, their N- and C-termini are highly divergent (17% and 27% sequence identity, respectively), thus providing an effective strategy to test

whether these intracellular domains differentially contribute to substrate or inhibitor affinities.

II.C Materials and Methods

Materials: Rat anti-DAT (MAB369) antibody was from Millipore and mouse anti-SERT antibody was from MAb technologies. Horseradish peroxidase (HRP)-conjugated anti-rat secondary and mouse anti-actin (SPM161) antibodies were from Santa Cruz and HRP-conjugated anti-mouse antibody was from Jackson ImmunoResearch Laboratories (115-035-003). Sulfo-NHS-SS-biotin and streptavidin agarose were from Thermo Scientific. GBR12909, citalopram, and fluoxetine were from Tocris. All other chemicals and reagents were from Sigma-Aldrich and Thermo Fisher Scientific and were of the highest grade available.

cDNA constructs: Chimeric hDAT and hSERT cDNAs were generated using the PCR-Ligation-PCR (PLP) approach (Ali and Steinkasserer, 1995), using hDAT pcDNA3.1 (+) and/or hSERT pcDNA3.1 (+) as templates, corresponding to the following coding regions: N-SERT/DAT (hSERT[1-78]/hDAT[60-620], DAT/C-SERT (hDAT[1-583]/hSERT[601-630]), SERT/DAT/SERT (hSERT[1-78]/hDAT[60-583]/hSERT[601-630]), N-DAT/SERT (hDAT[1-59]/hSERT[79-630]), SERT/C-DAT (hSERT[1-600]/hDAT[584-620]), DAT/SERT/DAT (hDAT[1-59]/hSERT[79-600]/hDAT[584-620]). The resulting chimeric cDNAs were

digested and cloned back into their parental transporters at the following restriction sites: HindIII/PfIMI (N-SERT/DAT, SERT/DAT/SERT), HindIII/AgeI (N-DAT/SERT, DAT/SERT/DAT), or ClaI/XbaI (DAT/C-SERT, SERT/C-DAT, SERT/DAT/SERT, DAT/SERT/DAT). All chimeric constructs were confirmed by Sanger sequencing (Genewiz).

Cell culture and generation of pooled stable cell lines: Human dopaminergic SK-N-MC cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in MEM supplemented with 10% fetal bovine calf serum, 100 units/mL penicillin/streptomycin, 2 mM L-glutamine, 37°C, 5% CO₂. Cells were transfected with wildtype or chimera cDNAs using Lipofectamine 2000 according to manufacturer's instructions with the following modifications: Cells were seeded into 6 well plates at a density of 1×10^6 one day prior to transfection, and were transfected with 3 µg plasmid DNA/well using a lipid:DNA ratio of 2:1. To generate stable cell lines, 48 hours following transfection, cells were selected using 0.5 mg/mL G418. Stably transfected cells were pooled and cell lines were maintained under selective pressure using 0.2 mg/mL G418.

Uptake assays: [³H]DA and [³H]5HT uptake: 7.5×10^4 (DA uptake) or 5.0×10^4 (5HT uptake) cells/well were seeded onto 96 well plates, one day prior to the assay. Cells were washed twice with room temperature Krebs-Ringers-HEPES

buffer (KRH: 120 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.2 mM CaCl₂, 10 mM HEPES, pH 7.4), and transport assays were conducted as described below. All transport reactions proceeded for 10 min, 37°C and were terminated by washing thrice with ice-cold KRH buffer. Cells were solubilized in scintillation fluid, 15 min, room temperature and [³H] accumulation was quantified using a Wallac Microbeta scintillation plate counter (Perkin Elmer). Triplicate points were measured for each data point and non-specific uptake was defined in the presence of either 10 μM GBR12909 (DA uptake) or 10 μM paroxetine (5HT uptake). Data were analyzed using Excel and GraphPad Prism software. Cocktail solutions were counted in parallel and accumulated [³H] was less than 10% of the total [³H] added for all assays.

Kinetics: [³H]DA (dihydroxyphenylethylamine 3,4-[ring-2,5,6,-³H]; Perkin Elmer) and [³H]5HT (5-hydroxytryptamine [³H]-; Perkin Elmer) 10x cocktails were prepared by diluting a 1/20th volume radiolabeled substrate into 60 μM stock solutions of unlabeled DA or 5HT in KRH containing 0.18% glucose and 10 μM each pargyline and sodium ascorbate (KRH/g/p/a). 60 μM [³H]substrate solutions were serially diluted into KRH/g/p/a to generate [³H]DA or [³H]5HT cocktails at 10X the indicated final substrate concentrations. Cells were preincubated (20 min, 37°C) with KRH buffer supplemented with 0.18% glucose (KRH/g) and uptake was initiated by adding a 1/10th volume of 10x concentrated [³H] substrate cocktails.

Inhibitor dose response curves: Cells were pre-incubated in KRH/g (30 min, 37°C) with the indicated drugs at the indicated concentrations. Uptake was initiated by adding a 1/10th volume of 500 nM [³H]DA or 250 nM [³H]5HT 10x cocktail, for final assay concentrations of 50 nM (DA) or 25 nM (5HT).

AMPH and fenfluramine dose response: Cells were pre-incubated in KRH/glucose buffer (20 min, 37°C). Uptake was initiated by adding a 1/10th volume of either 500nM [³H]DA or 250 nM [³H]5HT 10x cocktails, containing AMPH or fenfluramine, respectively, at 10x the indicated final concentrations.

Cell Surface biotinylation: Surface proteins were covalently labeled with sulfo-NHS-SS-biotin as previously described (Loder and Melikian, 2003; Holton et al., 2005). Briefly, cells were rapidly chilled by washing in ice-cold PBS, pH 7.4, supplemented with 1.0 mM MgCl₂, 0.1 mM CaCl₂ (PBS²⁺), and incubated with 1.0 mg/ml sulfo-NHS-SS-biotin in ice-cold PBS²⁺, twice, for 15 min at 4°C. Residual reactive NHS groups were quenched by repeated washing and incubating twice in PBS²⁺/100 mM glycine, 15 min, 4°C. Cells were washed three times in ice-cold PBS²⁺, lysed in RIPA buffer containing protease inhibitors and protein concentrations were determined using the BCA protein assay (Pierce). Biotinylated proteins from equivalent amounts of cellular protein were isolated by

batch streptavidin chromatography (overnight, 4°C) and bound proteins were eluted in 2X SDS-PAGE sample buffer, 30 min, room temperature. Either supernatants (WT DAT, DAT/C-SERT) or total cellular lysates (N-SERT/DAT, SERT/DAT/SERT) were used to normalize surface levels. Blots were probed with actin for normalization (N-SERT/DAT, SERT/DAT/SERT). Samples were resolved by SDS-PAGE and specific proteins were detected by immunoblotting with the indicated antibodies. Immunoreactive bands were detected with SuperSignal West Dura (Pierce) and were captured using a VersaDoc Imaging station (Biorad). Non-saturating bands were quantified using Quantity One software (Biorad). Relative surface expression was quantified as percent surface protein (WT DAT, DAT/C-SERT, N-DAT/SERT, DAT/SERT/DAT). Data were normalized to percent WT DAT (DAT/C-SERT), N-SERT/DAT (SERT/DAT/SERT), or N-DAT/SERT (DAT/SERT/DAT).

II.D RESULTS

SLC6 crystallographic studies have shed considerable light on both substrate translocation mechanisms and inhibitor binding sites (Yamashita et al., 2005; Penmatsa et al., 2013; Wang et al., 2015b; Coleman et al., 2016). However, it remains uncertain whether transporter intracellular N- and C-termini contribute to these fundamental transporter properties. To test the hypothesis that these cytoplasmic domains influence the transport mechanism and inhibitor binding, we

created DAT/SERT chimeras which retained either DAT or SERT transmembrane domains (i.e. “cores”), but differentially replaced DAT and SERT N- and C-termini with their homologous counterparts (see schematic, Fig. II-1A). We chose these domains because although DAT and SERT are highly homologous within their transmembrane domains, their N- and C-termini diverge significantly (17% and 27% amino acid conservation, respectively, Fig. II-1B). We first asked whether these domains contributed to substrate uptake kinetics for the DAT core chimeras. Each DAT core chimera exhibited saturable DA uptake (Figure II-2A), indicative of functional protein expressed at the plasma membrane. However, V_{\max} values for DAT/C-SERT and SERT/DAT/SERT were significantly lower than either WT DAT or N-SERT/DAT (Table II-1). V_{\max} is affected by both transporter surface expression and substrate translocation rates. To test whether reduced V_{\max} values were due to attenuated transporter surface expression, we performed surface biotinylation experiments comparing either WT DAT to DAT/C-SERT, or N-SERT/DAT to SERT/DAT/SERT. Note that the DAT and SERT antibody epitopes are localized on their respective N-termini, therefore allowing only for direct comparisons between transporters with identical N-termini. In addition, we found that the N-terminally directed anti-SERT antibody recognized a non-specific band in the supernatant (intracellular), but not surface, fraction recovered from SK-N-MC cell lysates, with similar mobility as SERT (not shown). This precluded using total or intracellular SERT signals for normalization. Therefore, for assays using the anti-SERT antibody, we

normalized chimera surface levels to actin as a loading control. The percent of DAT/C-SERT expressed at the surface, relative to its own total protein expression, was not significantly different than WT DAT ($p = 0.72$, Fig. II-2B). However, the absolute amount of DAT/C-SERT was significantly reduced, thus the absolute amount of DAT/C-SERT expressed at the cell surface was likewise significantly reduced compared to WT DAT ($51.4 \pm 6.0\%$ WT levels, Fig. II-2C), which accounts for the observed V_{\max} decrease. We also observed that SERT/DAT/SERT was expressed at significantly lower levels at the cell surface than N-SERT/DAT ($10.3 \pm 2.7\%$ of N-SERT/DAT levels; Fig. II-2D), fully accounting for its decreased V_{\max} , as opposed to compromised catalytic activity.

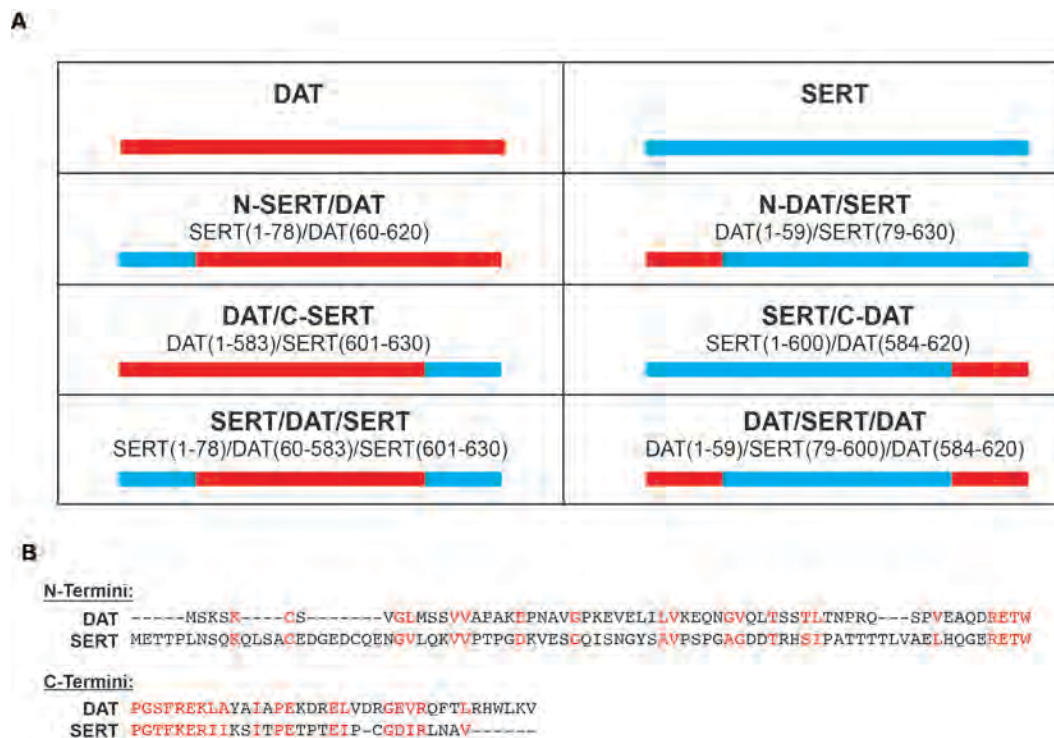


Figure II-1. A. DAT/SERT chimera schematic. DAT “core” and SERT “core” chimeric proteins were designed as depicted. DAT regions are indicated in red, SERT regions are indicated in cyan. **B. DAT and SERT N- and C-termini are highly divergent.** Sequence alignments of DAT and SERT N- and C-termini. Conserved residues (by identity, charge, or size) are indicated in red.

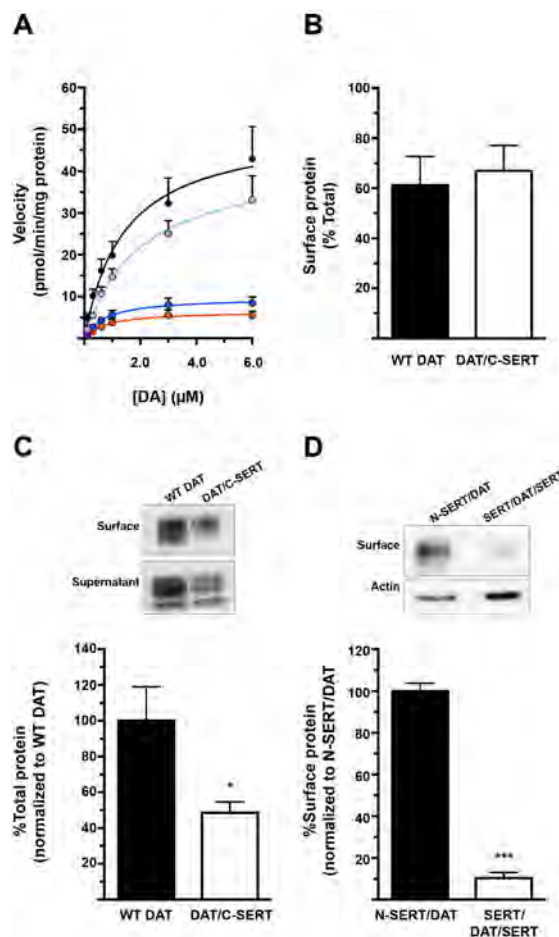


Figure II-2. DAT core chimera uptake kinetics and surface expression.

A. $[^3H]DA$ uptake assays. Saturation uptake kinetics were measured as described in *Experimental Procedures*, in SK-N-MC cells stably expressing either WT DAT (black), N-SERT/DAT (cyan), DAT/C-SERT (blue), or SERT/DAT/SERT (red). Average data are presented \pm S.E.M., $n = 11-16$. **B-D.** *Surface Biotinylation Assays.* Surface levels for the indicated constructs were determined by surface biotinylation, as described in *Experimental Procedures*. **B.** WT DAT vs. DAT/C-SERT surface levels. Average data \pm S.E.M. are presented, relative to the total amount of DAT chimera expressed, $p = 0.72$, Student's t test, $n = 3-4$. **C.** WT DAT vs. DAT/C-SERT total expression levels, calculated as the sum of biotinylated (surface) and supernatant (intracellular) fractions, *Significantly different from WT DAT, $p < 0.04$, Student's two-tailed t test, $n = 3-4$. *Inset:* Representative immunoblot for B and C. **D.** N-SERT/DAT and SERT/DAT/SERT surface expression, normalized to actin. ***Significantly different from N-SERT/DAT, $p < 0.001$, Student's two-tailed t test, $n = 3$. *Inset:* Representative immunoblots (Note: boxed bands were digitally extracted from a single blot exposure, probed for both the chimeras and actin, in parallel).

Table II-1. DAT-core chimeras:

[³H]DA uptake kinetics and inhibitor K_i values as determined by [³H]DA uptake inhibition.

Values are in nM unless otherwise indicated.

[3H]DA uptake kinetics					
	WT DAT	N-SERT/DAT	DAT/C-SERT	SERT/DAT/SERT	
Vmax (pmol/min/mg protein)	45.0 ±8.8	55.0 ±10.9	11.7 ±2.7 ^a	7.8 ±1.5 ^a	
Km (μM)	1.1 ±0.1	2.2 ±0.4 ^b	1.1 ±0.2	1.2 ±0.2 ^c	
Ki values					
Amphetamine	348.9 ±69.2	672.6 ±141.4 ^{d,e}	324.2 ±63.6	52.1 ±11.4 ^d	
Cocaine	289.9 ±63.9	214.0 ±50.8	204.3 ±4.4	190.3 ±20.5	
β-CFT	18.2 ±4.1	65.7 ±15.7 ^b	13.8 ±2.2	18.1 ±4.4 ^c	
β-CIT	0.42 ±0.14	0.20 ±0.02	N.D.	N.D.	
Methylphenidate	63.5 ±12.0	157.9 ±31.7 ^d	41.61 ±20.3	29.67 ±14.7 ^c	
GBR12909	2.1 ±0.22	2.8 ±0.6	2.5 ±0.4	1.9 ±0.2	

^aSignificantly different from WT DAT, p<0.01, one-way ANOVA with Dunnett's multiple comparison test.

^bSignificantly different from WT DAT, p<0.02, one-way ANOVA with Bonferroni's multiple comparison test.

^cSignificantly different from N-SERT/DAT, p<0.02, one-way ANOVA with Bonferroni's multiple comparison test.

^dSignificantly different from WT DAT p<0.05, one-way ANOVA with Bonferroni's multiple comparison test.

^eSignificantly different from SERT/DAT/SERT, p<0.001, one-way ANOVA with Bonferroni's multiple comparison test. n= 11-17 for [³H]DA kinetics and 3-11 for K_i values

To test whether DAT's N- and C-termini contribute to DA apparent affinity, we compared the WT DAT K_m value with those measured for the DAT core chimeras (Table II-1). Replacing the DAT N-terminus with the SERT N-terminus significantly decreased the apparent substrate affinity, as reflected by an increased K_m for DA (WT DAT: $1.1 \pm 0.1 \mu\text{M}$; N-SERT/DAT: $2.2 \pm 0.4 \mu\text{M}$, $n = 11-16$, Table I), whereas substituting the DAT C-terminus with that of SERT (DAT/C-SERT) had no effect on apparent DA affinity compared to WT DAT (Table II-1, $p = 0.99$). Surprisingly, replacing both DAT N- and C- termini with those of SERT restored the K_m back to WT DAT levels, thereby rescuing the loss of apparent affinity induced by substituting the DAT N-terminus alone (Table II-1). We next asked whether the N-terminus contributes to apparent substrate affinity in general, or to apparent DA affinity specifically, by measuring each chimera's sensitivity to AMPH, a competitive DAT substrate. As illustrated in Table II-1, similar to our findings with DA, N-SERT/DAT exhibited a significant increased K_i for AMPH, compared to WT DAT, indicating loss of apparent substrate affinity, whereas DAT/C-SERT showed no change in AMPH sensitivity compared to WT. Moreover, SERT/DAT/SERT exhibited a significant decrease in K_i for AMPH ($52.1 \text{ nM} \pm 11.4 \text{ nM}$, $n = 11$, $p < 0.05$), indicating an increased apparent affinity.

Given that DAT N- and C-termini differentially impacted apparent substrate affinity, we next extended our analyses to test whether these domains also

contribute to competitive inhibitor affinities. We first focused on cocaine and other tropane-derived congeners. Despite the impact that N-terminal substitution imposed upon apparent substrate affinity, none of the DAT core chimeras exhibited a significant difference in K_i values for cocaine, as compared to WT DAT (Table II-1). In contrast, similar to what we observed for DAT substrates, N-SERT/DAT exhibited significantly lower apparent affinity than WT DAT for the high-affinity DAT inhibitor β -CFT (WIN35,428; $n = 4$). Moreover, substituting the SERT C-terminus had no effect on apparent β -CFT affinity, and substituting both the DAT N- and C- terminus rescued the loss in apparent affinity observed with the N-terminal substitution (Table II-1). We further tested whether the apparent affinity of another high affinity tropane, β -CIT (RTI-55), was similarly impacted by the DAT N-terminal substitution. Interestingly, despite the structural similarity between β -CIT and β -CFT, N-SERT/DAT exhibited no difference in apparent affinity for β -CIT as compared to WT DAT (Table I, Student's two-tailed t-test, $p = 0.33$, $n = 3-7$). Cocaine is a relatively low affinity inhibitor with equimolar potency across monoamine transporters, whereas both β -CFT and β -CIT are non-selective, high affinity inhibitors for both DAT and SERT. Therefore, we next tested whether methylphenidate (MPH) and GBR12909, high affinity inhibitors with selectivity for DAT over SERT, were similarly affected by DAT N- and C-terminal substitutions. Similar to what we observed with DAT substrates, N-SERT/DAT exhibited significantly decreased sensitivity to MPH, whereas MPH apparent affinities for DAT/C-SERT were not significantly different from WT DAT

($p = 0.99$) and SERT/DAT/SERT rescued the loss in sensitivity observed for N-SERT/DAT (Table II-1). In contrast, substituting DAT N- and C-termini with SERT termini had no effect on DAT core chimera sensitivity to the high affinity DAT inhibitor, GBR12909 ($p = 0.37$).

We next tested whether reciprocal DAT substitutions onto the SERT core would similarly impact apparent substrate and inhibitor affinities. WT SERT and SERT core chimeras exhibited robust, saturable 5HT uptake when expressed in SK-N-MC cells (Fig. II-3A) and no significant differences in V_{\max} values were observed among the chimeras as compared to WT SERT, although N-DAT/SERT exhibited a trend towards decreased 5HT transport V_{\max} ($p = 0.07$).

