Modeling Molecular And Physiological Contributions To Dopamine-Associated Neuropsychiatric Disorders

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This dissertation is dedicated to my parents and my partner. I couldn't have done this without their endless love and unwavering support

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ABBREVIATIONS

Dopamine ....DA

Norepinephrine ....NE

Substantia Nigra ....SN

Ventral Tegmental Area ....VTA

Dorsal Striatum ....DS

Nucleus Accumbens... NAcc

Ventral Striatum...VS

Prefrontal Cortex .... PFC

G Protein-Coupled Receptor .... GPCR

Dopamine D2 Autoreceptor D2AR

Dopamine Transporter .... DAT

Methylphenidate ....MPH

Amphetamine .... AMPH

L-3,4 dihydrophenylalanine .... L-DOPA

Parkinsons Disease..PD

Serotonin 5-HT

Tyrosine Hydroxylase ....TH

Amino Acid Decarboxylase ....AADC

Vesicular Monoamine Transporter 2 ....VMAT2

Γ-Amino Butyric Acid ....GABA

Glutamate .... GLU

Metabotropic GLU Receptor...mGLUR

Chapter 1 – Dopaminergic Neurotransmission

1.1 Introduction

Dopamine (DA) has been the subject of intense investigation since its discovery as a neurotransmitter over 50 years ago. Though previously considered as simply a precursor for the sympathetic neurotransmitter norepinephrine (NE) (Hornykiewicz, 2002), DA is now understood to play a direct, modulatory role in the elaboration of many behaviors and cognitive states, such as movement (Salamone, 1992), motivation (Wise, 2004), reward (Koob, 1996), attention (Robbins et al., 1998), habit formation (Wickens et al., 2007) and executive function (Robbins, 2003). The bulk of brain DA projections originate primarily from neurons located in the substantia nigra (SN), specifically the pars compacta (SNPc), and the ventral tegmental area (VTA) (Lindvall and Bjorklund, 1978). These projections target cortical and sub-cortical areas: In the rodent, the SNPc DA neurons project to the dorsal striatum (DS) whereas DA neurons from the VTA project to the nucleus accumbens (NAcc) included in the ventral striatum (VS); minor, albeit significant VTA DA fibers also project to the prefrontal cortex (PFC) (Bjorklund and Dunnett, 2007). Molecularly, DA acts on five G protein-coupled receptors (GPCRs) (D1-D5Rs) that are located on post-synaptic target neurons, to initiate multiple signaling cascades that can alter levels of second messengers, modulate neuronal activity, and regulate gene expression (Missale et al., 1998). Importantly, and of relevance to this dissertation, DA also functions in an autocrine fashion, exerting presynaptic (at the

terminal fields in target regions) and somatodendritic (at DA soma and dendrites in the

SNPc and VTA) control of DA neurons themselves, via actions on the D2 autoreceptor

(D2AR) (Ford, 2014). Postsynaptic D2Rs signal through $G_{i/o}$ proteins and is generally

inhibitory in action with respect to neuronal excitability (Surmeier et al., 2010). Similarly,

D2ARs negatively modulate DA neuron activity (Lacey et al., 1987), but also reduce

terminal (Benoit-Marand et al., 2001) and somatodendritic release (Beckstead et al.,

2004) and DA synthesis (Wolf and Roth, 1990). D2AR signaling also diminishes DA

signaling via elevating surface expression of the DA transporter (DAT) (Bolan et al.,

2007), the primary high-affinity mechanism for extracellular DA clearance (Giros and

Caron, 1993). DA reuptake through DAT is tightly regulated through a complex

assemblage of DAT-interacting proteins, thereby providing dynamic control of DA

inactivation (Eriksen et al., 2010). DAT is the target for multiple psychostimulant drugs

of abuse such as cocaine, methylphenidate (MPH) and amphetamine (AMPH) that

elevate extracellular DA levels through their antagonistic actions at DAT (although they

differ in their mechanisms of action, which will be discussed in further detail below)

(Gowrishankar et al., 2014). Hence, it is not surprising that disruptions in DA

homeostasis are implicated in multiple neuropsychiatric disorders. In this chapter, I will

discuss the mechanisms essential for DA neurotransmission, illuminate the importance

of D2AR and DAT to these processes, how alterations in DAT increase risk for brain

disorders, and the efforts in the Blakely lab to model DAT-dependent disruptions in DA

homeostasis for translational insights.