Assessment of 5HT K_m values revealed no differences between WT SERT and any of the SERT core chimeras (Table II-2), suggesting that, unlike DAT, the SERT N-terminus does not contribute to apparent substrate affinity. To test whether this is specific to 5HT, or applies to other SERT substrates, we measured the apparent affinity for fenfluramine, a SERT-selective amphetamine derivative. As seen in Table II-2, substituting the DAT N-, C-, or both termini had no effect on the SERT core chimera fenfluramine K_i values, as compared to WT SERT. We similarly tested a panel of chemically diverse SERT inhibitors, including the tropane compounds cocaine and β -CIT, the SSRI escitalopram, and

the tricyclic antidepressant, imipramine. Replacing the DAT N- and/or C-termini onto SERT had no effect on the potency of these compounds, as compared to WT SERT (Table II-2), suggesting that while the DAT N-terminus contributes to apparent substrate affinity and potency for a subclass of inhibitors, SERT can tolerate N-terminal substitutions without compromising either apparent substrate or inhibitor affinities.

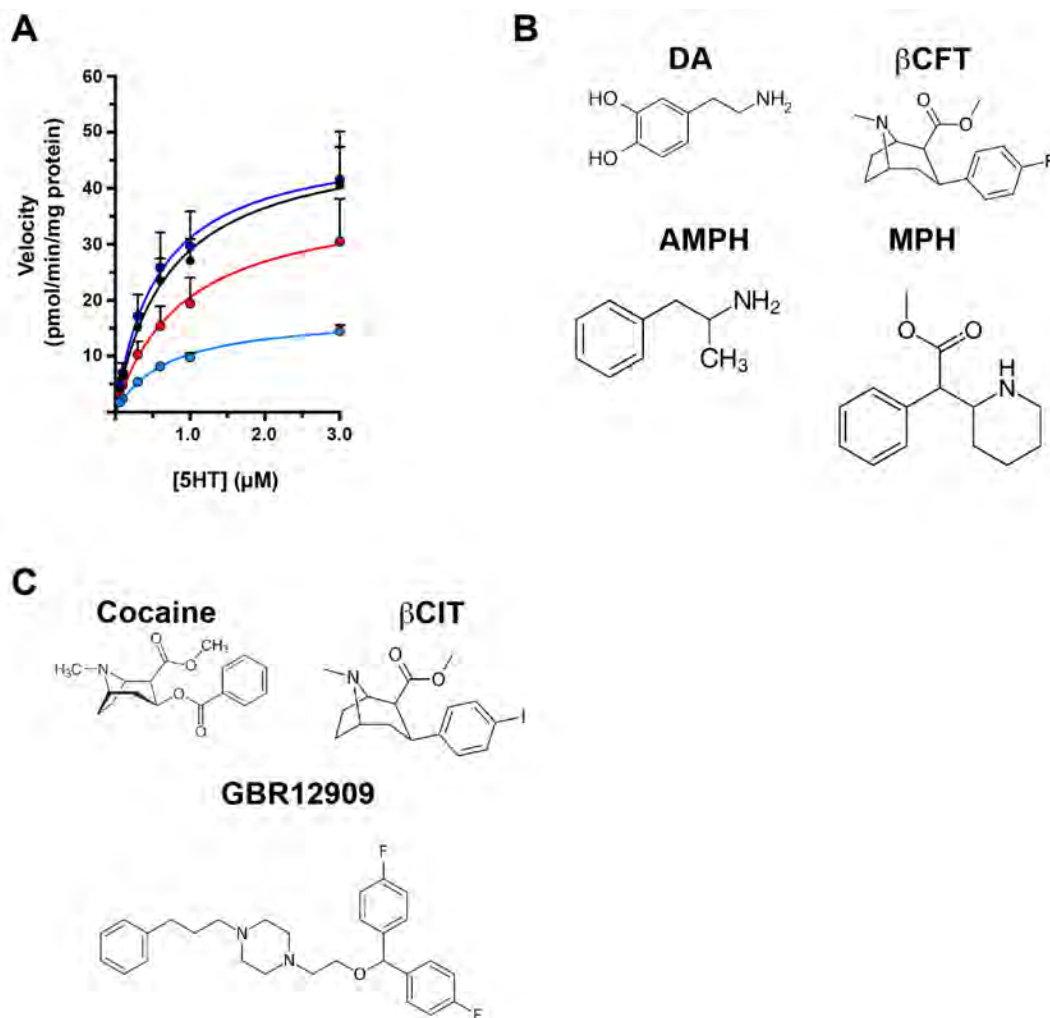


Figure II-3. A. SERT-core chimera uptake kinetics. [^3H]5HT uptake assays. Saturation uptake kinetics were measured as described in *Experimental Procedures*, in SK-N-MC cells stably expressing either WT SERT (black), N-SERT/DAT (cyan), SERT/C-DAT (blue), and DAT/SERT/DAT (red). Average data are presented \pm S.E.M., $n = 5-6$. **B-C. DAT substrate and inhibitor chemical structures.** Chemical structure of compounds whose affinities were (B) or were not (C) affected by DAT N-terminal substitution with the SERT N-terminus.

Table II-2. SERT-core chimeras
 $[^3\text{H}]\text{5HT}$ uptake kinetics and inhibitor K_i values
 Values are in nM unless otherwise indicated

	WT SERT	N-DAT/SERT	SERT/C-DAT	DAT/SERT/DAT
$[^3\text{H}]\text{5HT}$ uptake kinetics				
V_{max} (pmol/min/mg protein)	50.2 \pm 8.6	17.7 \pm 1.5	49.7 \pm 9.7	39.3 \pm 10.1
K_m (μM)	0.75 \pm 0.1	0.71 \pm 0.1	0.62 \pm 0.1	0.89 \pm 0.1
K_i values				
Fenfluramine (μM)	1.5 \pm 0.4	1.8 \pm 0.6	1.4 \pm 0.3	1.6 \pm 0.4
Cocaine	153.3 \pm 16.2	160.0 \pm 41.6	118.6 \pm 25.7	161.7 \pm 34.3
β -CIT	0.28 \pm 0.0	0.35 \pm 0.05	0.23 \pm 0.1	0.24 \pm 0.01
Escitalopram	1.4 \pm 0.4	1.3 \pm 0.9	1.2 \pm 0.6	1.2 \pm 0.5
Imipramine	6.4 \pm 0.7	8.7 \pm 2.3	9.1 \pm 2.6	9.4 \pm 1.1

n= 5-6 for $[^3\text{H}]\text{5HT}$ kinetics and 4-7 for K_i value

II.E DISCUSSION

Monoamine transporter N- and C-termini encode multiple motifs critical for biosynthesis (Sitte et al., 2004; Farhan et al., 2007; Sucic et al., 2011; Sucic et al., 2013; Koban et al., 2015), surface regulation (Holton et al., 2005; Boudanova et al., 2008b; Sorkina et al., 2009), and AMPH-stimulated substrate efflux (Khoshbouei et al., 2004; Guptaroy et al., 2009; Sitte and Freissmuth, 2010; Sucic et al., 2010; Rickhag et al., 2013a). Given that coding variants in both the DAT and SERT terminal domains have been independently reported in patients with neuropsychiatric disorders (Prasad et al., 2005; Prasad et al., 2009; Sakrikar et al., 2012; Cartier et al., 2015), understanding how these domains may also influence substrate and inhibitor binding is critical. DAT N-terminal residues interact with intracellular domains that contribute to an intracellular transport gating mechanism, which modulates substrate translocation (Yamashita et al., 2005; Guptaroy et al., 2009; Sucic et al., 2010; Guptaroy et al., 2011; Khelashvili et al., 2015b). In particular, R60 (Kniazeff et al., 2008) T62 (Guptaroy et al., 2009), and R51 (Khelashvili et al., 2015b) interact with intracellular loops to help stabilize the DAT outward (R60, T62) and inward (R51) facing conformations. R60 and T62 are maintained in our chimeras, as they are conserved between DAT and SERT. However, R51 is not conserved between DAT and SERT, and observed differences in substrate/inhibitor apparent affinities may be due to loss of R51 influence on gating. This putative change in gating may be translated to the substrate binding pocket, and thereby result in decreased apparent affinities

for certain DAT substrates and inhibitors. An R51W coding variant identified in autism spectrum patients was recently reported (Cartier et al., 2015), which results in reduced DA efflux capacity, consistent with a role for R51 in transitioning between inward and outward facing conformations. Such results further raise the possibility that altered N-terminal mobility may have broader functional implications in neuropsychiatric disorders.

Our results using DAT/SERT chimeric proteins revealed that the DAT N-terminus influences the apparent affinity of substrates and a subset of DAT inhibitors, whereas SERT function and inhibition was insensitive to N- and C-terminal substitutions. N-SERT/DAT exhibited reduced apparent affinity for DAT substrates DA and AMPH, as well as the inhibitors MPH and β -CFT. Interestingly, the N-terminus did not contribute to apparent affinities for cocaine, GBR12909, or β -CIT. In contrast, a recent study using DAT/NET chimeras, found that replacing the DAT N-terminus with that of NET enhanced the DAT affinity for DA, whereas replacing the NET N-terminus with that of DAT reduced NET affinity for DA (Vuorenpaa et al., 2016). Consistent with our results, they also observed that C-terminal exchanges had no effect on DA affinity. Further, a study from Vaughan and colleagues found that N-terminal point mutations (S4A, C6A, S7A, S7D) in rat DAT exhibited reduced β -CFT binding (Moritz et al., 2013), further supporting that the DAT N-terminus contributes to the affinity of select inhibitors.

Taken together with our findings, these data are consistent with a model in which the DAT N-terminus may influence the stability of the ligand-bound conformation. Interestingly, we observed that this occurred over a broad range of structurally diverse compounds (see chemical structures, Figs. II-3B, C). However, although cocaine, β -CFT and β -CIT are all tropane-derivatives, only β -CFT apparent affinity was affected by the DAT N-terminal substitution. A previous report identified a DAT point mutation (D345N) that is insensitive to β -CFT, but retains sensitivity to cocaine inhibition (Chen et al., 2001), suggesting that binding of tropanes derivatives within DAT is not identical. Moreover, a dDAT crystal structure encoding point mutations that mimic hDAT displayed enhanced cocaine and β -CFT, but not β -CIT binding (Wang et al., 2015b), consistent with a model in which these compounds have differential binding properties for DAT. Cocaine and β -CIT are larger molecules than β -CFT. Cocaine's ester linkage (absent in β -CIT and β -CFT) increases its size compared to β -CFT, while the substitution of the larger halide, iodide, in β -CIT increases its size compared to β -CFT. This raises the possibility that the molecular size of these compounds exerts a large influence on their ability to bind within the S1 binding pocket. Consistent with this premise, DA, AMPH, and MPH, which are all small DA-related compounds, were all sensitive to the N-terminal substitution, whereas GBR12909, which is a large, extended piperazine derivative, was not impacted by the DAT N-terminal substitution.

The potential explanation for the observed different shifts in inhibitory potency is an energy change for reaching the inhibitor-transporter complex state, which does not affect every inhibitor in the same way. Thus, the intracellular termini substitution may have caused structural changes in the outer vestibule or changed the free energy hypersurface of the translocation process. Most inhibitors bind competitively to DAT and prevent the transition from outward-facing to inward-facing. It is conceivable that larger compounds interfere already in the initial part of the transition, while smaller inhibitors block the transporter movement along the transition path. The geometry of the outward facing conformation is most likely unchanged, as the K_i of the largest compounds did not change. More intensive structural and/or computational interrogations will be necessary to discern among these possibilities.

How does the N-terminus influence the coordination of substrates and inhibitors in the DAT binding pocket? DAT's adaptation to molecule size within its pocket was demonstrated by Gouaux and colleagues (Wang et al., 2015b) using the same dDAT crystal structures bound to substrates and inhibitors. In their study, they posit that the small substrates sit precisely within the pocket in appropriate position. The larger inhibitors, like tropanes and antidepressants, lock DAT in an outward facing conformation to exert their effects. A notable difference between their findings and ours is that, as stated previously, they do not find any

difference between inhibitor binding sites for cocaine and β -CFT; however, their study focused on dDAT whereas ours investigated hDAT. Although the binding sites of hDAT and dDAT share approximately 80% identity, dDAT exhibits ten-fold lower affinity for cocaine than hDAT (Porzgen et al., 2001). Another potential difference between our findings and the previous results is that although their constructs bind DA and inhibitors, they lack DA uptake function. The dDAT crystal structures omit much of EL2 and the N-terminus. Complete N terminal truncation (residues 1-66) results in a loss of DA uptake (Sorkina et al., 2009), truncation of residues 1-60 results in marked loss of transporter uptake velocity (Torres et al., 2003b), and a previous DAT truncation mutant lacking residues 1-59 exhibits some measurable uptake, although neither the kinetic constants nor expression levels were reported for Δ 1-59 DAT mutant (Gu et al., 2001). Work from our laboratory likewise suggests that truncations within the N-terminus significantly impair transporter expression and function (data not shown). Our current study has the benefit of using N-and C-terminal substitutions on an otherwise complete WT background, such that DA uptake could be assessed in robustly expressing transporters.

Another factor that may contribute to role of the DAT N-terminus in substrate/inhibitor affinities is the interaction of the DAT N-terminus with PIP₂ and IL4 (Hamilton et al., 2014; Khelashvili et al., 2015b). A recent molecular dynamics study examining the hDAT N-terminus on the dDAT background

reported a PIP₂-mediated interaction with hDAT N-terminal residues (K3, K5, and R51) with IL4 (Khelashvili et al., 2015b). This interaction appears to break salt bridges necessary for the conserved intracellular gate. These findings contrast with simulation studies examining hSERT, where the N-terminus moves further away from intracellular domains following transition to the inward-open state (Fenollar-Ferrer et al., 2014). It should be noted that PIP₂ binds to SERT at its intracellular loops (Buchmayer et al., 2013), but whether PIP₂ affects SERT N-terminal mobility remains untested. We did not detect differences in substrate/inhibitor apparent affinities among the SERT-core chimeras. The reported differential trajectories for DAT and SERT N-terminal domains during transition to the inward-open state may result in differential effects on substrate/inhibitor binding sites. These opposing trajectories may explain why we see differences in effects between DAT-core and SERT-core chimeras.

In summary, we have identified a putative role for the DAT N-terminus in influencing the substrate/inhibitor binding site, and a possible synergistic role for the DAT N- and C-termini to coordinate accessibility to the binding site. These findings will provide critical information to future structural studies that incorporate DAT intracellular domains in structural analyses.

Preface to Chapter III

**Dopamine transporter amino- and carboxy-termini are synergistically
required for Ack1-dependent endocytosis**

Author contributions

Carolyn Sweeney and Haley Melikian designed experiments

Carolyn Sweeney and Brian Alejandro performed experiments

Carolyn Sweeney, Brian Alejandro, and Haley Melikian analyzed data

Chapter III

Dopamine transporter amino- and carboxy-termini are synergistically required for Ack1-dependent endocytosis

Sweeney CG, Alejandro B, and Melikian HE

III.A Introduction

DAT's regulation over synaptic DA clearance is directly related to DAT availability at the plasma membrane, as evidenced by DAT genetic deletion with *DAT*^{-/-} mice (Giros et al., 1996; Gainetdinov et al., 1998) and DAT pharmacological blockade with numerous drugs, including the psychostimulants cocaine and AMPH (Kristensen et al., 2011; Vaughan and Foster, 2013; German et al., 2015). DAT function and surface expression are acutely regulated by PKC. PKC activation results in rapid DAT internalization coupled with reduced recycling rates (Daniels and Amara, 1999; Melikian and Buckley, 1999). Multiple reports from our lab and others independently implicate domains within both DAT intracellular N- and C-termini as required for constitutive and PKC-mediated DAT endocytosis (Holton et al., 2005; Boudanova et al., 2008b; Sorkina et al., 2009). Our laboratory reported that a C-terminal motif, FREKLAYAIA (587-596), is both necessary and sufficient for PKC-stimulated DAT endocytosis (Holton et al., 2005; Boudanova et al., 2008b). Alanine substitution of the FREK sequence, (4A mutant),

demonstrated enhanced basal internalization rates like those seen following PKC activation (Boudanova et al., 2008b). This study posed the idea that a negative regulatory mechanism, or “endocytic brake”, exists to stabilize DAT surface expression. Following PKC activation, the brake is released, and the transporter internalizes rapidly. The Sorkin laboratory reported that DAT’s N-terminal domain (residues 1-65) is required for negatively regulating DAT surface expression, and that the juxtamembrane residues (60-65) are most critical for maintaining DAT surface expression (Sorkina et al., 2009). Taken together, these studies demonstrate that both N- and C-termini are required for negatively regulating DAT surface expression. It remains unknown whether independent endocytic mechanisms exist at both termini or whether DAT uses one synergistic endocytic mechanism that requires both domains. To test the hypothesis that DAT N- and C-termini are synergistically required for regulated DAT endocytosis, I used DAT/SERT chimeras in which I replaced DAT N-, C-, or both termini with SERT terminal domains (Sweeney et al., 2017). These chimeras were previously used to demonstrate that DAT’s N- and C-termini synergistically mediate substrate and certain inhibitor affinities. DAT and SERT are homologous transporters, yet they share little sequence identity at their terminal domains. DAT and SERT N- and C-termini contain multiple phosphorylation sites, and serve as domains for protein-protein interactions (Eriksen et al., 2010; Zhong et al., 2012; Bermingham and Blakely, 2016). Both transporters undergo PKC-stimulated endocytosis, but only DAT requires Ack1 inhibition, downstream of PKC activation, for this process

(Wu et al., 2015). Additionally, our laboratory reported that the small, neuronal, Ras-like GTPase, Rin directly binds to DAT but not SERT, and it is required for regulated DAT endocytosis (Navaroli et al., 2011). Here, we tested internalization rates for each DAT-core chimera following PKC activation and Ack1 inhibition to test whether the N- and C-termini synergistically mediate PKC-stimulated endocytosis. Our findings demonstrate that both domains are collectively required for enhanced endocytosis following Ack1 inhibition. Further, our data suggest that DAT's N-terminus mediates a functional step between PKC-activation and Ack1-inactivation to drive regulated endocytosis. Additionally, we show that Rin acts downstream of PKC activation to mediate Ack1 dephosphorylation, however neither DAT's N- nor C-terminus is required for the DAT/Rin interaction. Further, we propose a novel strategy to identify proteins associated with DAT's N- and C-termini.

III.B Materials and Methods

cDNA constructs: DAT/SERT chimeras were generated as previously described (Sweeney et al., 2017). Bungarotoxin-binding site (BBS) DAT-core chimeras were generated as described in Wu et al. (Wu et al., 2017), with the following amino acid sequence inserted: GSSGSSGWRYYESSLEPYPDGSSGSSG. Underlined sequence refers to the BBS sequence, as flanked by linker sequence. All chimeric BBS-constructs were confirmed by Sanger sequencing (Genewiz).

Cell culture, transfection, and generation of pooled stable cell lines: Cells were maintained in either MEM (SK-N-MC cells) or DMEM (HEK293T and SK-N-DZ cells) supplemented with 10% fetal bovine calf serum, 100 units/mL penicillin/streptomycin, 2 mM L-glutamine, 1X non-essential amino acids (SK-N-DZ cells), 37°C, 5% CO₂. Cells were transfected with WT, chimera, or BBS-DAT cDNAs using Lipofectamine 2000 according to manufacturer's instructions with the following modifications: Cells were seeded into 6 well plates at a density of 1×10^6 (SK-N-MC) or 5×10^5 (HEK293T) one day prior to transfection, and were transfected with 3 µg (SK-N-MC) or 2 µg (HEK293T) plasmid DNA/well using a lipid:DNA ratio of 2:1. To generate stable cell lines, 48 hours following transfection, cells were selected using 0.5 mg/mL G418. Stably transfected cells were pooled and cell lines were maintained under selective pressure using 0.2 mg/mL G418 (SK-N-MC).

Uptake assays: [³H]DA uptake: All uptake assays were conducted as previously described in Chapter II (Sweeney et al., 2017).

Internalization assay: Relative internalization rates were measured by reversible biotinylation as previously described (Loder and Melikian, 2003; Gabriel et al., 2013; Wu et al., 2015). Briefly, cells were rapidly chilled by washing in ice-cold PBS, pH 7.4, supplemented with 1.0 mM MgCl₂, 0.1 mM CaCl₂ (PBS²⁺), and incubated with 1.0 mg/ml sulfo-NHS-SS-biotin in ice-cold PBS²⁺, twice, for 15 min

at 4°C. Residual reactive NHS groups were quenched by repeated washing and incubating twice in PBS²⁺/100 mM glycine, 15 min, 4°C. Cells were washed four times with warmed PBS²⁺ supplemented with 0.18% glucose and 0.2% BSA \pm 1 μ M PMA or 20 μ M AIM-100, and incubated 10min, 37°C to allow for internalization. Internalization was stopped by immediately moving the cells to ice and washing three times with ice cold NT buffer (150 mM NaCl, 20mM Tris, pH 8.6, 1.0mM EDTA, pH 8.0, 0.2% BSA). Remaining surface biotin was stripped from internalization samples and strip controls with two incubations of 100mM TCEP in NT buffer, 25min, 4°C. Cells were washed four times with PBS²⁺ and then lysed, and biotinylated proteins were isolated with streptavidin agarose beads and analyzed by SDS-PAGE followed by immunoblotting with anti-DAT (WT DAT and DAT/C-SERT) or anti-SERT (N-SERT/DAT and SERT/DAT/SERT) antibodies.