1.2 Dopamine – From Precursor to Neurotransmitter

DA was first synthesized in 1910, and suggested to just be a metabolic intermediate in the synthesis of NE, owing to its presence in small amounts in peripheral tissue heavily innervated by NE-secreting sympathetic nerve fibers (Blaschko, 1957). Focus on brain DA, however began with the discovery of the catecholamine hydroxytyramine (later to be designated as DA) in the brains of multiple mammals by Montagu (Montagu, 1957). Notably, Carlsson and colleagues conducting pioneering work using a novel spectroflourimetry technique and showed that reserpine, through the blockade of the vesicular monoamine transporter (VMAT2), depleted the rabbit brain of DA (and NE) (Carlsson et al., 1958), and that L-3,4-dihydroxyphenylalanine (L-DOPA; a precursor to DA and NE) restored brain DA levels (Carlsson et al., 1957). These observations led Carlsson and colleagues to suggest that DA was indeed an agonist in the central nervous system (CNS), and not just an intermediate to NE. Subsequently, Bertler and Rosengren showed that the majority of DA in the dog brain was found in the striatum, an area with very low levels of NE (Bertler and Rosengren, 1959). Prior knowledge of the lack of extra-pyramidal control over movement in Parkinsons disease (PD) patients (Ehringer and Hornykiewicz, 1960), that PD-like symptoms arise in subjects treated with reserpine (Hornykiewicz, 1962), the central stimulation of movement by L-DOPA (Carlsson et al., 1957), and finally the ability of L-DOPA to reverse movement deficits in PD patients (Birkmayer and Hornykiewicz, 1961) promoted an attribution of DA function to the control of movement. These early studies, however, on the role of DA as an agonist and neurotransmitter in its own right, were met with initial skepticism (Hornykiewicz, 2002), but galvanized efforts to determine sites of

DA localization, and establish mechanisms of CNS DA synthesis, storage, release and inactivation.

1.3 Cellular Localization of Brain Dopamine

The discoveries outlined above provided the impetus to further understand the localization of DA in the brain and to determine whether this localization matched that of NE. Histoflourescence techniques developed by Falck, Hillarp and co-workers enabled the sensitive visualization of monamines, including DA NE and serotonin (5-HT), in specific cell bodies in the midbrain and their axonal projections (Falck and Torp, 1961; Carlsson et al., 1962). Owing to the distinctly colored fluorescence emissions of the three neurotransmitters, these studies provided strong support for DA-specific nuclei and projections and validated the existence of a nigrostriatal DA pathway that had been inferred from the loss of striatal DA following lesion of the SN (Anden et al., 1964). Moreover, removal of projections into the striatum via axotomy in the above study by Anden and workers resulted in an accumulation of fluorescence associated with DA in the cell bodies in the SN. Following the advent of immunohistochemical techniques that utilized antibodies targeted against gene products specific to catecholamine synthesis in the 1970s, mapping of DA neurons was carried out in further detail resulting in the establishment of nine distinct cell groups of DAergic neurons in the midbrain, hypothalamus, olfactory tubercle and retina (A1-9) (Marin et al., 2005).

The work described in this thesis is focused primarily on DA neurons and their projections arising from the SN and VTA, thus warranting a detailed description of

DAergic pathways emanating from the midbrain (Figure 1). In most mammals, midbrain DA neurons are grouped into two major nuclei– SNPc and the VTA. The DA neurons from the SNPc project primarily to the DS - also termed the caudate-putamen or striatum in rodents but which is divided into the caudate nucleus and putamen in humans - forming the nigrostriatal DA pathway (Bjorklund and Dunnett, 2007). The studies outlined above established that this pathway is critical in the control of movement and subsequent efforts found that SNPc DA neurons are also essential in the development and maintenance of habits (Wickens et al., 2007). The DA neurons in the VTA send projections to limbic regions in the brain concerned with the control of affect such as NAcc included in the VS, the ventral hippocampus and the amygdala, known as the mesolimbic DA pathway (Bjorklund and Dunnett, 2007). This pathway involves primarily the perception and modulation of reward, motivation and salience (Berridge and Robinson, 1998), though studies have also identified roles for mesolimbic neurons in aversion (Pignatelli and Bonci, 2015). A subset of VTA DA neurons also project to cortical areas such as the orbitofrontal, cingulate and prefrontal cortex making up the mesocortical DA pathway (Fallon and Moore, 1978), where DA is thought to regulate cognition and executive function (Robbins, 2003). DA neurons, based on their site of origin (SNPc vs VTA) differ widely in their intrinsic properties and the behaviors they regulate and this diversity in DA neuron anatomy, physiology and function (Roeper, 2013) will be discussed in more detail later in this chapter. In addition to the SNPc and VTA, DA neurons are also found in the retrorubral field (RRF) in the midbrain (Gasbarri et al., 1996), within the dorsal raphe (a nucleus comprised of predominantly of 5-HT synthesizing neurons) (Stratford and Wirtshafter, 1990), which are thought to mediate
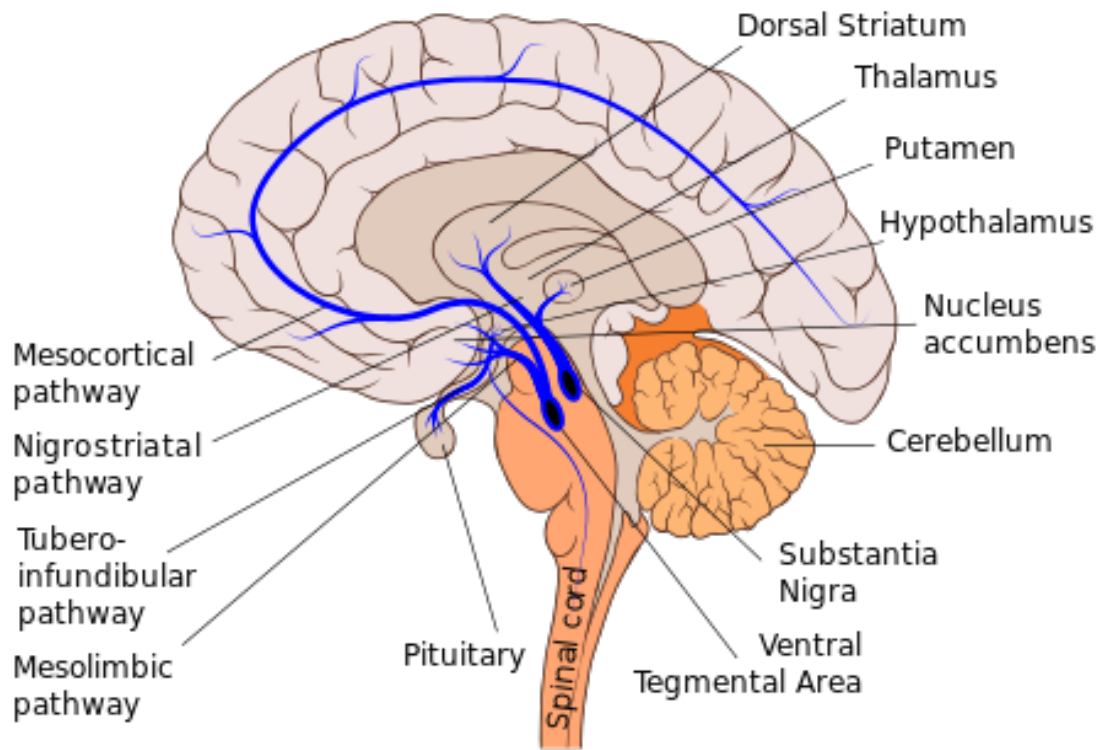
Figure 1. The dopaminergic pathways in the brain. Highlighted above are the 4 CNS DA pathways – Nigrostriatal, Mesolimbic, Mesocortical and Turbero-Infundibular. Image freely available via Wikimedia Commons.