BBS-DAT/chimera pulldowns: HEK cells transiently expressed BBS-DAT/chimera and HA-Rin at a ratio of 1:4. Cells were washed thrice with ice cold PBS and labeled with 120 μ M biotinylated α -bungarotoxin (BTX-b, Thermo Fisher), 2 hours, 4°C. Bungarotoxin was removed from the cells and disposed of in the satellite accumulation area. Next, cells were washed thrice with ice cold PBS to remove any excess BTX-b. Cells were lysed with cold co-immunoprecipitation lysis buffer, also known as “Magic Lysis Buffer”, (50 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10% glycerol, and 1 mM EDTA) containing protease inhibitors

(1.0 mM PMSF and 1.0 g/ml each leupeptin, aprotinin, and pepstatin) and Phosphatase Inhibitor Mixture V (EMD Millipore). Following a 30min lysis, lysates were transferred to centrifuge tubes, and spun down at max speed for 10min in the cold room. Lysate protein concentrations were assessed with BCA assay (ThermoFisher), and equal amounts of BTX-b labeled protein were rotated overnight with streptavidin M280 dynabeads at 4°C. The next day, beads were washed, gently, with cold Magic Lysis Buffer. Pulled-down proteins were resolved by SDS-PAGE, and immunoblotted with antibodies for DAT, SERT, and HA.

RT-qPCR: Total RNA was isolated using RNAqueous kit (Thermo) per manufacturers protocol. Following DNAase treatment (30 min, 37°C), cDNA was generated (Retroscript; Thermo) and mRNA levels were quantified by RT-qPCR, using an Applied Biosystems 7500 Real-Time System and TaqMan assays (Applied Biosystems). Each mRNA sample was analyzed in triplicate, and expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, using $2^{-\Delta C_t}$ method (Schmittgen and Livak, 2008).

Materials: Rat anti-DAT (MAB369) antibody was from Millipore, mouse anti-SERT antibody was from MAb technologies, and Rat anti-HA (3F10) was from Roche. Horseradish peroxidase (HRP)-conjugated anti-rat secondary and mouse anti-actin (SPM161) antibodies were from Santa Cruz and HRP-conjugated anti-

mouse antibody was from Jackson ImmunoResearch Laboratories (115-035-003). Biotinylated bungarotoxin and M280 dynabeads were from Thermo Scientific. PMA and AIM-100 were from Tocris. All other chemicals and reagents were from Sigma-Aldrich and Thermo Fisher Scientific and were of the highest grade available.

III.C Results

To test whether DAT's N- and C-termini synergistically mediate PKC-stimulated endocytosis, we measured endocytic rates in WT DAT and DAT-core chimeras (Sweeney et al., 2017) following PKC activation, using PMA, or downstream Ack1 inactivation using the Ack1-specific inhibitor AIM-100 (Fig. III-1). The DAT-core chimeras have DAT's N-, C-, or both termini replaced with SERT terminal domains. SERT is a homologous transporter, but shares little sequence identity with DAT at the intracellular terminal domains. We measured a significant increase in WT DAT internalization rates following PKC activation and Ack1 inhibition (Fig. III-1A), consistent with previous studies (Wu et al., 2015). DAT/C-SERT, where DAT's C-terminus is replaced with SERT's, displayed a significant increase in endocytic rates following both PKC activation and Ack1 inhibition, similar to WT DAT (Fig. III-1B). Surprisingly, substituting SERT's N-terminus onto DAT (N-SERT/DAT, Fig. III-1C) abolished PKC-stimulated DAT endocytosis, but maintained enhanced endocytic rates following Ack1 inhibition. Replacing both

intracellular termini with SERT's (SERT/DAT/SERT, Fig. III-1D) abolished enhanced endocytosis following both PKC activation and Ack1 inhibition. Taken together, these data suggest that DAT's N-terminus is required for PKC-stimulated endocytosis and that both N- and C-termini synergistically coordinate enhanced endocytosis following Ack1 inhibition. This interpretation initially seemed counterintuitive, given that previous reports from our lab and others demonstrate that PKC activation leads to Ack1 dephosphorylation (Linseman et al., 2001; Wu et al., 2015), and that Ack1 dephosphorylation is required for regulated DAT endocytosis (Wu et al., 2015). These findings raised the hypothesis that there is a molecule, which associates with DAT's N-terminus and acts downstream of PKC activation, to mediate Ack1 inactivation.

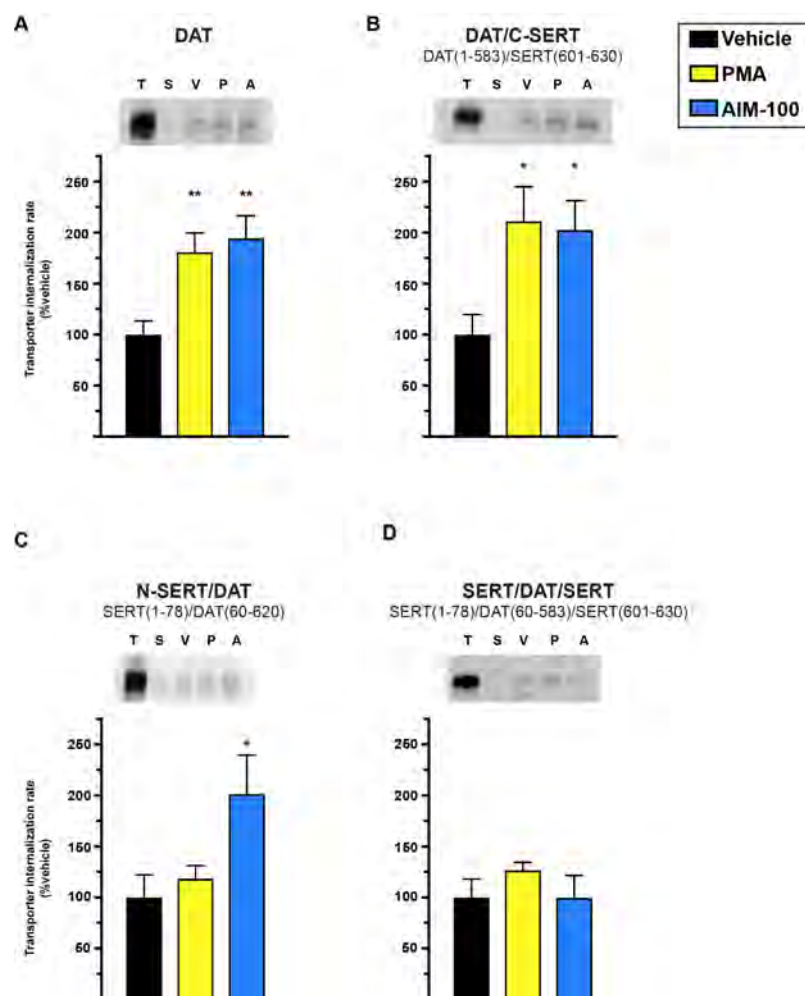


Figure III-1. DAT N-terminus is required for PKC-stimulated internalization and both N- and C-termini are required for the Ack1 endocytic brake. A-D. Internalization assays. Stable SK-N-MC cells expressing (A)WT DAT, (B)DAT/C-SERT, (C)N-SERT/DAT, or (D)SERT/DAT/SERT. Relative internalization rates were measured by reversible biotinylation over 10 minutes \pm 10 μ M PMA or \pm 20 μ M AIM-100, 37°C as described in *III.B Materials and Methods*. Top, representative immunoblot showing total surface transporter at time 0 (T), strip control (S), internalized transporter under vehicle treated (V), PMA treated (P), and AIM-100 treated (A) conditions. Bottom, averaged internalization rates \pm S.E.M., normalized to transporter's own vehicle treatment. **p<0.01, *p<0.05, significantly different from vehicle treated control, one way ANOVA, Dunnett's multiple comparisons test, n=7-13.

Our laboratory previously reported that Rin GTPase is required for PKC stimulated DAT endocytosis (Navaroli et al., 2011) and the Hoshino laboratory reported that PKC activates Rin (Hoshino and Nakamura, 2002), posing Rin as a possible candidate for promoting Ack1 inactivation downstream of PKC activation. To test this hypothesis, we overexpressed empty vector, HA-Rin, constitutively active HA-Rin(Q78L), and dominant negative HA-Rin(S34N) in HEK293T cells. We treated them $\pm 10\mu\text{M}$ PMA, 37°C, 30 minutes, and assayed Ack1 dephosphorylation (Fig. III-2). Dominant negative Rin significantly reduced PKC-stimulated Ack1 dephosphorylation compared to vector controls, indicating that Rin activity is required downstream of PKC to inactivate Ack1.

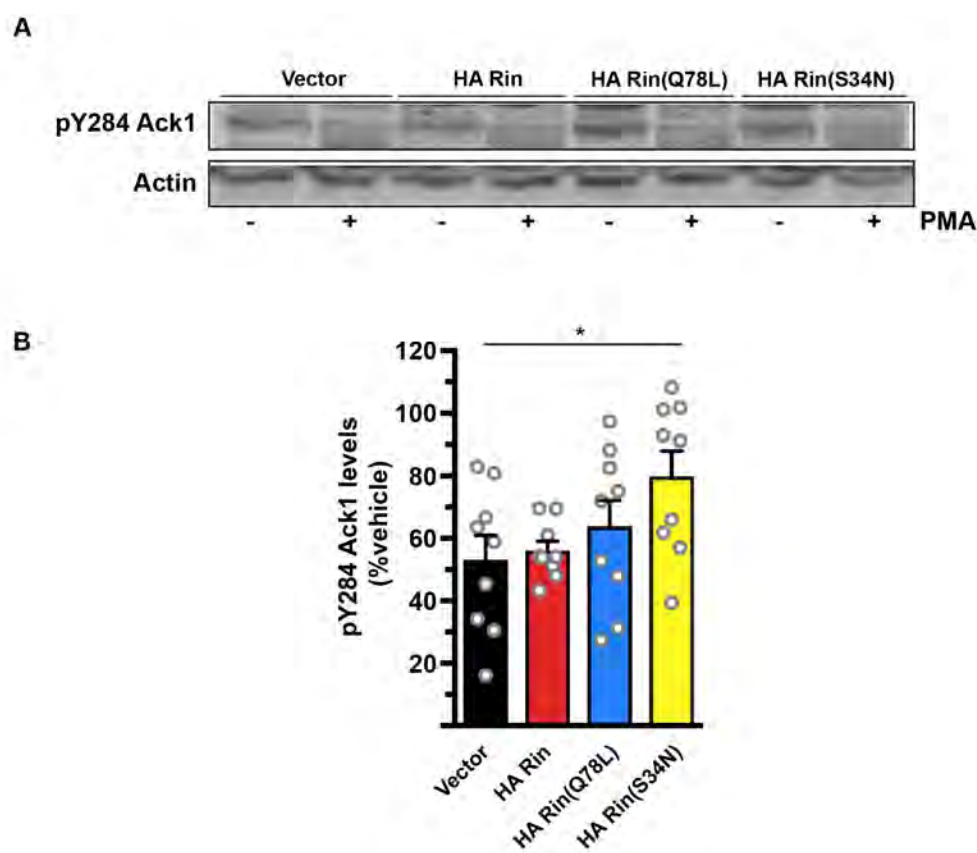


Figure III-2. Rin mediates Ack1 inactivation downstream of PKC activation. *Ack1 inactivation.* HEK293T cells were transiently transfected with Vector, HA Rin, HA Q78L Rin, or HA S34N Rin for 48 hours. Cells were treated \pm 10 μ M PMA, 37°C, 30 minutes. **(A)** Representative blot. **(B)** Average pAck1 expression relative to actin \pm S.E.M., normalized to own vehicle treatment. *Significantly different from vector control, $p < 0.05$, one way ANOVA, Dunnett's multiple comparisons test, $n = 9$.

Given our findings that DAT's N-terminus is required for PKC-stimulated endocytosis, and that Rin acts downstream of PKC to mediate Ack1 dephosphorylation, we hypothesized that Rin associates with DAT's N-terminus. Previous studies investigating DAT binding proteins rely upon co-IPs using large antibodies targeted against DAT's N- and C-terminus (Table I-1). These preparations isolate total cellular DAT populations, rather than specifically the surface population. To pulldown surface DAT protein, while keeping intracellular protein-protein interactions intact, we engineered WT DAT and DAT-core chimeras with a bungarotoxin binding site (BBS) in the second extracellular loop (EL2) (Fig. III-3A). The BBS-site was placed in EL2 because insertions into this domain do not disrupt DAT function and trafficking (Sorkina et al., 2006; Navaroli and Melikian, 2010; Wu et al., 2017). BBS-DAT displays functional [³H]DA uptake, and downregulates in response to PKC activation (Fig. III-3B), and BBS-DAT binds to Rin (Fig III-3C,D). Next, we tested whether DAT's N-terminus is required for the DAT-Rin interaction. Each BBS-DAT-core chimera expressed and came down following labeling with biotinylated bungarotoxin; albeit, BBS-SERT/DAT/SERT did not express well, consistent with our previous report (Sweeney et al., 2017). Interestingly, each BBS-DAT-core chimera pulled down HA-Rin, indicating that neither DAT's N- nor C-terminus is required for the DAT-Rin association, or that homologous domains within the SERT termini are sufficient to maintain the DAT-Rin interaction.

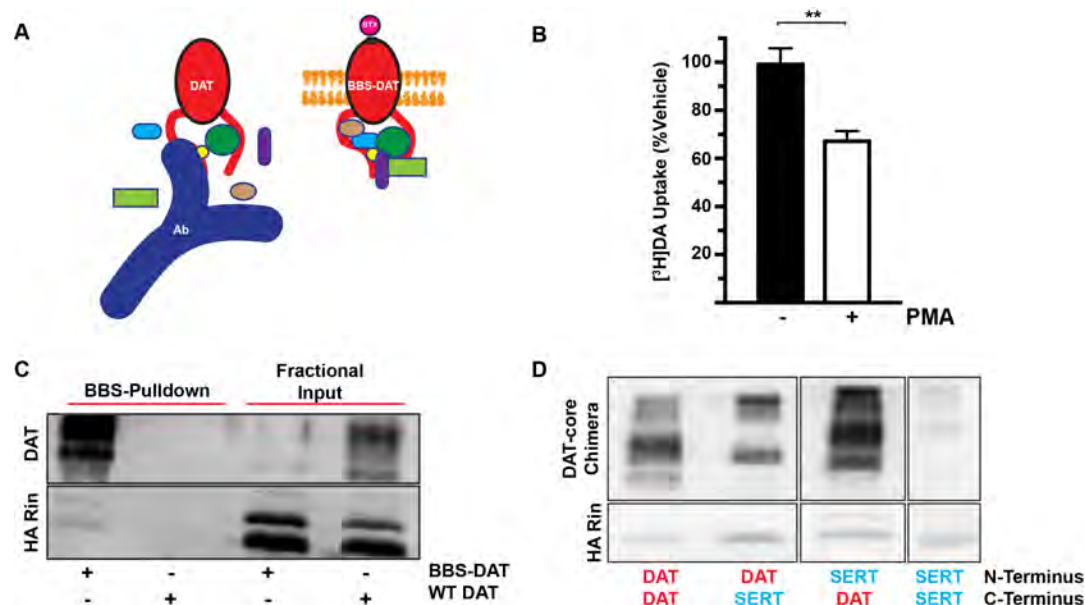


Figure III-3. Bungarotoxin binding site DAT (BBS-DAT) pulls down surface DAT and helps to maintain intracellular protein-protein interactions. (A) BBS-DAT schematic. Traditional DAT co-IP experiments use a large antibody targeted against DAT's intracellular N-terminus, which may disrupt intracellular interactions (left). BBS-DAT (right) uses an bungarotoxin binding site (BBS) inserted into DAT's second extracellular loop to exclusively pulls down surface DAT populations while keeping intracellular interactions intact. (B) BBS-DAT downregulates in response to PKC activation. $[^3\text{H}]\text{DA}$ uptake assay. SK-N-MC cells stably expressing BBS-DAT were treated $\pm 10\mu\text{M}$ PMA, 30 min, 37°C and uptake assay was performed as described in *III.B Materials and Methods*. PKC activation significantly reduced $[^3\text{H}]\text{DA}$ uptake compared to vehicle controls. **significantly different compared to vehicle control, $p < 0.01$, t-test, $n = 3$. (C) BBS-DAT specificity control. *BBS-DAT pulldown*. HEK293T cells transiently transfected with HA Rin and either BBS-DAT or WT DAT as described in *III.B Materials and Methods*. BTX labeling pulls down HA Rin with BBS-DAT but not WT DAT. (D) DAT's N- and C-termini are not required for DAT-Rin interaction. BBS-DAT core chimera pulldown with cells transiently transfected with respective BBS-chimera and HA Rin as described in *III.B Materials and Methods*. Each BBS-chimera pulled down HA Rin. Neither DAT N- nor C-terminus is required for the DAT/Rin interaction.

It should be noted that over the course of these studies, we discovered that SK-N-MC cells do not express endogenous Rin (Fig. III-4). While Navaroli et al., reported that every cell line they investigated contained Rin (*Rit2*) mRNA (Navaroli et al., 2011), this finding was in contrast with Zhang et al., who did not detect Rin mRNA in HEK293 or SK-N-MC cells. However, they did detect endogenous Rin mRNA expression in SK-N-DZ cells (Zhang et al., 2013). To test what cell lines contain endogenous Rin, we performed RT-qPCR using cDNAs isolated from human cell lines, HEK293T, SK-N-MC, and SK-N-DZ (Fig. III-4). We only detect Rin mRNA in SK-N-DZ cells, consistent with the findings of Zhang et al.

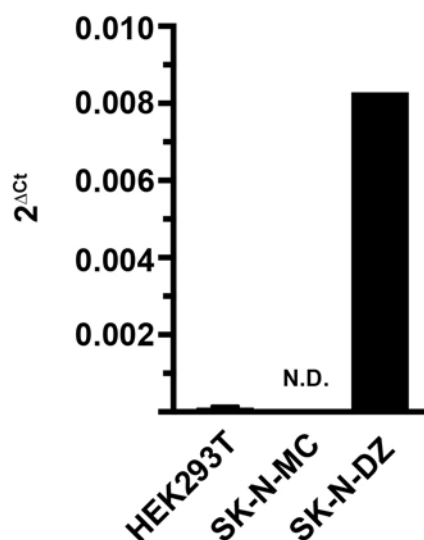


Figure III-4. SK-N-DZ but not HEK293 nor SK-N-MC cells express Rin. RT-qPCR from cDNA isolated from HEK293, SK-N-MC, and SK-N-DZ cells as stated in III.B. *Materials and Methods*. Relative Rin (Rit2) mRNA expression normalized to GAPDH levels and represented as $2^{-\Delta Ct}$. HEK293 and SK-N-MC cells do not express endogenous Rin, whereas, SK-N-DZ cells express measureable quantities of Rin.

III.D Discussion

Our laboratory reported that an “endocytic brake” negatively regulates DAT surface expression, and the DAT C-terminal domain, FREKLAYAIA (587-596), is both necessary and sufficient for PKC-stimulated endocytosis (Holton et al., 2005). Further, the FREK sequence (587-590) is required for the endocytic brake (Boudanova et al., 2008b). The Sorkin laboratory also reported a DAT endocytic negative regulatory mechanism, that requires juxtamembrane residues (60-65) at the N-terminus (Sorkina et al., 2009). These data suggest that DAT’s N- and C-termini each independently coordinate an endocytic brake or rather that both domains synergistically mediate the endocytic brake. In this chapter, I tested the hypothesis that both domains are synergistically required for regulating DAT surface expression.

Reports from our laboratory and others support that N- and C-terminal synergy is required for DAT regulatory mechanisms. AMPH-stimulated DA efflux requires coordination between DAT N- and C-termini. CaMKII (calcium/calmodulin-dependent protein kinase II) associates with the DAT C-terminus to mediate N-terminal phosphorylation (Khoshbouei et al., 2004; Fog et al., 2006a). Our laboratory recently reported that DAT N- and C-termini synergistically contribute substrate and select inhibitor affinities using the same DAT/SERT chimeras utilized in this chapter (this topic is discussed in depth in Chapter II) (Sweeney et al., 2017). Additionally, DAT is palmitoylated at its membrane-proximal C-

terminus (Foster and Vaughan, 2011; Moritz et al., 2015). Following PKC activation, palmitoylation decreases and N-terminal phosphorylation increases, and there is a reciprocal relationship between these two events. Taken together, these studies demonstrate that N- and C-terminal domains are synergistically required for AMPH-stimulated efflux, substrate affinity, and functional regulation; therefore, suggesting that other DAT functional mechanisms may require terminal synergy as well.

In this chapter, we demonstrate that DAT N- and C-termini synergistically coordinate regulated endocytosis. While N-SERT/DAT and DAT/C-SERT both showed enhanced endocytosis following Ack1 inhibition, swapping both DAT intracellular termini for SERT domains abolished enhanced endocytosis following PKC activation and Ack1 inactivation (Fig III-1D). This finding demonstrates that DAT uses both N- and C-termini for Ack1-dependent endocytosis.

WT SERT undergoes PKC-stimulated endocytosis (Qian et al., 1997; Samuvel et al., 2005), and its mechanism is distinct from DAT regulated endocytosis (Navaroli et al., 2011; Wu et al., 2015). When both SERT intracellular domains were swapped onto DAT, we abolished PKC-stimulated endocytosis. Although it is known that WT SERT undergoes PKC-stimulated endocytosis (Qian et al., 1997), having two “matching” SERT intracellular domains was not sufficient to drive PKC-stimulated endocytosis in a putatively SERT-dependent manner.