circadian activity (Cho et al., 2017) and sociability (Matthews et al., 2016). Outside of the midbrain, DA neurons found in the arcuate nucleus of the hypothalamus project to the median eminence, regulating prolactin release from the anterior pituitary gland, making up the tuberoinfundibular pathway (Hu et al., 2004). DA neurons are also found in the olfactory bulb, concerned with odor processing (Escanilla et al., 2009) and in the retina, involved in light/dark adaptations (Witkovsky and Schutte, 1991).

1.4 Dopamine Synthesis and Storage

Hypotheses related to a pathway for DA synthesis emerged from the work of Blaschko after the discovery DOPA decarboxylase, albeit only as a precursor in NE synthesis (Bhagvat et al., 1939; Blaschko, 1957). However, it wasn't until the discovery of NE and DA in equal amounts in chromaffin tissue and the subsequent demonstrations of rapid L-DOPA induced elevations in brain DA , that the first step in this pathway was thought to be rate limiting (Carlsson, 1960). As studies began to cement brain DA as a neurotransmitter in its own right, tyrosine hydroxylase (TH) was shown to be inhibited by DA (Nagatsu et al., 1964), later under physiological conditions that culminate in a pathway for DA synthesis (Carlsson et al., 1976). It is now widely known that DA synthesis initiates with hydroxylation of the amino acid tyrosine by TH, resulting in the formation of L-DOPA. L-DOPA is then converted to DA by aromatic acid decarboxylase (AADC) or DOPA decarboxylase (Figure 2). Multiple mechanisms in addition to feedback inhibition by DA and DA neuron activity have been shown to regulate DA synthesis (Kumer and Vrana, 1996). As the rate-limiting enzyme in DA synthesis, TH activity is now widely to infer overall DA synthesis rates. Indeed, among
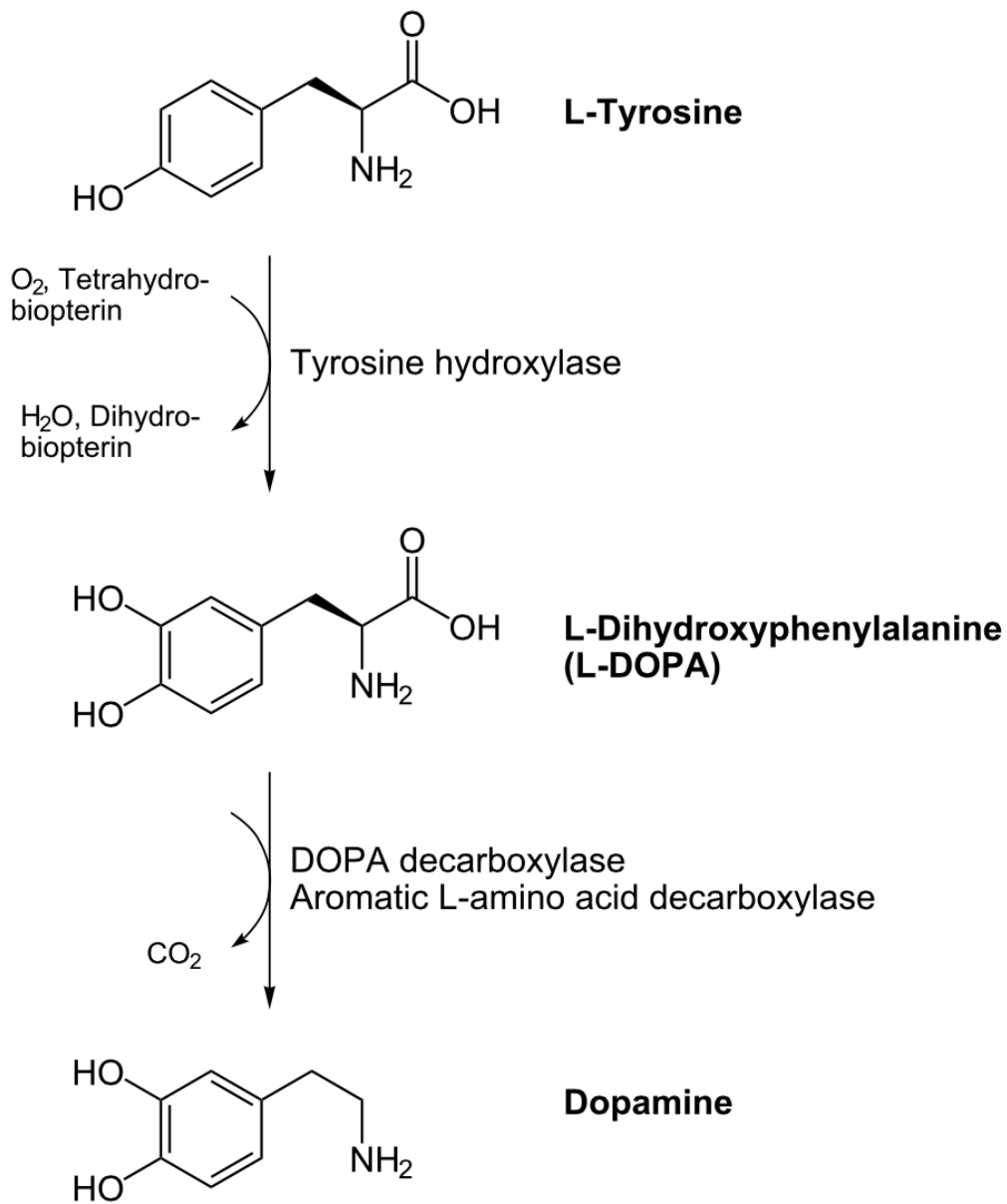
Figure 2. Dopamine synthesis. DA is synthesized from L-Tyrosine, which is converted to L-DOPA by TH. L-DOPA is converted to DA by DOPA Decarboxylase. Image freely available via Wikimedia Commons.