Rather, it appears that the SERT terminal domains may require intact SERT protein in order to drive PKC-stimulated internalization. Future studies using SERT-core chimeras may shed light as to the role of these domains in SERT endocytic mechanisms.

Interestingly, our N-SERT/DAT chimera did not internalize rapidly in response to PKC activation; however, it demonstrated significantly enhanced endocytic rates following Ack1 inhibition. We found this result surprising in light of reports from our group and others demonstrating that PKC activation inactivates Ack1, and Ack1 inactivation is required for PKC-stimulated DAT endocytosis (Linseman et al., 2001; Wu et al., 2015). How is the DAT N-terminus required for PKC-stimulated endocytosis but not enhanced endocytosis following the downstream Ack1 inactivation? We hypothesize that a molecule associated with the DAT N-terminus is required for PKC-stimulated Ack1 inactivation, and that it accelerates Ack1 dephosphorylation. We tested whether Rin, a protein which 1) associates with DAT but not SERT, 2) is activated by PKC, and 3) whose activity is required for PKC-stimulated DAT endocytosis, is required for Ack1 inactivation downstream of PKC. HEK293 cells overexpressing dominant negative Rin displayed significantly less Ack1 inactivation following PKC activation compared to vector controls. This result suggests that Rin activity is required downstream of PKC to inactivate Ack1.

Additionally, we found that neither the DAT N- nor C-terminus was required for Rin association with DAT. The most likely explanation for this finding is that DAT's intracellular loops are sufficient for the DAT/Rin interaction. However, Rin was initially discovered to be a DAT binding protein through a yeast 2-hybrid screen using the C-terminal sequence, FREKLAYAIA, as bait (Navaroli et al., 2011). Taken together, these data suggest that the DAT C-terminus is sufficient but not necessary for the DAT/Rin interaction. However, a limitation to the BBS-DAT pulldown is that it isolates DAT associated protein complexes rather than solely proteins directly interacting with DAT. Using this method, we cannot rule out the possibility that Rin, which binds to the plasma membranes via a polybasic domain (Heo et al., 2006), remains part of a DAT-associated complex even if potential direct interactions with N- or C-terminal domains is disrupted. Future FRET studies using CFP-tagged DAT-core chimeras will test this hypothesis. Further, additional studies, using the BBS-DAT chimeras will test additional candidates for N-terminally associated proteins required for Ack1 inactivation downstream of PKC activation. One such candidate is RACK1 (Receptor of activated protein kinase C 1). RACK1 associates with DAT's N-terminus (Lee et al., 2004; Franekova et al., 2008), and acts as an adapter protein to link PKC with its substrates (Adams et al., 2011). While RACK1 association with DAT was reported by two independent groups, the functional implications remain untested.

Our new BBS-DAT construct demonstrated that neither the DAT N- nor the C-terminus is required for the DAT/Rin interaction. However, in spite of this negative result, BBS-DAT will be a critical tool in identifying novel DAT binding partners. Previous studies testing DAT-associated proteins rely upon DAT co-immunoprecipitation using large antibodies targeted against the DAT N- or C-terminus. A limitation to this approach is that it requires bulky antibodies that can disrupt sensitive and dynamic protein-protein interactions. Additionally, these approaches investigate entire cellular DAT populations. Here, we are interested in proteins that interact with DAT specifically at the plasma membrane, and BBS-DAT will only pull down the surface DAT population. These studies are not the first to insert a bungarotoxin binding site in the DAT's EL2 (Hong and Amara, 2013). However, a notable difference between our construct and the Amara laboratory's construct is the use of our previously published linker sequence (Wu et al., 2015), which allows for epitope access at a wide range of temperatures. This access at 4°C is an important difference between our BBS-DAT and HA-EL2-DAT, which has the HA-tag only accessible at temperatures above 18°C (Sorkina et al., 2006).

Upon completion of our studies, we discovered that our SK-N-MC cells do not express endogenous Rin (Fig. III-4). While this finding suggests that Rin may not be in fact required for PKC-stimulated DAT internalization, we report that in intact DA nerve terminals, Rin is absolutely required for this process (Chapter IV,

Fig.IV-9). Therefore, we predict that another protein, perhaps the homologous GTPase Rit, compensates in the absence of Rin in certain heterologous expression systems; however, this remains untested. Future studies using heterologous expression systems to test DAT endocytic mechanisms should utilize cells that express endogenous Rin, such as the SK-N-DZ human neuroblastoma line.

In this chapter, we identified that DAT N- and C-termini synergistically mediate regulated endocytosis following Ack1 inactivation, Rin activity is required downstream of PKC to inactivate Ack1, and DAT's N-terminus mediates PKC inactivation of Ack1, but not through association with Rin. Further, we demonstrated use of a new BBS-DAT construct which can be used to test for future DAT interacting protein.

Preface to Chapter IV

**Conditional, inducible gene silencing in dopamine neurons
reveals a critical role for Rin GTPase in anxiety, presynaptic
dopaminergic function, and response to cocaine**

These data are unpublished and in preparation for manuscript submission

Author contributions:

Carolyn Sweeney, Gilles Martin, Andrew Tapper, and Haley Melikian designed experiments

Carolyn Sweeney, Patrick Kearney, Iris Rivera, and Gilles Martin performed experiments

Carolyn Sweeney, Patrick Kearney, Jenya Kolpakova, Gilles Martin, and Haley Melikian analyzed data

Jun Xie and Guangping Gao designed AAV-TRE-shRNA vector

University of Massachusetts Viral Vector Core packaged viral vectors for expression

Chapter IV

Conditional, inducible gene silencing in dopamine neurons reveals a critical role for Rin GTPase in anxiety, presynaptic dopaminergic function, and response to cocaine

Authors: Sweeney CG, Kearney PK, Rivera IV, Kolpakova J, Xie J, Guangping G, Tapper AR, Martin GE, and Melikian HE

IV.A Introduction

Dopamine (DA) is a critical modulatory neurotransmitter required for learning, motivation, movement, anxiety, and rewarding behaviors (Wise, 2004; Russo and Nestler, 2013). DA signaling dysfunction has profound clinical consequences, as evidenced by multiple neurological and neuropsychiatric disorders, including Parkinson's disease, ADHD, schizophrenia, and anxiety (Calhoon and Tye, 2015; Faraone et al., 2015; Grace, 2016; Poewe et al., 2017). The neuronal, Ras-like GTPase, Rin (Rit2) is highly enriched in DA neurons (Zhou et al., 2011); however, its role in DAergic signaling remains entirely unknown. GWAS studies implicate Rin as a risk allele for Parkinson's disease, autism spectrum disorder, essential tremor, schizophrenia, and bipolar disorder (Emamalizadeh et al., 2017). Rin is required for neurotrophic signaling and causes neurite outgrowth in PC6 cells (Shi et al., 2005b). Rin binds directly to the presynaptic, cocaine- and

AMPH-sensitive DA transporter (DAT), and Rin activity is required for regulated DAT endocytic trafficking in PC12 cells (Navaroli et al., 2011). Despite the putative linkage between Rin and DAergic activity, it is currently unknown whether Rin activity directly impacts DAergic physiology. To test the hypothesis that Rin expression in DA neurons is required for DAergic function, we used conditional, inducible Rin knockdown in DA neurons of adult mice, and assessed locomotor, anxiety, and cocaine-induced behavior.

IV.B Materials and Methods

Mice. $Pitx3^{IRES-tTA}/+$ were the generous gift of Dr. Huaibin Cai (National Institute on Aging), and were continually backcrossed to C57Bl/6 (Lin et al., 2012). Mice were maintained in 12hr light/dark cycle at constant temperature and humidity. Food and water was available ad libitum, and mice were maintained on either standard or doxycycline-supplemented chows (S3888, BioServ). Studies were conducted in accordance with UMASS Medical School IACUC Protocol A-1506 (H.E.M).

Stereotaxic viral delivery. Adult mice (minimum 5 weeks old) were anesthetized with an i.p. injection of 100mg/kg ketamine (Vedco Inc.) and 10mg/kg xylazine (Akorn Inc.). 20% mannitol (NeogenVet) was administered i.p. 15 minutes (minimum) prior to viral delivery, to increase viral spread (Burger et al., 2005).

Mice heads were shaved and placed in the stereotaxic frame (Stoelting Inc.). Heads were disinfected by washing three times with betadine followed by 70% ethanol. A small incision was cut to expose the skull, and a 30% H₂O₂ solution was used to clean the skull surface and visualize Bregma. 1 µl indicated viruses were administered bilaterally into the VTA (Bregma: anterior/posterior: -3.08mm, medial/lateral: ±0.5mm, dorsal/ventral: -4.7mm) at a rate of 0.2 µL/min over 5 minutes. Syringes were left in place for a minimum of 3 minutes post-infusion prior to removal. Mice were individually housed, for a minimum of six weeks before experiments were performed. Dox-treated mice were placed on dox-supplemented chow at minimum 4 days prior to stereotaxic injection and were maintained on +dox chow throughout recovery and experimental procedures.

Mouse Behavior. Open field test. Male and female mice were habituated to experimental room for a minimum of 30 minutes before conducting experiment. The room was kept in dim light, and white noise was used to maintain constant ambient sound. During test, a single mouse was placed in a 38.5x40cm box. Activity was recorded using a video camera for ten minutes. EthoVision software (Noldux) was used to determine total distance traveled and time spent in center versus periphery of the arena. Two-way ANOVA revealed no gender differences detected for either total distance (p=0.94) or time in center p=0.81, therefore data from both sexes were pooled. *Elevated Plus Maze (EPM).* Male and female mice were habituated to experimental room for a minimum of 30 minutes before

conducting the experiment. The room was kept in dim light throughout habituation and experimental procedures. The EPM apparatus consists of four arms- two open and two closed- 45cm above the floor. During the test, a single mouse was placed in the center of apparatus. The animal was allowed to explore open and closed arms of apparatus for 5 minutes. Time spent in open and closed arms and total number of entries into both sets of arms was recorded by MED-PC IV software (MED Associates, Inc.). Two-way ANOVA revealed no gender differences detected for time in open arms ($p=0.63$), therefore data from both sexes were pooled. *Cocaine induced locomotor activity.* Animals were placed in locomotor activity chamber (San Diego Instruments), and locomotor activity was recorded during a 45 minute habituation period. Next, each animal received a saline injection (10mL/kg), and locomotor activity was recorded for 90 minutes. This initial test served as a baseline for each mouse. Two days later, mice habituated to test chamber 45 mins, during which their locomotor activity was recorded. Next, each mouse received cocaine injection at either 15mg/kg or 30mg/kg, and we recorded their activity for 90 minutes.

Immunohistochemistry. Mice were transcardially perfused with ice-cold phosphate-buffered saline, pH 7.4 (PBS), followed by ice cold 4% paraformaldehyde/PBS (w/v). Brains were removed and dehydrated in 30% sucrose/PBS (w/v), 3-7 days, and 25 μ m sections were prepared using microtome. Tissue was blocked and permeabilized in IHC blocking solution (5%

normal goat serum, 1% H₂O₂, 0.1% Triton-X 100 in PBS), 1 hour, at RT. All antibodies were diluted in IHC blocking solution. Slices were incubated in indicated primary antibodies, 1 hr, RT, washed and incubated with the indicated secondary antibodies, 45 minutes, RT. Slices were washed, mounted on glass slides and allowed to air dry prior to mounting on glass coverslips using Prolong Gold Anti-fade with DAPI (Invitrogen). Slides dried overnight and widefield epifluorescent images were captured with a Zeiss Axiovert 200M scope using Retiga-R1 CCD camera (QImaging) and Slidebook 6 software (Intelligent Imaging Innovations).

Midbrain tissue isolation and RT-qPCR. Animals were sacrificed, brains were rapidly removed and either flash frozen on dry ice or sectioned (200µm) fresh on a Vibratome in ice cold cutting solution (2.5mM KCl, 1.25mM NaH₂PO₄, 20mM HEPES, 2mM thiourea, 5mM sodium ascorbate, 3mM sodium pyruvate, 92mM N-methyl-D-glucamine, 30mM NaHCO₃, 25mM D-glucose). 1mm diameter tissue punches were made in VTA/SNc or SNr, under magnification. For frozen tissue, 10µm coronal sections were made using cryostat (Leica Microsystems Inc.). A Veritas Microdissection System Model 704 (Arcturus Bioscience) was used to microdissect SNc/VTA- or SNr-enriched regions. Total RNA was isolated using RNeasy kit (Thermo) per manufacturers protocol. Following DNase treatment (30 min, 37°C), cDNA was generated (Retroscript; Thermo) and mRNA levels were quantified by RT-qPCR, using an Applied Biosystems 7500 Real-

Time System and TaqMan assays (Applied Biosystems). Each mRNA sample was analyzed in triplicate, and expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, using $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008).

Western blot. Protein was solubilized in RIPA (10mM Tris base pH 7.4, 150mM NaCl, 1mM EDTA, 0.1% (v/v) SDS, 1% (v/v) Triton-X 100, 1% (w/v) sodium deoxycholate) with protease inhibitors (1mM PMSF, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin, 1 μ g/mL aprotinin) for a minimum of 30 minutes at 4°C. Protein concentrations were determined by Pierce BCA protein assay. Indicated amounts of protein were incubated with sample buffer (1.0M Tris, pH 6.8, 5%SDS, 10% glycerol, 1% bromophenol blue, 50mM DTT) and rotated, 30 minutes at RT. Samples were analyzed by SDS-Page and immunoblot using indicated antibodies. Immunoreactive bands were quantified using Quantity One software (BioRad).

Cell transfection and cDNA. 5×10^5 HEK293T cells/well were seeded one night prior to transfection in 6 well plates. Cells were transfected with Lipofectamine 2000 (Thermo Fisher) per manufacturers protocols. Briefly, we used 4 μ g Lipofectamine/well at a ratio of 2:1 lipid to cDNA. For Rin knockdown experiments we used 0.66 μ g shRNA containing cDNA and 0.33 μ g RFP-mRin

reporter cDNA, and 1 µg rtTA plasmid. Mouse Rin in pGEX2T was a gift from Julian Downward (Addgene plasmid #55663)(Fritsch et al., 2013). It was PCR amplified and subcloned into TagRFP vector using HindIII and XbaI. Rin shRNAs and pGIPZ controls were obtained from Dharmacon. Mature antisense sequences for each shRNA are as follows: mRin837 (clone V3LMM_441839) TTATCTTCTTCCACAGGCT and mRin794 (clone V3LMM_441840) TCATAGGTGTGACGGACCT. *pscAAV-TRE-eGFP* and *pscAAV-TRE-miR33-shRNA-eGFP* plasmids. pTre3G promoter was isolated from pTre3G (Clontech) plasmid using EcoRI and Sall. The CB6 promoter in the pscAAV-CB6-eGFP was removed by MluI and BstXI digestion and replaced with the pTre3G promoter to generate pscAAV-TRE3g-eGFP plasmid. pscAAV-TRE3g-eGFP plasmid was digested with BglII and PstI as backbone, miR-33-shRNA was synthesized as insert to generate pscAAV-TRE3g-miR33-shRin-eGFP.

Slice preparation: Three-week-old male *Pitx3^{lRES-tTA/+}* mice or *Pitx3^{lRES-tTA/+} D1r.Td-tomato* mice were given bilateral VTA injections of either AAV9-TRE-eGFP or AAV9-TRE-shRin-eGFP and allowed 3-4 weeks to recover and for viral expression. Mice were deeply anesthetized with isoflurane, and killed by decapitation. To cut 200 µm (electrophysiology) or 300 µm (slice biotinylation)-thick coronal slices with a Vibroslicer (VT1200, Leica MicroInstruments; Germany), we rapidly removed and transferred the brain in a cold (~ +0.5°C) oxygenated (95% O₂ and 5% CO₂) cutting solution of the following composition:

92 mM NMDG, 2.5 mM KCl, 1.25 mM NaH_2PO_4 , 30 mM NaHCO_3 , 20 mM HEPES, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 0.5 mM $\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$, and 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Then, slices were immediately transferred in an incubation chamber containing aCSF (in mM): 119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4 , 24 mM NaHCO_3 , 12.5 mM glucose, 2 mM $\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$, and 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Electrophysiology: Following a minimum recovery time of 1 hr at room temperature, we transferred slices to a recording chamber where they were submerged and perfused with a carbogenated aCSF, at a constant rate of 1–2 mL/min. We visualized nucleus accumbens medium spiny neurons in infrared differential interference contrast video microscopy using a fully motorized microscope (Scientifica, Uckfield, UK) mounted with Olympus objectives (10x and 60x; Olympus Microscopy, Japan). Slice biotinylation: Striatal slices were collected and hemisected along the midline before recovering 40min at 31°C in oxygenated ACSF.

Electrophysiology. When recording spontaneous excitatory postsynaptic potentials (sEPSPs), we acquired 2 min gap-free current and voltage traces using borosilicate glass electrodes (1.5 mm OD, 4–6 M Ω resistance) filled with an internal solution containing (in mM): 120 Cs-methanesulfonate, 20 KCl, 10 HEPES, 2 K₂ATP, 2 K₂GTP, and 12 phosphocreatine. We acquired and filtered EPSPs at 10 kHz and 2 kHz, respectively, with an EPC-10 amplifier and Patchmaster, an acquisition software (HEKA, Elektronik, Germany). We analyzed

sEPSPs amplitude and frequency with Mini 6.0 software (Synaptosoft Inc, USA). The threshold was determined by the formula $\sqrt{\text{rms}} \times 3$ (~ 0.4 mV). We monitored series resistance by comparing EPSCs decay time before and after induction. We rejected recordings with changes of resting potential larger than 1 mV.

Slice biotinylation. Hemislices were treated $\pm 1\mu\text{M}$ PMA or $10\mu\text{M}$ AMPH in ACSF for 30min at 37°C with constant oxygenation, using contralateral hemisections as controls. Following drug treatment, slices were moved to ice and biotinylated with membrane-impermeant sulfo-NHS-SS-biotin (1mg/ml) for 45min. Residual biotin was quenched with two washes of ice-cold ACSF supplemented with 100mM glycine for 20min. Slices were then washed with ice-cold ACSF and lysed in RIPA buffer containing protease inhibitors by triturating sequentially through a 200 μL pipet tip, 22G and 26G tech-tips and rotating 30min at 4°C . Protein concentrations were determined using a BCA protein assay. Biotinylated proteins were isolated by pull down of 20 μg lysate with 30 μL of streptavidin agarose beads overnight. A total 20 μg lysate fraction of each lysate was also reserved. Isolated proteins were eluted from beads in denaturing SDS/PAGE sample buffer for 30min at room temperature with rotation. Samples were analyzed by SDS/PAGE and DAT was detected by immunoblotting. Surface DAT levels were determined by comparing biotinylated DAT to total DAT fractions.

Viral production. pscAAV-TRE-shRin-eGFP and pscAAV-TRE-eGFP were packaged into AAV9 viral particles by the University of Massachusetts Medical School Viral Vector Core.

Antibodies. Rat anti-DAT 1:2000 (MAB369, Millipore), mouse anti-actin 1:5000 (Santa Cruz), mouse anti-RFP 1:2000 (Thermo), rabbit anti-TH 1:500 (Millipore), goat anti-rat HRP 1:5000 (Santa Cruz), goat anti-mouse HRP 1:5000 (Jackson Laboratories).

IV.C Results

Conditional and inducible Rin knockdown with the Tet-OFF/ON system.

Conditional and inducible, rAAV-mediated gene delivery has proven an indispensable tool for pinpointing molecular mechanisms that operate within specific neuronal circuits. However, AAV-mediated gene-silencing studies have not progressed as rapidly as have transgene overexpression and gene ablation studies. Short, inverted repeat DNA sequences cause instability within the viral genome (Xie et al., 2017). This precludes effectively using Cre-lox technology due to the inverted sequences in both the shRNA and within lox sites. AAV packaging size constraints further eliminate many large, neuronal promoters to conditionally drive shRNAs (Wu et al., 2010). This obstacle is particularly difficult to circumvent in DA neurons, which are phenotypically distinguished by TH and

DAT, both of which have large promoters that are incompatible with AAV. Here, we circumvented these obstacles, by designing an rAAV vector that expresses an shRNA under the TRE promoter, facilitating use of the TET-OFF system (Das et al., 2016) to drive conditional Rin-directed shRNA expression in tetracycline transactivator (tTA)-expressing cell populations (Fig. IV-1).

First, we tested commercially available mouse Rin (mRin)-targeted shRNAs. We identified two mouse-specific, Rin-directed shRNA candidates (shRin#1 and shRin#2) using an RFP-mRin reporter in transfected HEK293T cells (Fig. IV-2A). Both shRNAs significantly knocked down RFP-Rin levels, and we continued our studies with the more efficacious shRNA (shRin#1, further referred to as shRin). shRin was cloned into pscAAV-TRE-eGFP vector. To test that shRin is efficacious in this vector, we coexpressed RFP-mRin, TRE-shRin, and rtTA (TET-ON) in HEK cells \pm doxycycline. In this system, shRin significantly reduced RFP-mRin levels in a dox-dependent manner (Fig. IV-2B), thus shRNAs cloned into the pscAAV-TRE-eGFP vector are both efficacious and inducible.