the many techniques used to monitor TH activity, L-DOPA accumulation (methodology discussed in further detail in Chapter 3) following the inhibition of AADC (Carlsson et al., 1972) remains a powerful and popular approach (Bello et al., 2011). Upon synthesis, DA is packaged into synaptic vesicles, a process both essential to electrically evoked neurotransmitter release (Eiden and Weihe, 2011) as well as to limit the neurotoxic actions of DA oxidation products (Guillot et al., 2008). The importance of the mechanisms employed within DA neurons to remove cytosolic DA will be more apparent when I discuss modes of DA inactivation and the ability of DAT to efflux DA. DA transport into synaptic vesicles is carried out by the vesicular monoamine transporter 2 (VMAT2) (Erickson et al., 1992), an H^+-ATPase antiporter (Reimer et al., 1998) that is energized by the vesicular H^+ gradient established by the vacuolar ATPase (Moriyama and Futai, 1990). The ATPase establishes a pH gradient within the synaptic vesicle, enabling VMAT2 to transport in to the vesicle lumen a molecule of DA for every two molecules of H^+ extruded into the cytosol (Rudnick et al., 1990). Mice completely devoid of VMAT2 are postnatally lethal, owing to a loss in feeding with reduction in DA, NE and 5HT tissue content (Wang et al., 1997; Mooslehner et al., 2001); conversely, animals overexpressing VMAT2 have an enhancement in vesicle size and a concurrent elevation in monoamine-associated behaviors (Lohr et al., 2014). Thus VMAT2 plays an essential role in the sequestration of DA into synaptic vesicles, enabling the release of DA on demand and keeping cytosolic DA levels in check (Lohr et al., 2017).

1.5 Electrophysiological Properties of Dopamine Neurons

The identification of distinct DAergic nuclei, capable of synthesizing and storing DA within the brain enabled pioneering studies on the electrophysiological properties of DA neurons (Bunney and Aghajanian, 1977). It has since been found using *ex-vivo*, slice whole-cell patch clamp and *in vivo* electrophysiology techniques that DA neurons exhibit three different states of activity – a hyperpolarized, inactive state, a "tonic" state of spontaneous activity and a "phasic", depolarization-dependent, burst-firing mode of activity (Grace and Bunney, 1983). Depending on the region however (SNPc vs. VTA, or lateral to medial gradients within these regions), the existence of these different modes of activity and the mechanisms shaping it are found to be different (for a detailed review, see (Gantz et al., 2018). The inactive, hyperpolarized state of DA neurons is dependent upon local control by inhibitory γ-aminobutyric acid (GABA) secreting interneurons and by GABAergic afferents onto DA cell bodies and dendrites (Grace and Bunney, 1985). Tonic spontaneous firing is controlled by intrinsic mechanisms involving multiple ion channels in the DA cell bodies ensuring pacemaking activity (Grace and Bunney, 1984). Phasic, burst firing is initiated by extensive afferent input, and is often succeeded by a pause in activity, followed by the resumption of spontaneous firing (Overton and Clark, 1997). DA neurons receive input from both GABAergic and glutamatergic afferents from a variety of cortical, subcortical and brainstem nuclei. GABAergic interneurons within the midbrain and from the striatum (DS and VS), rostrotegmental nucleus (Kaufling et al., 2009), the ventral pallidum (Grace et al., 2007) and the substantia nigra pars reticulate (Morikawa and Paladini, 2011) provide powerful inhibitory control of DA neuron activity via the activation of ionotropic (GABA-A) and