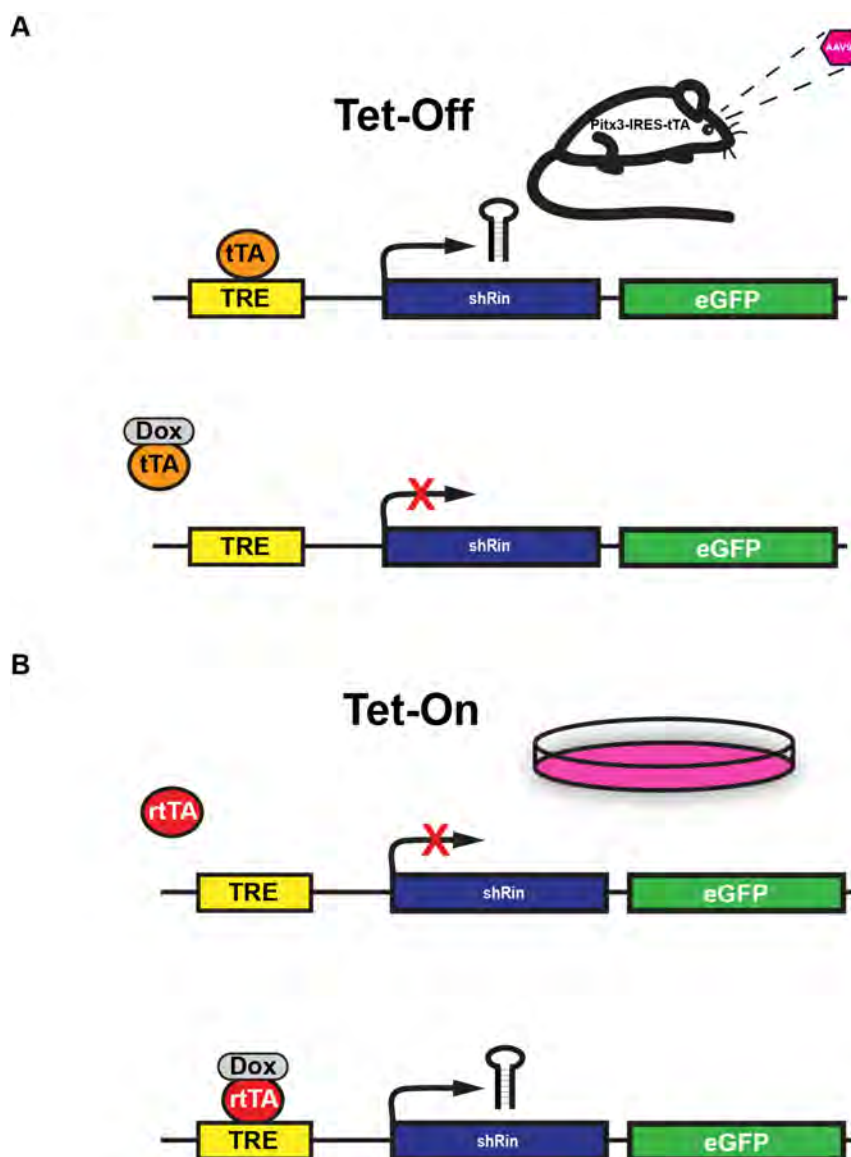


Figure IV-1. Tet-OFF/ON system for conditional and inducible shRNA expression. rAAV (TET-OFF/ON) vector uses a tetracycline responsive element (TRE) to drive Rin-targeted shRNA expression selectively in cells expressing the Tet transactivator (tTA). **(A)** For *in vivo* experiments, we drive TRE-shRIN-eGFP expression in *Pitx3^{IRES-tTA}/+* mice (TET-OFF). shRNA expression was suppressed by maintaining mice on a doxycycline (dox) diet. **(B)** For *in vitro* cell experiments, we use rtTA-expressing vector to drive expression of TRE-shRin-eGFP (TET-ON). shRNA expression was driven in the presence of dox in cell media.

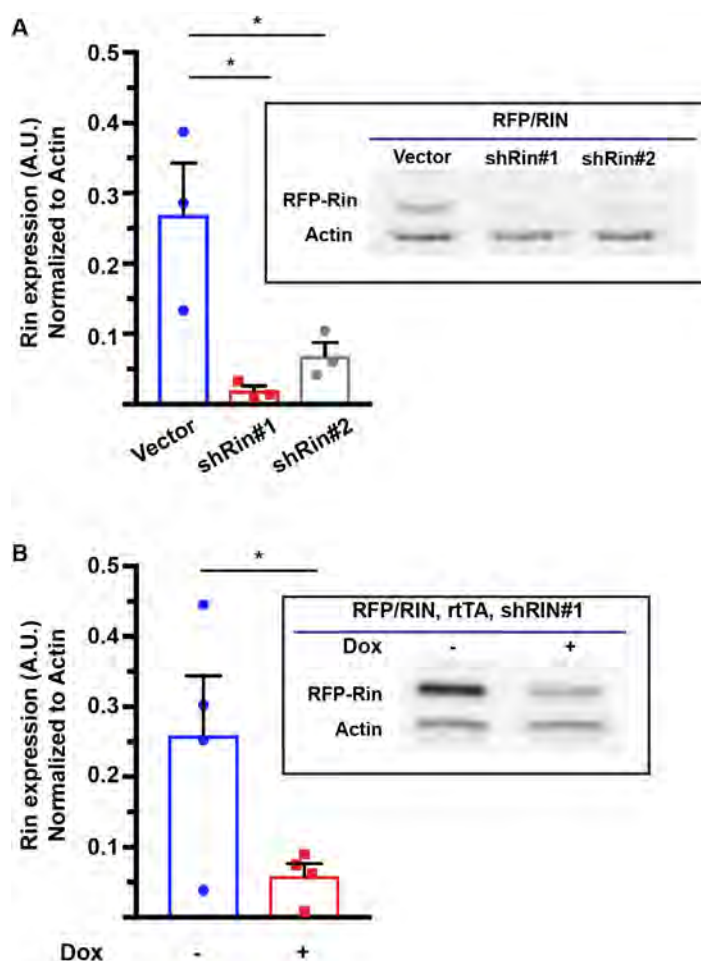


Figure IV-2. Testing Rin-targeted shRNAs. (A) Screen for efficacious mRin-directed shRNA. HEK293T cells were transiently transfected with RFP-Rin reporter and either vector (pGIPZ) or Rin-directed shRNAs and RFP-Rin protein levels were assessed 48 hours post-transfection by immunoblot, normalized to actin. Both shRin#1 and shRin#2 significantly reduced Rin expression as compared to vector controls. *Significantly different from vector control, $P < 0.04$, one way ANOVA, Dunnett's multiple comparisons test, $n = 3$. **(B)** Test for inducible shRNA expression. Cells were co-transfected with RFP-Rin reporter, rtTA, and TRE-shRIN#1 cDNAs, and were treated ± 500 ng/mL dox for 48 hours. RFP-mRin expression was assessed by immunoblot, normalized to actin. The TRE-shRin construct exhibited significant inducibility. *Significantly different from (-)dox control, $P = 0.02$, one tailed t test, $n = 4$.

Rin expression increases throughout postnatal development and stabilizes around 3-4 weeks post birth (Lee et al., 1996; Spencer et al., 2002). We did not want Rin knockdown to interfere with Rin development. Therefore, we tested Rin mRNA expression in WT mice at 4, 7, and 11 weeks old. We saw no significant difference in Rin expression in mice from 4-11 weeks in age, (Fig, IV-3, $p=0.79$, One way ANOVA, $n=4-6$) indicating that midbrain Rin expression is stable in adolescent and adult mice, and that knockdown experiments should not interfere with the reported Rin developmental period

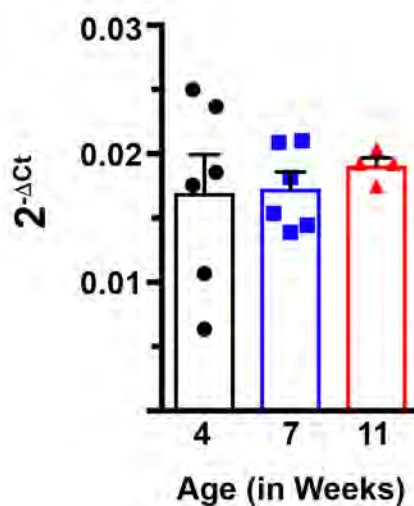


Figure IV-3. Midbrain Rin mRNA levels do not change after 4 weeks of age. (qPCR Analysis. Mouse midbrain was isolated and Rin expression was measured by qPCR, normalized to GAPDH internal controls. We saw no significant difference in Rin expression at 4, 7, and 11 weeks of age. One way ANOVA, $P=0.78$, $n=4-6$).

AAV-mediated Rin knockdown in *Pitx3*^{IRE2-tTA}/+ mouse dopaminergic neurons. *Pitx3* is a transcription factor exclusively expressed in postnatal DA neurons (Smidt et al., 1997; Maxwell et al., 2005). *Pitx3*^{IRE2-tTA}/+ mice were previously demonstrated to express a tetracycline transactivator (tTA) in TH+ VTA and SNc midbrain neurons (Lin et al., 2012). We bilaterally injected AAV9-TRE-shRin-eGFP into *Pitx3*^{IRE2-tTA}/+ VTA, and maintained mice ± 200 mg/kg doxycycline for 6 weeks post-injection. GFP reporter expression was clearly visible in TH+ cell bodies in the VTA and SNc in (-)dox mice (Fig. IV-4). GFP expression was noticeably higher in VTA compared to SNc, consistent with reports that *Pitx3* expression is 6X greater in VTA than in SNc (Korotkova et al., 2005). In contrast, GFP expression was markedly suppressed in mice kept on (+)dox diet (Fig. IV-4). Rin mRNA expression was significantly decreased in SNc-enriched tissue from (-)dox mice, as compared to (+)dox mice (Fig. IV-5A), and no change in Rin expression was detected in the neighboring, non-DAergic SNr (Fig. IV-5B, $p=0.92$). Decreased Rin expression was not likely due to compromised DA neuron viability, as there was no significant change in TH or DAT mRNA expression in SNc enriched tissue (Fig. IV-5C,D, $p=0.86$ and 0.53 respectively).

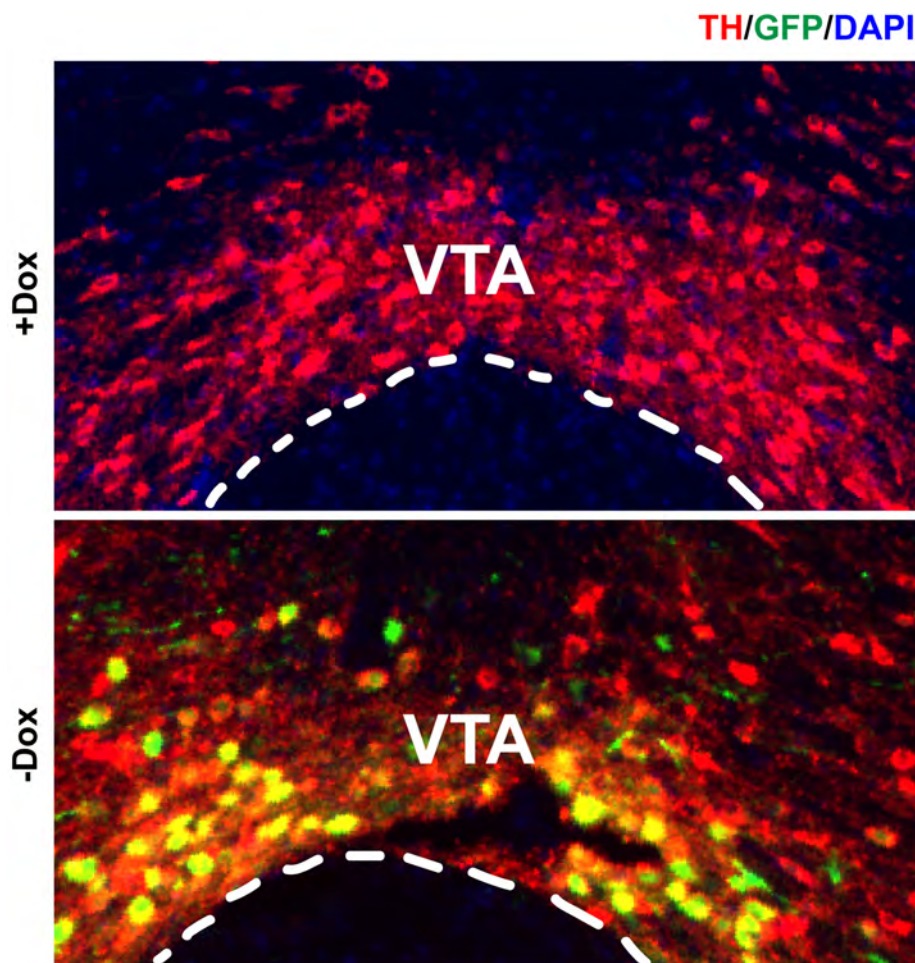


Figure IV-4. Conditional gene expression in *Pitx3*^{IRES-tTA}/+ DA neurons. *Pitx3*^{IRES-tTA}/+ mouse VTA was bilaterally injected with AAV9-TRE-GFP and mice were maintained \pm dox. Brains were sectioned 6 weeks post-injection, stained for TH (red) and imaged for TH and GFP (green) co-localization. Dox diet suppressed GFP expression in TH+ cells. Intrachannel exposure times and post hoc image manipulations are identical.

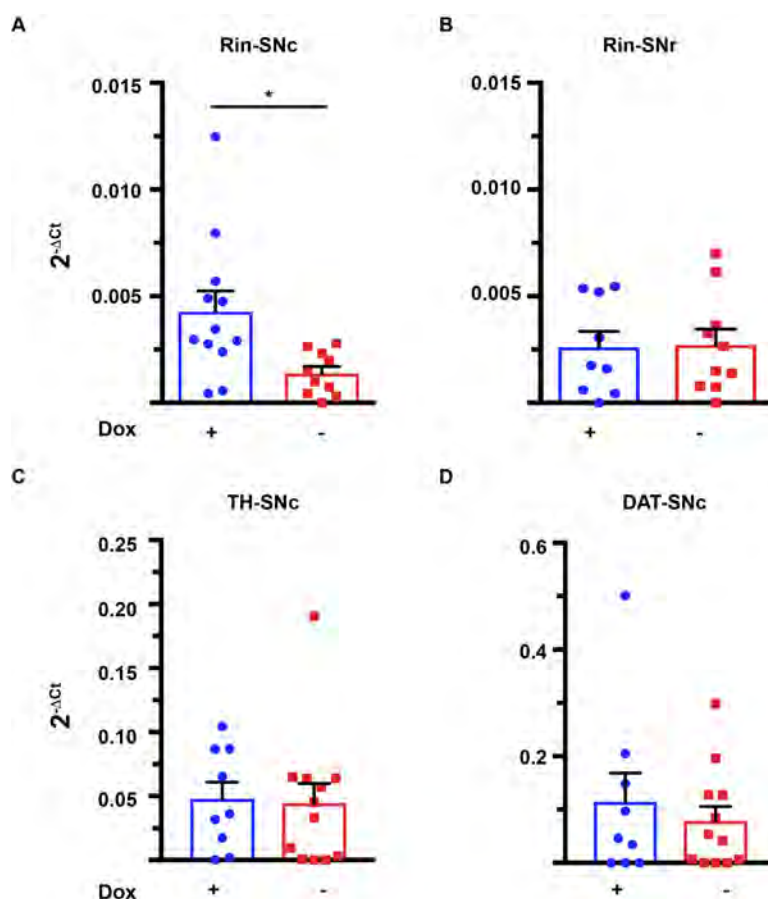


Figure IV-5. Rin knockdown in SNc does not impair DAT or TH expression. qPCR Analysis. AAV9-TRE-miR33-shRin was bilaterally injected into Pitx3IRES2-tTA/+ VTA, and mice were maintained \pm dox diet, 6 weeks. The indicated brain regions were harvested by tissue punch and laser capture microscopy, and Rin expression was measured by qPCR, normalized to GAPDH internal controls. Note significant Rin knockdown in SNc (**A**), but not in the neighboring, non-DAergic, substantia nigra pars reticulata (SNr) (**B**), one tailed t-test, $P=0.92$. Rin knockdown had no effect on (**C**)TH (TH-SNc, one tailed t-test, $P=0.87$) or (**D**)DAT (DAT-SNc, one tailed t-test, $P=0.52$). *Significantly different from (+)dox control, $P<0.02$, one tailed t test, $n=10-12$.

DAergic Rin expression impacts baseline anxiety, but not locomotion. The DAergic system is central to a variety of rodent behaviors including locomotion (Beninger, 1983). To test whether Rin expression in DAergic neurons impacts baseline locomotor behavior, we used an open field test (OFT) in which mice explore a novel arena for ten minutes. We measured total distance traveled in male and female mice injected with AAV9-TRE-shRIN-eGFP \pm dox. There was no significant effect on sex (Two way ANOVA, sex effect, $F_{1,26}=0.058$, $P=0.94$) therefore we grouped both sexes together for analysis. Rin KD in DA neurons did not affect total distance traveled (Fig. IV-6A, t-test, $P=0.67$); however, Rin KD mice spent significantly more time in the field center (Fig. IV-6B). Mice avoid open spaces, and increased time spent in an open field is indicative of reduced anxiety, suggesting that Rin KD in DAergic neurons results in an anxiolytic phenotype. As a complementary approach to the OFT, we used the elevated plus maze (EPM) to test whether Rin KD in DAergic neurons reduces baseline anxiety. The elevated plus maze is the gold standard for studying anxiety-related phenotypes in rodents. Mice are free to explore two opposing open arms or two opposing closed arms. Mice prefer to spend time in closed arms; therefore, an increase in time spent in open arms is indicative of an anxiolytic-like response in the animal. Once again, sex had no effect on time spent in open arms (Two way ANOVA, sex effect, $F_{1,26}=0.24$, $P=0.63$); therefore, we grouped the two sexes together for analysis. Rin KD mice spent significantly more time in the open arms as compared to (+)dox controls (Fig. IV-6C), consistent with an anxiolytic-like

phenotype. To test whether the dox diet increases anxiety in mice, *Pitx3*^{RES2-tTA}/+ mice were maintained \pm dox for four weeks prior to test in the elevated plus maze. Once again, we saw no effect on sex (Two way ANOVA, sex effect, $F_{1,28}=2.60$, $P=0.13$) so we grouped males and females together. We saw no significant difference in time spent in open arms between (+)dox and (-)dox mice (data not shown, t-test, $n=14-16$, $P=0.75$), demonstrating that our observed anxiety effects are not due to the dox diet. Taken together, these data demonstrate that Rin expression in DA neurons influences baseline anxiety-like behaviors, but does not impact baseline locomotor activity.

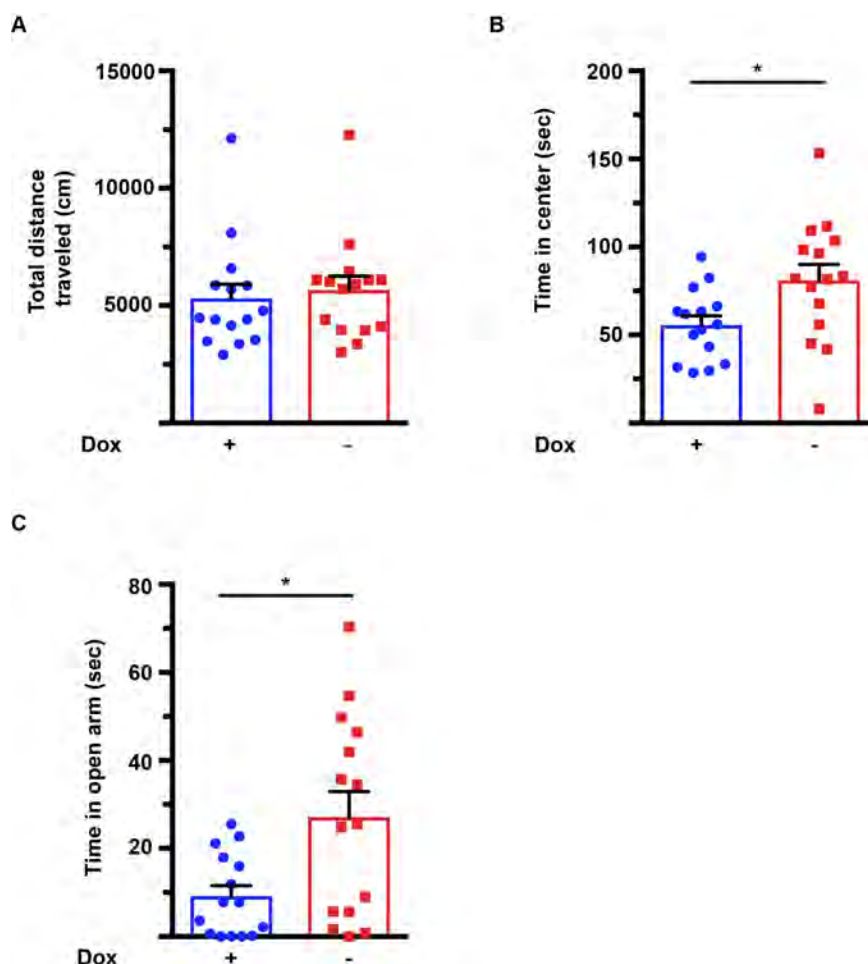


Figure IV-6. Rin knockdown in DA neurons does not affect baseline locomotor activity but reduces generalized anxiety. AAV9-TRE-miR-shRin-eGFP was injected into *Pitx3*^{IREs2-tTA}/+ VTA, and mice were maintained \pm dox diet, 6 weeks. Locomotor behavior and anxiety were assessed in males and females equivalently. Open field test. (A) Total distance traveled was not impacted by Rin KD, two tailed t-test, $P=0.67$, $n=15$. (B) Mice spent significantly more time in the center of the field following Rin KD as compared to (+)dox controls. *Significantly different from (+)dox control, two tailed t-test, $P=0.02$, $n=15$. (C) Elevated plus maze. Rin KD mice spent more time in open arms compared to (+)dox controls. *Significantly different from (+)dox mice, two tailed t-test, $p<0.01$, $n=15$.

DAergic Rin expression differentially impacts male and female locomotor response to acute cocaine injection. Cocaine is a potent DAT inhibitor, and acute cocaine treatment increases extracellular DA levels. Additionally, the Nestler laboratory found increased Δ FosB binding to the Rin promotor in mouse NAc following chronic cocaine treatment, suggesting that cocaine may impact Rin expression levels and/or signaling (Renthal et al., 2009). To test the hypothesis that Rin expression in DA neurons impacts locomotor response to acute cocaine injection, we tested male and female mouse locomotor activity in a beam break assay. On day one, mice habituated to the locomotor chamber for 45 minutes, and then received a saline injection (10mL/kg). Two days later, mice habituated to the locomotor chamber for 45 minutes and then received either 15mg/kg or 30mg/kg cocaine injection, and locomotor activity was recorded for 90 minutes. In (+)dox control male mice, a 15mg/kg injection was subthreshold, but they displayed significantly increased locomotor activity following a 30mg/kg cocaine dose (Fig. IV-7A,C). In contrast, following DAergic Rin KD, male mice displayed significantly increased locomotor activity following 15mg/kg and 30mg/kg cocaine injection (Fig. IV-7B,C). These data indicate that in male mice, Rin KD in DA neurons enhances sensitivity to cocaine. Female (+)dox control mice showed significant increase in locomotor activity at both 15 and 30mg/kg cocaine injection (Fig. IV-8A,C). This finding is consistent with previous reports that females are more sensitive to cocaine than males (Becker and Koob, 2016). Following Rin KD in DA neurons, female mice did not display enhanced

locomotor activity to either 15 or 30mg/kg cocaine (Fig. IV-8B,C). To test whether the observed effects are not due to the dox diet alone, *Pitx3*^{lRES2-tTA}/+ were maintained on \pm dox for four weeks, and were tested at 15mg/kg cocaine. We saw no significant difference between males \pm dox (Two tailed t-test, P=0.82) and females \pm dox (Two tailed t-test, P=0.85). Therefore, the dox diet does not contribute to the observed effects on cocaine-induced locomotor activity. These data indicate that DAergic Rin KD in female mice abolished locomotor response to acute cocaine injection.

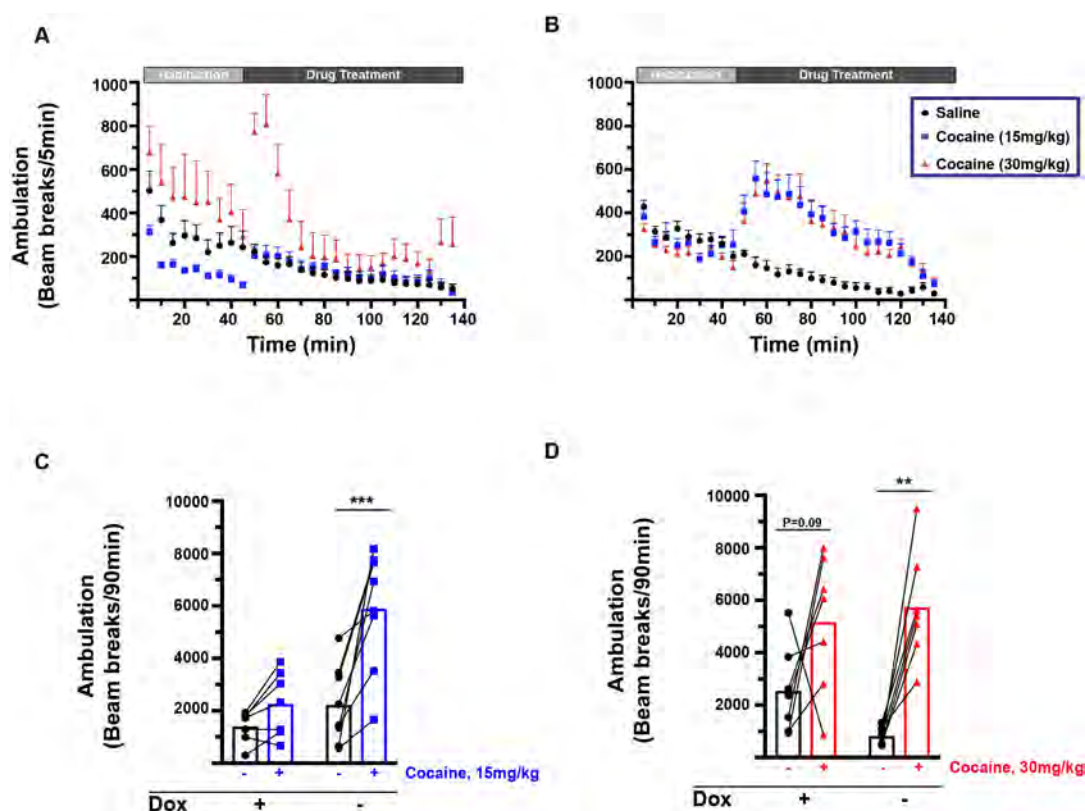


Figure IV-7. Rin KD in DA neurons enhances sensitivity to cocaine locomotor effects in males. AAV9-TRE-shRin-eGFP was bilaterally injected into male *Pitx3*^{*lRES2-tTA*}/+ VTA, and mice were maintained \pm dox diet, 6 weeks. **(A,B)** Locomotor activity time course. Mice, maintained **(A)** (+)dox and **(B)** (-)dox, habituated to locomotor chambers for 45min before injection with saline, and then activity was recorded for 90min. The assay was repeated two days later and mice received either **(C)** 15mg/kg cocaine, or **(D)** 30mg/kg cocaine during the test period. Rin knockdown in DA neurons (-dox) significantly increased sensitivity to 15mg/kg cocaine (Two way ANOVA, drug effect, $F(1,13)=25$, Bonferroni's multiple comparisons test, *** $P<0.001$) while both groups (+dox and -dox) had increases in locomotor activity following injection with 30mg/kg cocaine (Two way ANOVA, drug effect, $F(1,12)=19.61$, ** $P<0.01$).

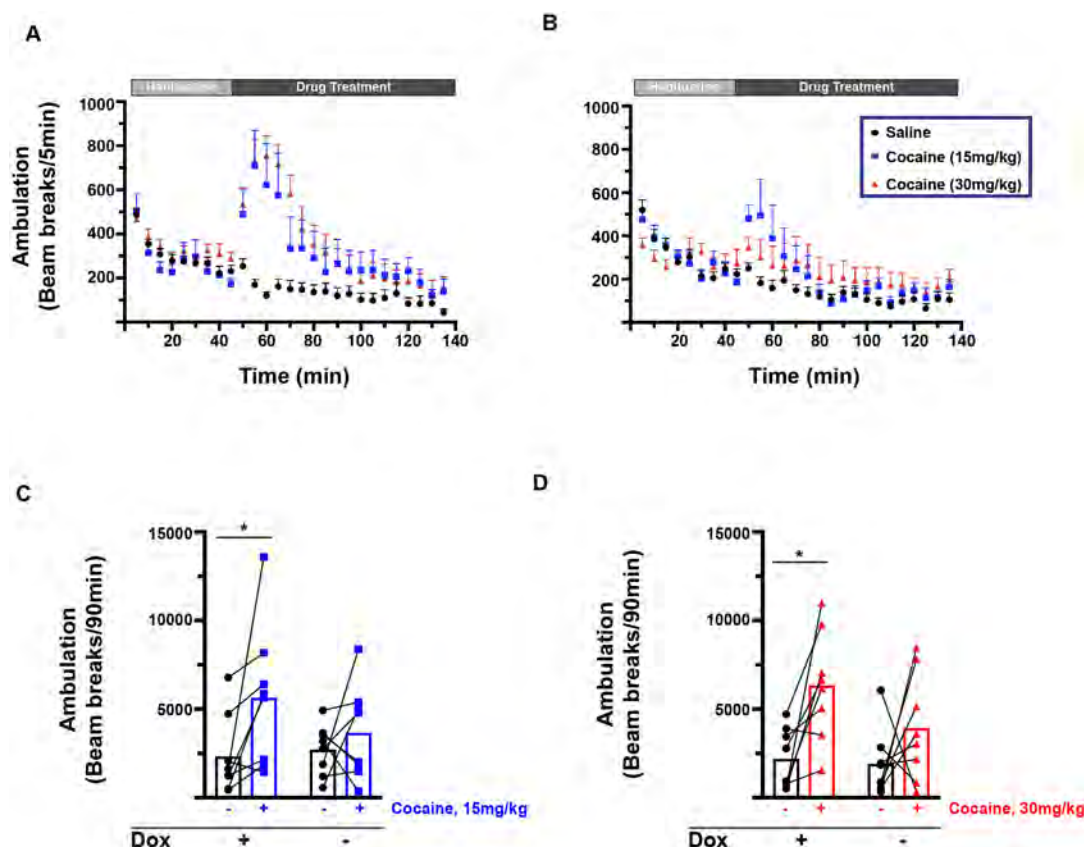


Figure IV-8. Rin KD in DA neurons reduces cocaine locomotor effects in females. AAV9-TRE-shRin-eGFP was bilaterally injected into female *Pitx3^{lIRES2-IT1}/+* VTA, and mice were maintained \pm dox diet, 6 weeks. **(A,B)** Locomotor activity time course. Mice, maintained **(A)** (+)dox and **(B)** (-)dox, habituated to locomotor chambers for 45min before injection with saline, and then activity was recorded for 90min. The assay was repeated two days later and mice received either **(C)** 15mg/kg cocaine, or **(D)** 30mg/kg cocaine during the test period. 15mg/kg cocaine significantly increased locomotor activity in control (+dox) (Two way ANOVA, drug effect, $F(1,14)=5.54$, Bonferroni's multiple comparisons test, $*P<0.05$) as did 30mg/kg (Two way ANOVA, drug effect, $F(1,14)=9.09$, Bonferroni's multiple comparisons test, $*P<0.05$). Rin KD in DA neurons reduced female cocaine-induced locomotor response at 15mg/kg (Two way ANOVA, drug effect, $F(1,14)=5.54$, Bonferroni's multiple comparison test, $P=0.94$) and 30mg/kg (Two way ANOVA, drug effect, $F(1,14)=9.09$, Bonferroni's multiple comparisons test, $P=0.36$).

Rin knockdown in DA neurons blocks protein kinase C stimulated DAT endocytosis, and reduces total DAT content. We found that Rin knockdown in DA neurons reduces baseline anxiety in male and female mice, and it produces dimorphic phenotypes in response to acute cocaine treatment. We wanted to test what mechanisms are required for these observed effects. Our laboratory previously reported that Rin binds to DAT, and its activity is required for PKC stimulated DAT endocytosis in PC12 cells. To test whether Rin is required for PKC-stimulated DAT endocytosis in bona fide DA nerve terminals, we expressed AAV9-TRE-shRin-eGFP or AAV9-TRE-eGFP control in *Pitx3*^{lRES2-tTA/+} mice. We performed a slice biotinylation assay in acute, coronal striatal slices \pm PKC activation with PMA. In NAc, DAergic Rin KD blocked PKC-stimulated DAT internalization compared to eGFP controls (Fig. IV-9A). DAT rapidly internalizes following AMPH exposure; however, it is unknown whether this mechanism requires Rin activity. Following Rin knockdown in DA neurons, both eGFP control and Rin knockdown mice demonstrate AMPH-stimulated DAT internalization in NAc, indicating both that Rin is not required for AMPH-stimulated DAT internalization, and that Rin KD in DA neurons does not globally block all endocytic mechanisms (Fig. IV-9A). In DS, we see a trend (t-test, $p=0.08$) for blocked PKC-stimulated internalization following Rin knockdown in DAergic terminals; however, the effect is not significant (Fig. IV-9B). Consistent with our findings in the NAc, Rin KD had no effect on AMPH-stimulated DAT endocytosis

(Fig. IV-9B). Taken together, Rin in DA neurons is required for PKC-stimulated endocytosis but not AMPH stimulated endocytosis in NAc.

We next asked how blocking PKC-stimulated DAT endocytosis affects total DAT populations. We hypothesized that while PKC-stimulated DAT trafficking was blocked during a six-week Rin KD, the neuron might compensate for loss of regulated trafficking by altering total DAT levels in a post-transcriptional mechanism. To test this hypothesis, we isolated the striatum from male mice expressing AAV9-TRE-shRin-eGFP, (\pm)dox, and probed for total DAT content. Following DAergic Rin KD, we observed significantly less DAT in striatal tissue (Fig. IV-10). Given that we observed no change in DAT mRNA levels in mouse midbrain following Rin knockdown, these data suggest that loss of total DAT in Rin knockdown animals happens post transcriptionally, and may indicate a compensatory mechanism in the absence of PKC-stimulated DAT endocytosis.

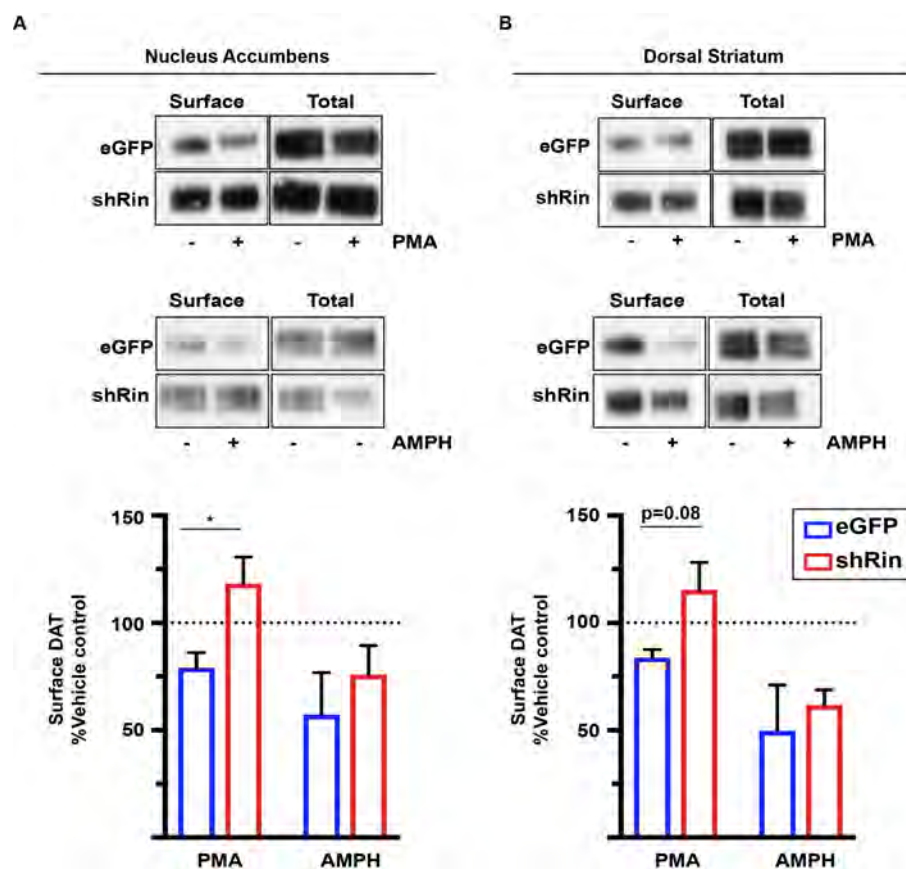


Figure IV-9. Rin KD in DA neurons blocks PKC-stimulated DAT endocytosis in NAc. Striatal slice biotinylation assay. Acute striatal slices were made from mice expressing AAV9-TRE-eGFP or AAV-TRE-shRin-eGFP for 4 weeks. **(A)** NAc. Above, representative blots from slices treated $\pm 1\mu\text{M}$ PMA or $10\mu\text{M}$ AMPH. Below, quantification of NAc data. **(B)** Dorsal Striatum. Above, representative blots from PMA or AMPH treated slices. Below, quantification of dorsal striatum data. Rin expression in DAergic nerve terminals is required for PKC-stimulated internalization but not AMPH-stimulated DAT trafficking in NAc. * $p < 0.05$, Student's t test, $n = 2-7$.

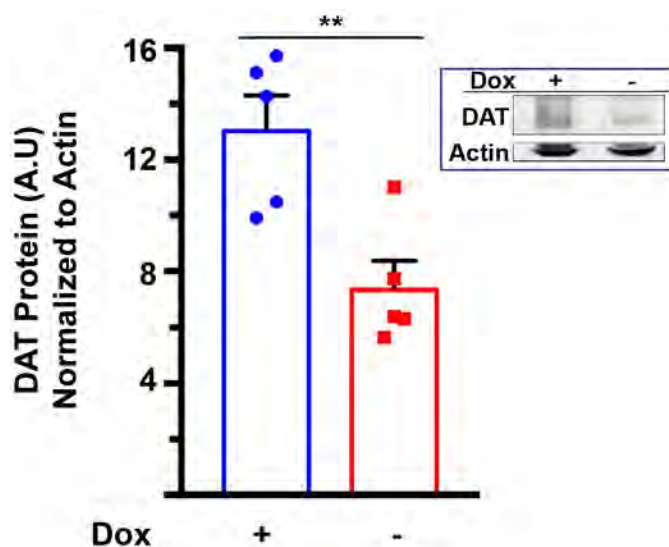


Figure IV-10. Rin KD in DA neurons reduces total striatal DAT. Total striatal DAT. Mice expressed AAV9-TRE-shRin-eGFP for a minimum 6 weeks \pm dox. Total striatal protein was isolated and assayed by SDS-PAGE followed by quantitative immunoblot. Rin KD mice (-dox) expresses less DAT in the striatum. * $p < 0.05$, Student's t test, $n = 5$.

DAergic Rin KD reduces spontaneous EPSP in NAc. We next asked what is the physiological output following Rin KD in DA terminals. DAT expression is required for regulating extracellular DA levels, and further, spontaneous glutamatergic activity in the striatum is modulated by DA (Cepeda et al., 1993; Nicola and Malenka, 1998). We hypothesized that Rin KD and loss of PKC-stimulated DAT endocytosis impacts excitatory transmission in the NAc. We measured sEPSP in MSNs in current clamp from mice injected with either AAV9-TRE-eGFP or AAV9-TRE-shRin-eGFP in VTA. Cocaine did not affect the sEPSP amplitude following cocaine treatment in either control or Rin KD mice (Fig. IV-11A). However, in eGFP control mice, cocaine significantly increased the sEPSP interevent interval following cocaine exposure. Rin KD mice displayed no difference in sEPSP interevent interval following cocaine exposure (Fig. IV-11B). When examining the cumulative distribution of interevent intervals from eGFP animals and Rin KD mice, we observed a rightward shift in measurements from Rin KD mice (Fig. IV-11C). This finding suggests that Rin KD in DA nerve terminals reduces the sEPSP frequency.

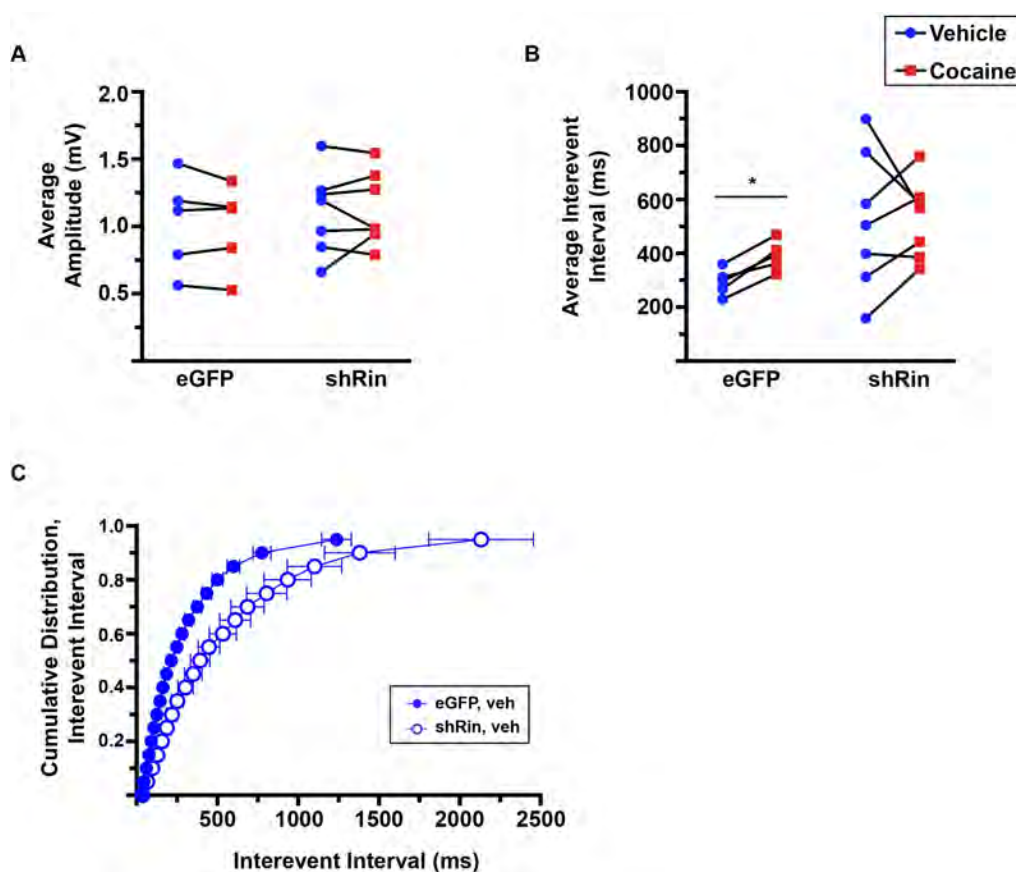


Figure IV-11. Rin KD in DA neurons reduces spontaneous excitatory postsynaptic potentials (sEPSP) frequency in NAc. *Pitx3^{IRES-tTA}/Drd1a-tdTomato* mice expressed AAV9-TRE-eGFP or AAV9-TRE-shRin-eGFP for 4 weeks. MSNs were kept in current clamp, and sEPSPs were measured $\pm 10\mu\text{M}$ cocaine. **(A)** Amplitude of sEPSPs. **(B)** Interevent interval of sEPSPs. **(C)** Cumulative distribution of sEPSP frequency from eGFP controls vs. Rin KD mice. * $P < 0.05$, Wilcoxon test, $n = 5-7$.