metabotropic (GABA-B) receptors (Liss and Roeper, 2008). Conversely, DA neuron activity is initiated and maintained by glutamatergic input from cortical, thalamic and hindbrain nuclei, and also by glutamatergic neurons within the SNPc or VTA that function as interneurons, a much more recent finding (Dobi et al., 2010). DA neurons express both AMPA and NMDA-type glutamate (GLU) receptors, ionotropic receptors that control DA activity and synaptic plasticity (Bonci and Malenka, 1999; Overton et al., 1999), and metabotropic Glu receptors (mGluRs) that regulate activity through G protein-dependent signaling cascades at slower timescales (Fiorillo and Williams, 1998).

The transition from tonic to phasic (or burst) firing in DA neurons is triggered by reward prediction error (RPE), a cognitive response that is associated with reward learning. Pioneering studies from Schultz and many others have found that phasic firing of DA neurons is initiated when a subject (rodent or monkey) encounters an unexpected reward; however, DA neuron firing is absent when the animal encounters the same reward in the future and is suppressed when the reward is omitted altogether (Hollerman and Schultz, 1998; Schultz, 1998; Roesch et al., 2007). This difference in expected vs. received reward is known as the RPE, a now well-characterized phenomenon intrinsic to DA neurons in both the SNPc and VTA (Schultz et al., 2017) that encodes features informing reward value such as probability (Fiorillo et al., 2003), magnitude (Bayer and Glimcher, 2005; Tobler et al., 2005) and preference (Kobayashi and Schultz, 2008). Recent studies involving optogenetics, which enables spatiotemporal control of DA neurons through genetically-encoded, light-sensitive ion channels, have furthered our understanding of the importance of phasic DA neuron

activity (Cohen et al., 2012). Tsai and colleagues were the first to demonstrate that phasic DA neuron activity is essential to the encoding of conditioned behaviors, thereby attributing causality to behaviors thought to be mediated by DA (Tsai et al., 2009). Studies since then have further explored the importance of phasic DA in encoding the various components of RPEs, locomotor responses and other aforementioned behaviors (Watabe-Uchida et al., 2017).

Tonic DA firing has received far less attention compared to phasic activity. It is widely known that pace-making activity establishes a basal extracellular DA tone (Grace, 1991), but only recently have studies attributed a more specific role for tonic DA in constraining phasic DA activity and attributed specific roles for tonic DA in behavior. Tye and colleagues showed that an imbalance in tonic/phasic DA results in the inhibition of hindbrain control of phasic DA neuron activity in the VTA, resulting in elevated DA release (Tye et al., 2013). Behaviorally, tonic DA in the striatum has also been shown to be important in controlling instrumental avoidance via positive RPEs (Dombrowski et al., 2013). In the VTA, through control by the ventral pallidum (Floresco et al., 2003), tonic activity maintains response to novelty-associated information (Lisman and Grace, 2005; Grace et al., 2007). More recently, tonic activity of DA projections to the PFC has been associated with sustaining behavioral flexibility (Ellwood et al., 2017).

1.6 Dopamine Release

Among the many studies of significance to establish DA as a neurotransmitter was the demonstration of the neurotransmitter's activity-dependent release. Portig and workers first found elevations in DA in the caudate nucleus upon stimulation of the cat substantia nigra via superfusion (Portig and Vogt, 1969). In addition to providing support for the existence of DAergic pathways in the brain, specifically the nigrostriatal pathway in this case, these findings gave rise to multiple studies integral to our understanding the molecular mechanisms of DA release and the development of new methods to assay activity-dependent DA release. Of all the methods available to assay DA release, the use of radiolabeled superfusion techniques ([^{3}H]DA, discussed in further detail in Chapter 2) has been a highly quantitative and efficient way of determining DA release from brain tissue (Mergy et al., 2014b). This technique involves the incubation of extracted striatal or cortical tissue with [^{3}H]DA. Following the uptake of the radioisotope into DA terminals via DAT and its packaging into synaptic vesicles by VMAT2, vesicular DA release can be stimulated electrically, or via perfusion of high potassium (KCl) or 4-aminopyridine (4-AP, a K$^+$ channel blocker). The resultant perfusate can be collected and the amount of [^{3}H]DA determined using scintillation spectrometry, providing a measure of the capacity for DA release from the DA terminals in the tissue (Figure 3). This technique however, does not report the release of endogenous DA. To determine endogenous DA release, complementary approaches are often implemented that can quantify DA with specific physiochemical methods, as with high performance liquid chromatography (HPLC) (Luderman et al., 2015) or electrochemical detection of DA by oxidation (Bucher and Wightman, 2015). Moreover, superfusion methods are no
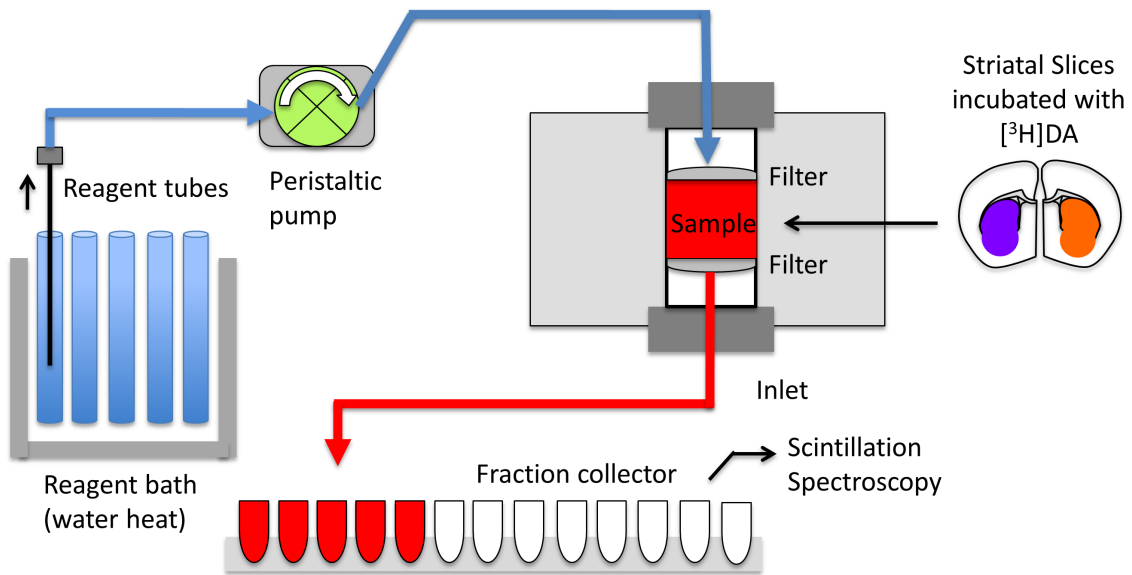
Figure 3. Radiolabelled dopamine superfusion. Striatal Slices are first incubated with [^{3}H]DA and placed in the sample chamber. Reagents to evoke DA release are then perfused through the chamber and the perfusate is collected. [^{3}H]DA in the perfusate fractions are analyzed for radioactivity as a proxy for DA release via scintillation spectroscopy.

spatiotemporally precise and are typically performed only on DA terminals in excised tissue. In order to circumvent around these limitations, Ralph Adams and colleagues first studied the electrochemistry of a variety of biogenic amines and found that monoamines including DA can be easily oxidized (to dopamine-o-quinone, in the case of DA) in response to applied voltage, yielding an oxidation current that is proportional to DA concentration (Kissinger et al., 1973). Many labs since have implemented this approach, and of the multiple variations of electrochemical techniques used to detect DA, either amperometry and fast-scan cyclic voltammetry are most widely used. Amperometry involves the use of a carbon fiber electrode held at a constant voltage higher than the oxidation potential of DA, thereby generating a current that corresponds to the amount of DA being oxidized at the electrode (Adams, 1976). This technique however, is fraught with issues of selectivity and is mostly used in cell culture preparations where the chemical species is clearly evident, such as with chromaffin (Petrovic et al., 2010) or mast cells (Manning et al., 2012), and the size of the preparation allows one to detect vesicular release events (Mosharov and Sulzer, 2005). To improve on analyte specificity, electrodes can be coated with resins (e.g. Nafion) that prevent oxidizeable molecules distinct from DA from reaching the electrode (Hashemi et al., 2009). Owing to selectivity issues, amperommetry has been adapted to be used to measure clearance of oxidizable monoamines via high-speed chronoamperometry (HSC), by creating an electrode-pipette assembly, with the pipette being filled with a known concentration of analyte. Following pressure ejection of the analyte, the clearance or decay of the oxidation current detected at the electrode can be used a direct measure of monoamine clearance. This technique has been utilized by many to