IV.D Discussion

DAergic transmission is integral to locomotor behavior both in rodents (Marshall and Berrios, 1979; Starr and Starr, 1986), and in humans, where DA neuronal death underlies the movement anomalies in PD (Lotharius and Brundin, 2002). Moreover, a recent report revealed that NMDA-mediated DA neuron phasic bursting is required to elicit anxiety in response to aversive conditioning (Zweifel et al., 2011). Rin was reported as a risk allele for several DA-associated disorders, including PD, ET, ASD, schizophrenia, and bipolar disorder (Emamalizadeh et al., 2017), many of which have a significant anxiety component. However, whether Rin impacts DAergic function and contributes to disease pathogenesis remains completely unknown. We found that Rin expression in DA neurons is involved in baseline anxiety-related behavior, but had no effect on baseline locomotor activity (Fig. IV-6). Additionally, we report that Rin KD in DA neurons enhances cocaine's locomotor effects in males (Fig. IV-7) but eliminates cocaine's locomotor effects in females (Fig. IV-8). Although Rin is required for NGF-mediated signaling via MAP kinase (Shi et al., 2005b), it is unlikely that the observed phenotypes were due to changes in DA neuron viability, as TH and DAT expression were unaffected by Rin KD (Fig. IV-5). DA neurons are heterogeneous, and have distinct target areas that drive these behaviors (Parker et al., 2016). Thus, Rin knockdown may preferentially impact a DA neuron subpopulation, leading to the differential phenotypes we observed.

Rin is required for PKC-stimulated DAT endocytosis in PC12 cells (Navaroli et al., 2011), and here we demonstrate that it is also required for regulated DAT endocytosis in intact DAergic nerve terminals in NAc, however not in DS (Fig. IV-9). This result suggests that these two DAergic nuclei have distinct regulatory DAT trafficking mechanisms. This hypothesis is consistent with the differential DAT trafficking regulation in NAc vs DS, reported in *Vav2*^{-/-} mice (Zhu et al., 2015). AMPH stimulated DAT trafficking was unaffected following Rin KD in DAergic terminals (Fig. IV-9). This finding demonstrates that Rin is not required for AMPH-stimulated DAT endocytosis, which is consistent with previous reports that PKC-stimulated and AMPH-stimulated DAT endocytosis are distinct mechanisms (Saunders et al., 2000; Boudanova et al., 2008a). Moreover, this result demonstrates that Rin KD did not broadly disrupt all endocytic mechanisms within the neuron.

Rin KD and loss of regulated DAT endocytosis resulted in reduced total DAT within the striatum of male mice (Fig. IV-10). Increased cocaine sensitivity is correlated with reduced DAT availability at the plasma membrane (Nelson et al., 2009), suggesting that increased cocaine hyperactivity at a 15mg/kg dose in Rin KD mice may result from reduced DAT availability. Interestingly, we observed that in female mice, Rin KD reduced hyperactivity following both 15 and 30mg/kg cocaine (Fig. IV-8). It is unknown whether Rin KD reduced DAT levels in female

striatum. Females are more sensitive to cocaine (Calipari et al., 2017). Further, female mice in estrous are more sensitive to cocaine's rewarding effects and have increased DAT N-terminal phosphorylation compared to females in dioestrous (Calipari et al., 2017). This finding suggests that hormonal signaling in females not only contributes to increased cocaine sensitivity, but also that this sex hormone effect impacts DAT regulation. Therefore, loss of DAT regulation in Rin KD mice might account for the differences we observed cocaine-induced hyperlocomotion between males and females (Fig. IV-7,8). It is unknown whether females undergo regulated DAT endocytosis. Future studies testing PKC-stimulated trafficking in female mice will test whether DAT endocytic mechanisms differ between males and females.

We observed that Rin KD resulted in decreased sEPSP frequency with no change in amplitude (Fig. IV-11), suggesting that Rin KD in DAergic neurons modulates presynaptic excitatory signaling. Given that Rin KD was restricted to DA neurons, I predict that the changes in excitatory signaling arise from changes in DA signaling within the NAc. Both D1 and D2-like receptor activation decreases glutamate release onto MSNs within the NAc (Tritsch and Sabatini, 2012). Therefore, I hypothesize that reduced sEPSP frequency arises from increased extracellular DA. In support of this hypothesis, Rin KD mice express reduced DAT levels (Fig. IV-10), which may result in reduced DA reuptake.

Future studies using FSCV will test whether Rin KD impacts DA release and clearance.

Additionally, control mice show increased sEPSP interevent interval following cocaine treatment, but Rin KD mice show no change in interevent interval following cocaine exposure (Fig. IV-11). Interestingly, a similar trend was seen in a previous report using DAT KD mice, which express 90% less DAT (Wu et al., 2007). DAT KD mice show no change in sEPSC frequency following cocaine treatment, whereas WT mice showed decreased sEPSC frequency with cocaine treatment. These studies provide further support that reduced DAT expression impacts excitatory signaling in NAc following cocaine treatment. Additional parallels between Rin KD mice and DAT KD mice is observed following acute cocaine injection. DAT KD mice display increase hyperactivity in response to cocaine injection, consistent with our Rin KD mice (Tilley et al., 2007). In contrast to the hypothesis that our observed behaviors arise from increased extracellular DA in the NAc, we observed that Rin KD resulted in reduced anxiety in male and female mice (Fig. IV-6). Increased DA signaling and reduced glutamatergic firing within the NAc is associated with increased anxiety (Russo and Nestler, 2013). How can we observe increased cocaine sensitivity and reduced anxiety in Rin KD mice? I predict that the effect we observe on anxiety arises from a brain region other than the NAc. The VTA projects to multiple brain regions including the amygdala, which is commonly associated with anxiety-

related behaviors (Calhoon and Tye, 2015; Zarrindast and Khakpai, 2015). Future studies using retrograde AAV serotypes will be informative in isolating VTA-NAc pathway dependent behaviors versus amygdala-VTA behaviors.

Conditional and inducible gene expression control has proved an indispensable approach to interrogate the nervous system, and to elucidate the neuronal basis for normal and pathological physiology and behavior. Our successful application of TET-OFF technology, in combination with higher integrity shRNA-encoding AAV, is likely to have broad utility in neurobiological studies, and there are multiple conditional tTA mice available with which to apply this approach. Although we observed a striking and significant ability to suppress Rin expression in DA neurons, it should be noted that we occasionally observed GFP reporter expression in TH⁺ cells of dox-treated mice (not shown). This is reflected in the significantly more variable Rin expression values obtained by qPCR (Fig. IV-5A) compared to (-)dox mice, and may be due to the short half-life of dox, and inability to control mouse dox dosing via *ad libitum* route. Alternatively, it may reflect a longer GFP protein half-life, in comparison to Rin mRNA. Nevertheless, the dox regimen was sufficient to suppress the observed anxiolytic phenotypes and acute cocaine responses.

Taken as a whole, these studies are the first to demonstrate Rin GTPase's role *in vivo*. Further, we confirm that in bona fide DA neurons, Rin is required for PKC-stimulated DAT endocytosis. This finding correlates PKC-stimulated DAT trafficking with baseline anxiety and locomotor response to cocaine, making this study the first to investigate the physiological impact of PKC-stimulated DAT endocytosis.

Chapter V

Discussion and Future Direction

DA neurotransmission is critical for movement, reward, cognition, and anxiety (Wise, 2004; Schultz, 2007; Russo and Nestler, 2013). DAergic dysregulation results in numerous neuropsychiatric diseases such as PD, ADHD, and ASD. Therefore, understanding DAergic regulation is necessary to understand complex behaviors and to create novel therapeutics for neuropsychiatric disease. DA reuptake through DAT is the primary mechanism for terminating DA signaling events. Therefore, DAT is a critical regulator of DAergic signaling cascades. DAT availability at the plasma membrane is required for DA reuptake and is tightly regulated through presynaptic signaling pathways, which affect DAT function and surface expression through endocytic regulation. DAT is the molecular target for AMPH and cocaine. Further, point mutations in DAT are linked to DTDS, PD, ADHD, BPD, and ASD, suggesting that changes within DAT function can have profound consequences in DAergic behavior. Within this thesis I discuss how DAT terminal domains regulate DA uptake (Chapter II) and regulatory trafficking pathways (Chapter III). Further, taking advantage a known DAT endocytic mechanism, I blocked PKC-mediated DAT endocytosis *in vivo* to test the physiological impact of regulated endocytosis.

V.A DAT N-terminus is required for substrate and small inhibitor affinity

Multiple lines of evidence demonstrate that DAT's N-terminus is required for many DAT mechanisms including AMPH-stimulated efflux (Khoshbouei et al., 2004), regulated phosphorylation (Bermingham and Blakely, 2016), negative endocytic regulation (Sorkina et al., 2009), and protein binding (Table I-1). Further, a point mutation in DAT's N-terminus (R51W) was identified in a patient with ASD (Cartier et al., 2015). Therefore, the DAT N-terminus is critical for DAT regulation, and may play an important role in DAergic behavior. We identified that the DAT N-terminus is required for substrate (DA, AMPH) and certain inhibitor (β CFT, MPH) affinities but not affinity for cocaine, GBR12909, or β CIT (Table II-1). These data point to the N-terminus' role in shaping the substrate/inhibitor binding pocket. The DAT N-terminus serves as an intracellular gate, which moves in relation to DAT intracellular loops during the translocation process, to expose the translocated DA to the cytosol (Cheng and Bahar, 2015). The juxtamembrane N-terminal domain required for N-terminal gating (RETWK, 60-65) is conserved between DAT and SERT, and therefore conserved in our N-SERT/DAT chimera (Fig. II-1). Therefore, affinity loss from N-terminal substitution is not due to changes in the N-terminal intracellular gate. Additionally, when we replaced both SERT intracellular domains onto DAT (SERT/DAT/SERT), we rescued the affinity losses displayed by N-SERT/DAT. These data suggest that DAT and SERT N- and C-termini may "recognize" one another within the cytoplasm, and therefore work together to stabilize the DA

binding pocket. DAT and SERT's N-terminal domains are mobile during the substrate translocation process (Fenollar-Ferrer et al., 2014; Khelashvili et al., 2015a; Khelashvili et al., 2015b); however, it is unknown how DAT's N- and C-termini interact with one another during this process. Molecular dynamic simulations and intramolecular FRET were used to determine how the SERT N- and C-termini move with respect to one another during the substrate translocation process (Fenollar-Ferrer et al., 2014). Whether DAT's terminal domains interact with one another when DAT is in an open conformation, as is predicted for SERT, remains untested. Future studies modeling both terminal domains together will be informative in asking this question, and will reveal DA translocation mechanisms, and perhaps provide mechanistic insight into other N- and C-terminal synergistic mechanisms such as substrate efflux.

V.B DAT N-terminus is required for PKC-stimulated DAT endocytosis

As stated previously, DAT's N- and C-termini are required for negative endocytic regulation (Holton et al., 2005; Boudanova et al., 2008b; Sorkina et al., 2009). Reports from our lab and others independently implicate DAT's N- and C-termini as required for stabilizing DAT surface expression. Therefore, I wanted to test whether these two domains synergistically work together to support regulated DAT endocytosis. It is known that DAT rapidly internalizes following PKC activation, and Ack1 inactivation is required downstream of PKC to stimulate

enhanced DAT endocytosis (Wu et al., 2015). Using DAT/SERT chimeras (Fig. I-1) (Sweeney et al., 2017), I tested endocytic rates following PKC activation and Ack1 inhibition. WT DAT, N-SERT/DAT, and DAT/C-SERT all showed increased endocytic rates following Ack1 inhibition; however, SERT/DAT/SERT did not. These data demonstrate that individually, neither DAT's N- nor C-terminus is required for rapid endocytosis following Ack1 inhibition. This raises two hypotheses: 1) that Ack1 inactivation independently stimulates mechanisms associated with N- and C-termini for rapid endocytosis or 2) that Ack1 inactivation stimulates a molecule(s) that associates with both N- and C-termini to stimulate rapid endocytosis. Given that N- and C-termini synergistically mediate substrate affinity (Sweeney et al., 2017), AMPH-stimulated efflux (Fog et al., 2006b), and the reciprocal relationship between palmitoylation and phosphorylation (Moritz et al., 2015), I hypothesize that both terminal domains synergistically work together to mediate enhanced endocytic rates following Ack1 inhibition. An intriguing hypothesis is that Ack1 itself mediates N- and C-terminal synergy, and once dephosphorylated, it disassociates from DAT. However, preliminary results from our lab refute this hypothesis. Ack1 does not co-IP with DAT (Sweeney and Melikian, unpublished data), and Ack1 and DAT do not colocalize as measured through immunocytochemistry (Marshall and Melikian, unpublished data). Therefore, I predict that another molecule mediates this N- and C-terminal synergy. I tested whether Rin is the mediating molecule. Given the fact that 1) Rin is required for PKC-stimulated DAT endocytosis, 2) Rin is

activated by PKC, and 3) Rin activity is required for PKC-stimulated Ack1 dephosphorylation, it appeared to be a strong candidate to mediate N- and C-terminal synergy. However, Rin associated with each DAT-core chimera (Fig. III-3), demonstrating that neither DAT's N- nor C-terminus is required for DAT/Rin interaction. However, we did not test whether Rin's association with DAT-core chimeras is differentially affected by PKC activation or Ack1 inhibition. It may be that at steady state, Rin associates with each DAT-core chimera, and that stimulating regulated endocytosis affects the association. Future studies testing DAT/Rin association in the presence of PMA or AIM-100 will provide further mechanistic insight to the DAT/Rin interaction. Additionally, other factors may mediate the hypothesized DAT terminal synergy. BBS-DAT-core chimeras will serve as an excellent tool to screen for DAT N- and C-terminal binding proteins at the cell surface. Previous studies screening for DAT associated proteins have relied upon GST-tagged peptides or yeast 2-hybrid screens with DAT terminal peptides as bait (Table I-1). While these approaches have been critical for identifying DAT associated proteins, they do not take into account the entire DAT protein. Further, they preclude the possible identification of proteins that require both N- and C-termini for DAT association. BBS-DAT can be used both in cellular systems and *in vivo* to identify novel DAT binding partners in the future.

V.C DAT microdomain localization and regulated endocytosis

DAT localizes to distinct microdomains within the plasma membrane. Further, localization to these domains and/or mechanisms associated with DAT microdomain targeting correlate with mechanisms associated with regulated endocytosis. Our lab previously reported that DAT interacts with Rin, and that DAT/Rin colocalization is enhanced in lipid rafts (Navaroli et al., 2011). Additionally, Rin is required for PKC-stimulated DAT downregulation (Fig. IV-9, Navaroli et al., 2011). These findings raise two questions: 1) Does Rin target DAT to lipid raft microdomains? and 2) is DAT's presence within these microdomains domains required for PKC-stimulated internalization? To test the former, one can compare DAT localization within lipid rafts in mice \pm Rin KD. A loss of DAT localization to lipid rafts in Rin KD mice would suggest that Rin is required to target DAT to these domains. Data from the Yamamoto lab correlate DAT microdomain localization to regulated DAT endocytosis. They showed that DAT associates with the lipid raft-enriched, integral membrane protein, Flot-1, and that Flot-1 is required to maintain DAT within membrane microdomains. Further, they demonstrated that Flot-1 is required for PKC-stimulated endocytosis (Cremona et al., 2011). These findings support the hypothesis that PKC-stimulated DAT internalization requires DAT to be localized within lipid raft domains. Additional support linking membrane localization to DAT trafficking mechanisms comes from studies with the ADHD-associated DAT mutant, R615C. 615C DAT is enriched in non-raft compartments, and it does not undergo PKC-stimulated internalization (Sakrikar et al., 2012). It should be noted that the

absence of PKC-stimulated internalization is due to increased basal endocytic rates. In fact, our lab reported that a constitutively active Ack1 mutant, S445P, can rescue the enhanced basal endocytic rates associated with the 615C mutant, and therefore restores PKC-stimulated internalization. Does S445P-Ack1 restore basal DAT endocytic rates by restoring 615C DAT localization to lipid rafts? This hypothesis remains untested.

In Chapter III, I demonstrate that N-SERT/DAT does not undergo PKC-stimulated endocytosis, but maintains Ack1-dependent endocytosis. Further, I predicted that a molecule associated with the DAT N-terminus must mediate this effect. However, given what we know about DAT localization to lipid rafts, it raises the alternative hypothesis that N-SERT/DAT is mislocalized, and expression in different compartments within the plasma membrane may result in the loss of PKC-stimulated endocytosis. To test this hypothesis, one can first test whether N-SERT/DAT colocalize with a lipid raft marker, such as CTxB, and whether its localization is different from WT DAT.

Ultimately, the studies linking DAT membrane localization to regulated endocytosis are correlations. It remains unknown whether loss of regulated endocytosis following Rin KD or Flot-1 KD arise due to loss of direct interaction with these proteins, or rather due to loss of localization. Does DAT need to be in the right place at the right time to undergo regulated endocytosis, or rather, does

it need to directly interact with raft-associated proteins for regulated endocytosis? These two ideas are not mutually exclusive, and further studies linking known DAT regulatory mechanisms to DAT membrane localization will provide further insight regarding how membrane localization impacts DAT function and regulation.

V.D Rin is not expressed in certain heterologous expression systems – Implications for data interpretation

Our laboratory previously reported that Rin directly binds to DAT, and that Rin is required for PKC-stimulated DAT endocytosis (Navaroli et al., 2011). This study used RT-PCR to demonstrate that Rin is expressed in multiple heterologous expression systems, including non-neuronal cell lines, which was surprising given that Rin is only expressed in neuronal tissue. However, throughout the course of my graduate work, I found that 1) I could not detect Rin in SK-N-MC cells and HEK293T cells using RT-qPCR and 2) the Rin antibody we were using (27G2 clone) detected an immunoreactive band that is not Rin. These findings raised questions regarding Rin's putative role in DAT endocytic regulation. When reevaluating Navaroli et al.'s work, several findings remain true in light of this new information. 1) Rin binds to DAT but not SERT. Rin was originally identified as a DAT binding protein through a yeast 2-hybrid screen using FREKLAYAIA (587-596) as bait. Additionally, FRET analysis using YFP-Rin and CFP-DAT

showed significant interaction between the two proteins, but no interaction between Rin and SERT nor Rin and GAT1. 2) Rin activity is required for PKC-stimulated DAT endocytosis. Internalization assays in PC12 cells expressing constitutively active and dominant negative Rin mutants showed that Rin activity is required for PKC-stimulated DAT internalization. However, Rin is a Ras-like GTPase, and within this family, there is significant homology within effector domains. Therefore, we cannot rule out that a dominant negative Rin sequesters effectors necessary for other GTPases that are required for DAT endocytic regulation. Ultimately, we are interested in DAT endocytic mechanisms in DAergic terminals rather than cellular expression systems. When we knockdown Rin in DAergic nerve terminals, we block PKC-stimulated DAT endocytosis (Fig IV-9). Therefore, Rin is absolutely required for PKC-stimulated DAT endocytosis. However, if Rin is not expressed in all cell systems, how can we say that it is truly required? Rin shares significant homology with the ubiquitously expressed Rit. We do not know whether Rit is required for PKC-stimulated internalization, or that Rit can compensate for Rin in cellular systems. Our laboratory has efficacious Rit-targeted shRNAs (Sweeney and Melikian, unpublished data), which can be used to test this possibility. Alternatively, DAT may use different regulatory endocytic pathways in different neuronal populations. In our studies, Rin KD blocked PKC-stimulated DAT internalization in NAc but not DS (Fig. IV-9). Similarly, Vav2 is required for regulating DAT surface expression in NAc but not DS (Zhu et al., 2015). Taken together, these data demonstrate that DAT requires

different endocytic regulatory mechanisms in nigrostriatal pathways than in mesolimbic pathways. Therefore, more studies are needed to study DAT regulatory mechanisms in DA terminals to test the hypothesis that different forms of regulation exist within different DAergic neuronal populations.