determine DA and 5-HT clearance (Zahniser et al., 1998; Daws et al., 2005), and also used in Chapter 3, to assay DA clearance (Figure 4). In addition to amperommetry, others have employed ramps of voltage, as with fast-scan cyclic voltammetry (FSCV), to generate a more precise oxidation-reduction fingerprint of the analyte. FSCV involves the application of a waveform (predominantly of a triangular shape) at a high sampling rate (e.g.-0.4V to +1.3V at 400 V/sec, repeated at 100 ms intervals in the case of DA) that results in a cyclic voltammogram specific for the substrate in question, with peak oxidation currents typically used for quantification. Numerous labs have employed FSCV *ex vivo* in striatal slices preparations (Calipari et al., 2014) and *in vivo*, in either anesthetized (Hashemi et al., 2012) or freely moving rodents (Carelli and Wightman, 2004), non-human primates (Alikaya et al., 2018) and more recently humans (Kishida et al., 2016; Moran et al., 2018). Typically the latter effort involves the surgical implantation of carbon fiber microelectrodes into brain regions of interest to determine fluctuations in endogenous DA following electrical stimulation, or more recently, using optogenetics to specifically evoke DA release from genetically-specified DA projections (McElligott, 2015). Although FSCV is spatiotemporally precise and is widely used, it does not account for absolute values of DA or in measurements of baseline extracellular DA. To obtain measurements of absolute values of extracellular DA (and baseline DA), investigators often resort to *in vivo* microdialysis methods (Mergy et al., 2014b). This technique involves the surgical implantation of a semi-permeable dialysis probe. Perfusion fluid is circulated inside the probe across which small molecules are exchanged. The dialysate removed from the brain during perfusion can then be assayed for analyte concentrations using HPLC or mass spectrometry. Although microdialysis
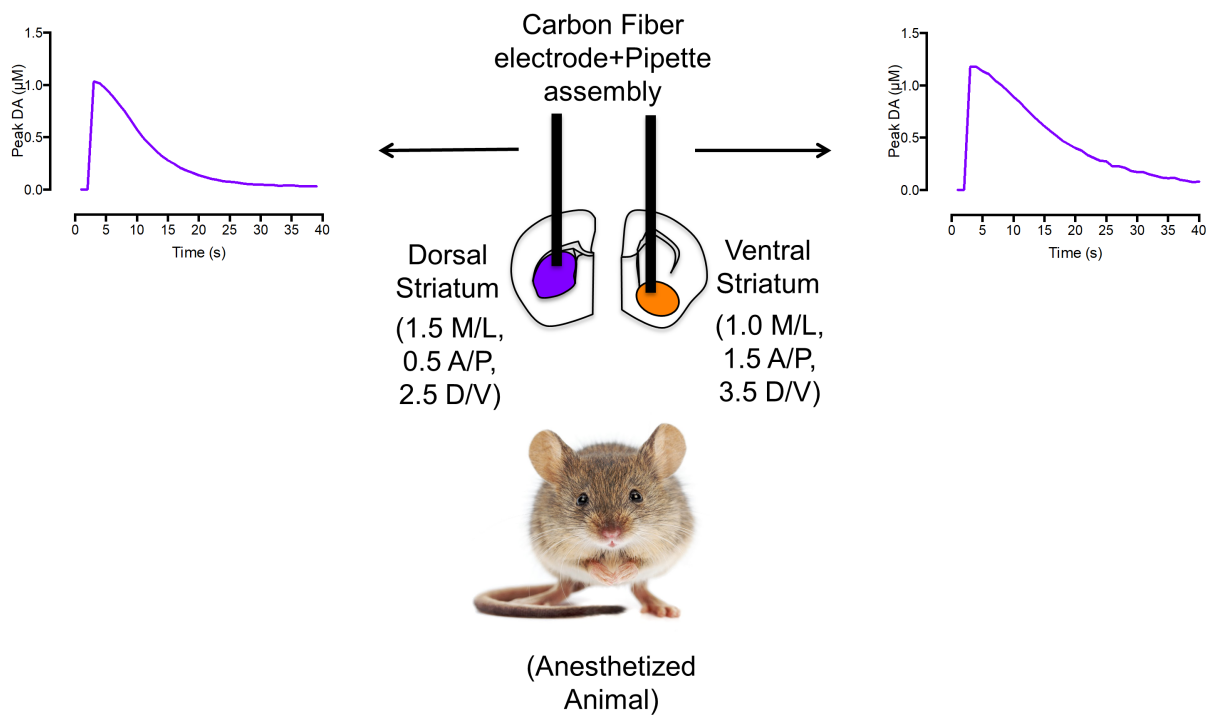
Figure 4. Dopamine high speed chronoamperometry. Animals are anesthetized and a carbon fiber and pipette assembly, with the pipette containing 200 µM DA is lowered into either the dorsal or ventral striatum using the coordinates indicated. DA is pressure ejected, resulting in a peak oxidation current, following which DA clearance can be measured.

enables the determination of baseline levels of DA and fluctuations of extracellular DA in response to stimulation or the application of drugs impacting the DAergic system, this approach is not as temporally precise as FSCV with time points typically gathered every 1-10 minutes. Recent studies led by the Kennedy group however, have coupled segmentation flow and capillary electrophoresis with laser-induced fluorescent detection to enable ultra-fast sampling of perfusate in second timescales, with the promise of greater temporal resolution (Wang et al., 2016).

Following the packaging of DA into synaptic vesicles (as discussed above) the neurotransmitter can be released via depolarization-elicited vesicular fusion mechanisms (Sulzer et al., 2016). Studies have also recently shown that vesicular packaging of DA is also activity-dependent, interestingly through the actions of the vesicular glutamate transporter (VGLUT2), also expressed in a subset of DA neurons (Aguilar et al., 2017). DA synaptic vesicles are found not only in the projections (in the DS, VS and PFC), but also in significantly smaller quantities in the somatodendritic compartments of midbrain DA neurons (Sesack et al., 1995; Lewis et al., 1998; Sesack et al., 1998). Somatodendritic DA release has been shown to dampen DA neuron activity (Cheramy et al., 1981; Rice et al., 1997)by signaling through the D2AR on neighboring DA soma and dendrites (Beckstead et al., 2007). D2AR stimulation results in a reduction in neuronal firing through the activation of G protein-coupled inwardly-rectifying potassium channels (GIRK) by Gβγ-mediated signaling that can be detected as an outward current (D2AR-IPSC or D2AR-mediated inhibitory postsynaptic current) in DA cell bodies when measured via whole-cell patch clamp electrophysiology (Beckstead et al., 2004). D2AR activation has also been found to inhibit P and N/Q-type

calcium channels on midbrain DA neurons (Cardozo and Bean, 1995). Finally, somatodendritic DA release can either inhibit or activate GABAergic synapses onto DA neurons, through actions on D2- or D1-type heteroreceptors expressed presynaptically on GABA terminals (Cameron and Williams, 1993; Matsui et al., 2014).