V.E PKC-stimulated DAT endocytosis *in vivo*

Initial reports that DAT functionally downregulates in response to PKC activation were published over 20 years ago (Bermingham and Blakely, 2016). This led to numerous papers investigating the functional downregulation and endocytic trafficking mechanisms following PKC activation. However, to date, the endogenous source of PKC activation in DA terminals remains unknown. Previous studies relied heavily upon direct PKC activation with phorbol ester treatment (PMA). However, this approach directly bypasses endogenous receptor activation, and therefore bypasses other secondary messenger cascades associated with receptor activation. PKC is activated by G_q -coupled receptor activation. G_q signaling activates phospholipase C (PLC), which in turn cleaves PIP_2 to generate DAG and IP₃, which respectively activate PKC and increase intracellular Ca^{2+} . What are the endogenous G_q -coupled receptors on DAergic terminals and does their activity regulate DAT surface expression? Muscarinic acetylcholine receptors (mAChRs, M1-M5) are distributed throughout the striatum. Both M1, M3, and M5 subtypes are G_q -coupled; however, only M5 is expressed on DAergic terminals (Vilaro et al., 1990; Weiner et al., 1990; Shin et

al., 2015), making it a strong candidate for endogenous PKC activation to drive DAT endocytosis. Preliminary data from our laboratory, using a nonselective mAChR activator at a subthreshold concentration in conjunction with an M5 positive allosteric modulator, suggest that M5 activation stimulates regulated DAT trafficking in DAergic terminals (Kearney and Melikian, unpublished data). mGluR5 is another G_q -coupled candidate to activate DAT endocytic signaling. mGluR5 is a Group I metabotropic glutamate receptor. Page et al. nonselectively activated Group I mGluRs in striatal synaptosomes, which caused decreased DA uptake, and this effect was blocked with co-incubation with a mGluR5 specific antagonist (Page et al., 2001). These data demonstrate that DA uptake velocity is sensitive to mGluR5 activation. While these data must be repeated in intact striatal preparations, it points to an mGluR5-dependent mechanism for reducing DA uptake.

If M5 and mGluR5 are the endogenous sources of regulated DAT endocytosis, it raises interesting hypotheses regarding the physiological importance of PKC-stimulated DAT endocytosis. For example, M5-induced DAT trafficking would suggest that DAT is critical for regulating DAergic tone during cholinergic signaling events in the striatum. To this same extent, mGluR5-stimulated DAT endocytosis would point to DAT's importance in regulating homeostatic DA levels during glutamatergic signaling. Both cholinergic and glutamatergic DAT regulation can be tested using channelrhodopsin conditionally expressed in

cholinergic neurons or glutamatergic terminals in the striatum. Using blue light to stimulate either ACh or Glu release, we can test whether cholinergic or glutamatergic stimulation stimulates DAT surface redistribution. Moreover, if these studies point to M5- and mGluR5-mediated regulated DAT endocytosis, I propose blocking this pathway with our Rin KD model to further test the physiological pathway and impact behind PKC-stimulated DAT endocytosis.

V.F What is the physiological importance of PKC-stimulated DAT endocytosis?

DAergic signaling is critical for key brain functions including movement initiation, learning, and reward. DAT regulates DA signaling events through reuptake into the presynaptic neuron. DAT must be available at the plasma membrane in order to regulate DA signaling. PKC-stimulated DAT endocytosis dynamically regulates DAT surface expression, and therefore regulates DAT availability at the plasma membrane. However, the physiological consequence of PKC-stimulated DAT endocytosis remains entirely unknown. To test the hypothesis that PKC-stimulated DAT endocytosis is required for DAergic behavior, we knocked down Rin conditionally in adult DA neurons. Our laboratory previously identified Rin as a DAT binding protein that is required for PKC stimulated endocytosis (Navaroli et al., 2011), and here we demonstrated that Rin is required for PKC-stimulated endocytosis in NAc DAergic terminals (Fig. IV-9). We also demonstrated that

PKC-stimulated DAT endocytosis is not required for baseline locomotor behavior (Fig. IV-6A); however, it contributes to baseline anxiety as measured by EPM and OFT (Fig. IV-6B,C). Additionally, we showed that PKC-stimulated DAT endocytosis differentially affects male and female locomotor response to acute cocaine injection. Blocking regulated DAT internalization enhanced sensitivity to cocaine in males (Fig. IV-7); however, it eliminated cocaine-induced locomotor response in females (Fig. IV-8). This study design relies on Rin knockdown to block PKC-stimulated DAT endocytosis. While I can correlate regulated DAT-trafficking to the observed behaviors, I cannot say that it is absolutely causative. To directly assess the physiological importance of PKC-stimulated DAT endocytosis *in vivo*, we require knock in mice expressing regulated DAT trafficking deficient mutants. N-SERT/DAT does not internalize rapidly in response to PKC activation (Fig. III-1), lending itself as an interesting endocytic loss-of-function mutant. Additionally, R615C DAT displays an endocytic gain-of-function phenotype in cells. It internalizes rapidly in the absence of PKC activation (Sakrikar et al., 2012). These two mutants will provide the opportunity to test the physiological impact of DAT endocytosis on DAergic behaviors. I predict that if these mutants phenocopy our behavioral data following Rin KD, it will help to support the conclusion that regulated DAT endocytosis is required for locomotor response to acute cocaine injection and generalized anxiety.

V.G Rin and Behavior

Rin is a Ras-like GTPase expressed solely in neurons (Lee et al., 1996; Wes et al., 1996). It is activated in response to growth factor signaling, and it binds CaM (Lee et al., 1996; Wes et al., 1996; Shi et al., 2005b). We know very little about Rin function, and all Rin functional studies have been performed *in vitro* or in heterologous expression systems. Rin signaling *in vivo* remains untested, and the physiological importance of Rin signaling in the brain is entirely unknown. Multiple lines of evidence suggest that Rin activity is key in DAergic neurons. GWAS studies implicate Rin as a risk allele for DAergic neurological disorders (PD, schizophrenia, ASD, essential tremor, BPD, and speech delay) (Emamalizadeh et al., 2017). Further, Rin expression is enriched in DAergic brain regions (Zhou et al., 2011). Finally, our laboratory identified that Rin binds to DAT, and is required for regulated DAT endocytosis. Therefore, we conditionally knocked down Rin in DAergic neurons to test whether Rin is required for DAergic behavior.

Rin KD and anxiety

Anxiety is characterized by apprehension, sustained arousal, and risk avoidance (Tovote et al., 2015). In rodents it is often measured in avoidance assays, in which more anxious animals avoid open spaces (Carola et al., 2002). To test whether Rin KD in DAergic neurons affected anxiety-related behavior, we used

the EPM and OFT. We saw that Rin KD in male and female DAergic neurons produced an anxiolytic phenotype (Fig. IV-6). While amygdala, BNST, hippocampal, and cortical regions are classically associated with anxiety related behavior, increasing evidence demonstrates that midbrain DAergic neurons are key in modulating anxiety responses (Calhoon and Tye, 2015; Zarrindast and Khakpai, 2015). Optogenetic stimulation of DAergic VTA neurons causes animals to spend less time in open arms in EPM (Gunaydin et al., 2014). This effect was also seen by activating DAergic terminals within the PFC, suggesting that increased activity in VTA-PFC projections has an anxiogenic effect. Conditional NMDAR KO in DAergic neurons revealed that NMDA-mediated DA neuron firing is required to elicit anxiety in response to aversive conditioning (Zweifel et al., 2011). Leptin receptor is expressed in VTA DAergic neurons. Conditional leptin receptor (*Lepr*) KO in DAT-Cre mice reduced open arm entries in EPM, and increased DA neuron burst firing (Liu et al., 2011). Further, microinjection of D1 antagonist in amygdala attenuated this phenotype, demonstrating that a DAergic VTA-amygdala pathway contributes to anxiety behavior.

VTA DAergic neurons express M5 mAChRs (Vilario et al., 1990), which are G_q -coupled, and therefore activate PKC signaling cascades. Potentiating cholinergic signaling in the VTA by local physostigmine infusion resulted in anxiogenic response in rats (Small et al., 2016). It is tempting to hypothesize that cholinergic activation of PKC signaling in DA neurons causes both regulated DAT trafficking

and anxiogenic response. In Rin KD mice, PKC-stimulated DAT trafficking is blocked and these mice have an anxiolytic response. These data provide a correlation between DAT trafficking in midbrain DAergic neurons and anxiety related behavior. KOR is expressed in DAergic neurons, and KOR signaling increases DAT surface expression (Kivell et al., 2014). Interestingly, conditional KOR KO in DAT-cre mice has an anxiolytic effect as measured by increased time in the center during OFT and reduced latency to enter the lit compartment in a light/dark box (Van't Veer et al., 2013). These data further correlate a known DAT trafficking mechanism with an anxiety-related phenotype. Whether our observed anxiolytic phenotype following Rin KD is due to impaired DAT trafficking, and therefore impaired homeostatic DA regulation or, rather, due to other changes related to loss of Rin signaling remains unknown. As discussed previously, future studies with DAT trafficking-defective mutants will help to test whether regulated DAT trafficking is required for anxiety-response in mice.

In our studies, we knocked down Rin in VTA and SNc DAergic neurons. These nuclei send projections throughout the basal ganglia and cortical regions. Therefore, we cannot determine the circuitry required for reduced anxiety in our Rin KD mice. To test whether Rin KD in DAergic VTA or SNc nuclei is required for anxiolytic response, I propose using AAV2 to infect specifically VTA or SNc. This viral serotype results in a more focal infection with reduced viral spread compared to the AAV9 used in our studies. Further, both VTA and SNc send

projections to multiple brain regions. I propose that future studies using retrograde viruses to infect the projection sites, such as NAc, PFC, amygdala, or hippocampus will be critical to interpret the brain circuitry mediating our findings.

Rin and cocaine

Sexual dimorphism within the DAergic system has been well documented (Becker et al., 2012). Cocaine is a powerful psychostimulant, and it affects females differently than males. Females binge more, escalate drug use more quickly, and progress to addictive behavior more rapidly (Becker et al., 2012). In our study, control females were sensitive to an acute 15mg/kg cocaine injection, whereas in males, this dose was subthreshold (Fig IV-7, IV-8). Further, Rin KD differentially affected male and female locomotor response to cocaine. In male mice, we observed enhanced sensitivity to cocaine-induced locomotor response at 15mg/kg (Fig. IV-7). In female mice, Rin KD abolished locomotor response to cocaine (Fig. IV-8). These data raise interesting questions as to how Rin regulates DAergic neuronal firing, and how this effect is different in male versus female mice. It is known that DA neuron firing rates fluctuate over the course of the estrous cycle, with increased firing in estrous and dioestrous females compared to proestrous (Becker et al., 2012). It should be noted, that we did not control for estrous cycle in our female mice. Further, we do not know how DAergic Rin KD impacts DA neuron firing. Future studies will test how Rin KD affects DA neuronal firing in both male and female mice.

Given that Rin KD blocked PKC-stimulated DAT endocytosis, and that cocaine is a potent DAT inhibitor, it raises the hypothesis that cocaine-dependent phenotypes may arise from loss of regulated DAT trafficking. Further, given the differential effect in cocaine response following Rin KD in males and females, I hypothesize that DAT endocytosis may be differentially regulated in males and females. The Nestler group recently reported increased DAT N-terminal phosphorylation in estrous females compared to males and dioestrous females, supporting the hypothesis that sex-dependent DAT regulatory mechanisms exist (Calipari et al., 2017). Here, we demonstrate that Rin is required for PKC-stimulated DAT endocytosis in male NAc; however, we do not know if it is required for regulated DAT endocytosis in females. Future studies in females testing regulated DAT endocytosis \pm Rin expression using our Rin KD model will provide insight as to whether DAT is regulated differently in males and females.

Rin in NAc DAergic terminals

Our electrophysiological studies demonstrate DAergic Rin signaling modulates excitatory transmission in NAc. We observed that DAergic Rin KD in NAc terminals resulted in reduced sEPSP frequency but not amplitude suggesting a presynaptic response (Fig. IV-11). It is known that DA modulates excitatory signaling from NAc glutamatergic terminals (Tritsch and Sabatini, 2012).

Therefore, I hypothesize that DAergic Rin KD impacts extracellular DA levels, and therefore, impacts DAergic modulation at glutamatergic presynaptic sites. Whether the hypothesized changes in extracellular DA result from loss of DAT availability resulting from impaired PKC-stimulated DAT endocytosis remains unknown. *Ex vivo* studies using fast scan cyclic voltammetry (FSCV) will directly test extracellular DA concentrations and clearance rates. Alternatively, alterations in DA signaling may result from changes in DA neuron firing following Rin KD. Studies testing the DAergic neuron firing properties in Rin KD mice will answer this question. Taken as a whole, we know that DAergic Rin KD blocks PKC-stimulated endocytosis, and impacts spontaneous excitatory transmission. These findings support our original hypothesis that blocking PKC-stimulated endocytosis will prevent maintenance of DA homeostasis, and will impact striatal neurotransmission.

Appendix VI

Is PKC-stimulated DAT internalization required for locomotor sensitization to amphetamine?

VI.A Introduction

DAT dynamically traffics to and from the plasma membrane. Following PKC activation, DAT's internalization significantly increases, resulting in less DAT at the plasma membrane. PKC-stimulated DAT internalization requires Rin activity and Ack1 dephosphorylation (Navaroli et al., 2011; Wu et al., 2015); however, the physiological impact of this mechanism remains unknown. Work previously described in this thesis correlates PKC-stimulated DAT internalization with cocaine induced hyperlocomotion and anxiety-related behavior (Fig IV-6, 7, 8). While these studies implicate PKC-stimulated DAT internalization in regulating psychostimulant-induced behavior and anxiety, it is unknown whether PKC-stimulated DAT internalization is required for locomotor sensitization to psychostimulants. Repeated exposure to drugs of abuse results in augmented motor effects. This effect is known as locomotor sensitization (Steketee and Kalivas, 2011)

VI.B Materials and Methods

Stereotaxic viral delivery. DAT-cre mice were bilaterally injected with AAV2 as described in Chapter IV. Virus was allowed to express for four weeks before behavioral analysis.

Virus and cDNAs. Ack1-HA (WT and S445P) were cloned into pcDNA3.1+ as described previously (Wu et al., 2015). To flank the sequence with loxP and lox2722 sites, they were subcloned into pAAV-EF1a-DIO-hChR2(H134R)-EYFP (gift of Karl Deisseroth. Addgene plasmid #20298) using *Ascl* and *NheI*. Next, double floxed WT and S445P Ack1 were cut using *Accl* and *EcoRI* and blunted. They were then ligated into pAAV-CB6, which was blunted following linearization with *EcoRI*. These viral vectors were packaged into AAV2 by the University of Massachusetts Medical School viral vector core.

Locomotor sensitization to amphetamine. Mice received daily injections of 2mg/kg AMPH for 10 days. On days 1, 4, 7, and 10, the mice first habituated to a locomotor activity chamber for 45 mins before receiving AMPH injection. Upon receiving the AMPH injection, their locomotor activity was recorded for 90 minutes. The mice then underwent a wash out period for two weeks. On day 25, we recorded the mice's locomotor activity and injected them with saline as done previously on days 1, 4, 7, and 10. Typically, this assay would have saline controls run in parallel; however, this data set does not include saline controls (Fig. VI-1).

To test the hypothesis that PKC-stimulated DAT internalization is required for induction of locomotor sensitization to AMPH, I overexpressed constitutively Ack1 in NAc of DAT-cre mice. We et al. previously demonstrated that Ack1 dephosphorylation is required for PKC-stimulated DAT internalization, and overexpression of a constitutively Ack1 (S445P) blocks PKC-stimulated DAT internalization (Wu et al., 2015). I bilaterally injected AAV2 expressing double floxed, inverted WT-Ack1, S445P-Ack1, or GFP control into VTA of DAT-cre mice. Following four weeks of viral expression, I tested mice for LMS to AMPH.

VI.C Results and Discussion

DAT-cre mice were bilaterally injected in the VTA with AAV2 expressing double-floxed, inverted WT- or S445P-Ack1 or GFP, the virus expressed for 4 weeks before starting locomotor sensitization experiments. Mice received daily AMPH injections (2mg/kg) for ten days, and we measured their locomotor activity on days 1, 4, 7, and 10 as described in Fig VI-1. On day 25, the mice received a challenge AMPH dose. GFP, WT-Ack1, and S445P-Ack1 overexpressing showed no differences in locomotor activity during LMS induction on days 1, 4, 7, and 10 (Fig. VI-2). However, on day 25, following the two week washout, WT-Ack1 overexpressing mice showed significantly reduced locomotor activity during the last thirty minutes of the locomotor assay (Two way ANOVA, Ack1 effect, $F(2,297)=47.45$). This finding raises an interesting possibility regarding Ack1

expression in DA neurons during maintenance of LMS. WT-Ack1 overexpression was significantly reduced in the final 30 minutes following AMPH injection compared to GFP infected mice. Therefore, WT-Ack1 overexpression alone, alters AMPH-stimulated locomotor activity following a sensitization protocol. Therefore, the Ack1 overexpression serves as a baseline for interpreting Ack1 mutant overexpression. Constitutively active S445P-Ack1 showed significantly increased locomotor activity in the final 30 minutes following AMPH injection compared to WT-Ack1. These data suggest that Ack1 activity increases locomotor AMPH-stimulated locomotor effects following LMS. AMPH is a potent, competitive DAT substrate. As discussed in Chapter I, AMPH affects dopamine transporter activity in multiple ways including blocking DA reuptake, increasing DAT endocytic rates, and inducing DA reverse transport via DAT. Our lab previously reported that Ack1 inactivation is required for PKC-stimulated internalization, and that a constitutively active Ack1 (S445P) blocks PKC-stimulated DAT internalization. Further, this Ack1 mutant rescues a gain-of-function trafficking dysregulated DAT mutant (R615C). The data presented here suggest that blocking regulated DAT trafficking through S445P-Ack1 overexpression amplifies locomotor activity following sensitization. Taken together with the findings in Chapter IV, it suggests that PKC-stimulated DAT trafficking is required for responding to psychostimulant induced behavior. It should be noted that the studies presented within this appendix are preliminary. Animal groups are small (n=5-7), and there may be additional AMPH-dependent

behavioral effects that are not reported due to lack of power. Further, saline controls were not conducted as depicted in Fig. VI-1. While additional experiments are necessary to complete this data set, it provides rationale for investigating the role of regulated DAT endocytosis in the sensitizing effects of psychostimulants.

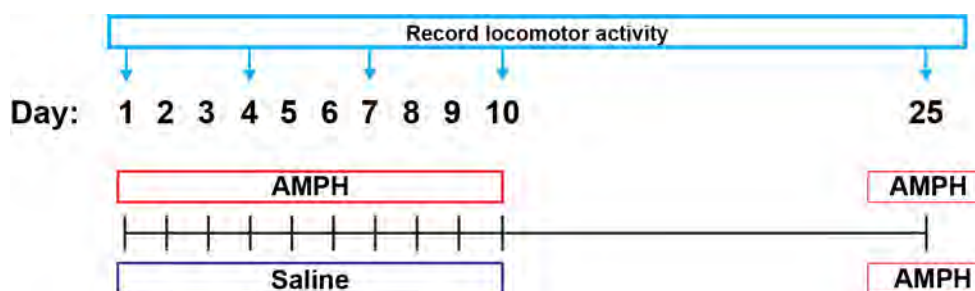


Figure VI-1 Locomotor sensitization to AMPH paradigm. Animals receive daily injections of either AMPH (2mg/kg) or saline (10mL/kg) for ten days. Their locomotor activity is recorded on days 1, 4, 7, and 10. Then the animals undergo a two week washout period, after which all animals receive a single 2mg/kg AMPH injection. An animal is considered sensitized to AMPH if it moves significantly more during day 25 compared to day 1.

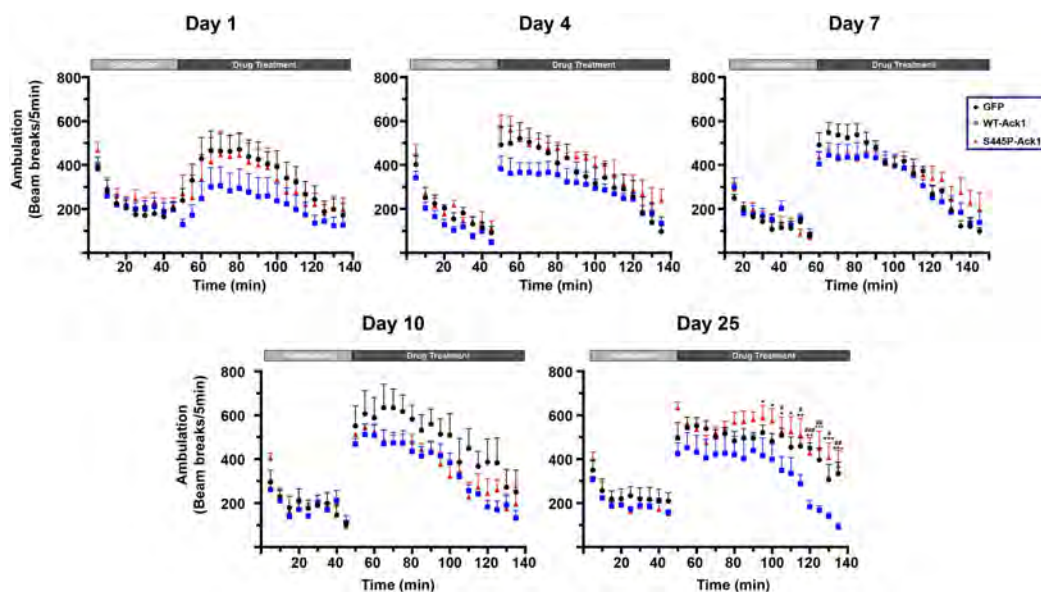


Figure VI-2. Ack1 overexpression amplifies locomotor sensitization to AMPH. DAT-cre mice bilaterally injected with AAV2 expressing GFP or double floxed, inverted WT-Ack1 or S445P-Ack1 in the VTA. Mice underwent LMS AMPH protocol as described in Materials and Methods. On Day 25, WT-Ack1 mice demonstrate significantly reduced AMPH-stimulated locomotor activity compared to GFP and S445P-Ack1 (Two way ANOVA, Ack1 effect, $F(2,297)=47.45$, Dunnett's multiple comparison test, $\#P<0.05$, $\#\#P<0.01$, GFP significantly different from WT-Ack1. $*P<0.05$, $**P<0.01$, $***P<0.001$, S445P-Ack1 significantly different from WT-Ack1).

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