The majority of the studies on DA release and the mechanisms controlling release have been pursued in the target regions where DA projections lie. Electrical and optogenetic stimulation of DA terminals in the striatum result in DA release, however a more complex picture arises when trying to determine the source of this input (Cachope and Cheer, 2014). The striatum receives glutamatergic input from the cortex, thalamus, amygdala and the hippocampus (Sesack and Grace, 2010; Stuber et al., 2012) and studies have shown that stimulation of these inputs can evoke DA release through the activation of AMPA and NMDA receptors on DA terminals (Imperato et al., 1990). mGLURs are also found on DA projections and their direct actions have also been demonstrated to regulate DA release (Cheramy et al., 1991; Desce et al., 1991; Galli et al., 1991; Krebs et al., 1991). Interestingly, stimulation of cholinergic interneurons in the striatum and subsequent release of acetylcholine (ACh) can also enhance DA release in the striatum via the activation of either nicotinic ionotropic or muscarinic metabotropic receptors found on DA axons (Threlfell and Cragg, 2011). Further complexity arises owing to the fact that a subset of DA neurons also co-release DA and GLU (Stuber et al., 2010), however it is unknown if the stimulation of DA release occurs through the activation of GLURs; or the activation of the cholinergic interneurons (Zhang and Sulzer, 2012). Finally, as is the case with somatodendendritic DA release, axonal DA release can also be inhibited by the activation of D2ARs (Benoit-Marand et al., 2011), through

the inhibition of vesicular fusion and DA biosynthesis (Wolf and Roth, 1990).

It is important to mention that DA neurons, in addition to releasing DA, have also been shown to package and release GLU and GABA, whereby they can mediate fast excitatory or inhibitory neurotransmission in target neurons, as opposed to the slower, modulatory action typical of DA. Fast excitatory transmission upon DA terminal excitation in MSNs was observed as early as 1976 (Kitai et al., 1976), but it was only with the advent of optogenetic techniques that enable specific DA terminal excitation that studies have been able to delineate if and how this happens (Hnasko and Edwards, 2012). Studies since have shown that GLU is transported into vesicles via VGLUT2 that helps to acidify the vesicle interior, thereby enabling more efficient packaging of cationic DA into the same vesicles (Hnasko et al., 2010); however whether DA and GLU are packaged into the same vesicles in all DA neurons positive for VMAT2 and VGLUT2, and why, is still a matter of debate. Additionally, studies have also shown the capacity for GABA release from DA projections, with the GABA transporter (GAT) on DA terminals mediating reuptake of local GABA, which can then be packaged into vesicles, possibly via VMAT2 (Tritsch et al., 2012; Tritsch et al., 2014; Tritsch et al., 2016). Post-synaptically, studies have found that this GLU release induces an EPSC in striatal MSNs (Hnasko and Edwards, 2012), and the GABA release induces an IPSC (Tritsch et al., 2012), although the magnitude of this release and its co-occurrence, in addition to DA release are yet to be determined. Whether this GLU and/or GABA release occurs concurrently with DA release upon terminal excitation, and if the mechanisms controlling it (i.e. D2AR-mediated inhibition) are the same, remains to be determined. Additionally, questions remain as to the post-synaptic populations (i.e. D1 vs. D2-MSNs or

cholinergic/GABA interneurons) or compartments (soma vs. dendrites) targeted by GLU and/or GABA. Moreover, it is also unknown if GLU or GABA release exists in the somatodendritic compartment making release of neurotransmitters from DA neurons an exquisitely complex field of study.

1.7 Dopamine Reuptake and Inactivation

Following activity-dependent release, DA can be cleared from extrasynaptic space by DAT. This concept was suggested to be essential for neurotransmitter inactivation as early as 1960, when Axelrod discovered the reuptake of catecholamines into adrenergic nerves (Glowinski and Axelrod, 1964). Two decades later, DAT was implicated in DA reuptake through its isolation in the rat brain (Giros and Caron, 1993). The expression of DAT cDNA in heterologous cell systems demonstrated high-affinity transport of radiolabeled DA, blocked by psychostimulants that directly target the transporter. Recycled DA can be repackaged for future vesicular release (Gowrishankar et al., 2014). A full elaboration of DAT activity and its contributions to DA signaling are discussed later in this chapter. Additionally, cytosolic DA, upon its synthesis or reuptake can be inactivated via its metabolism by monoamine oxidase (MAO) and catechol-o-methyltransferase (COMT), albeit via different mechanisms (Kopin, 1985). MAO converts cytosolic DA to 3-4-dihydroxyphenyl acetic acid (DOPAC) and COMT results in the formation of 3-methoxytyramine (3-MT), that can both be metabolized to homovanillic acid (HVA).

1.8 Regional Diversity in Dopamine Signaling

The development of techniques to assay DA neuron activity, release and inactivation, in addition to advancing our understanding of the core molecular components of DA signaling, also enabled the discovery and understanding of intrinsic diversity in midbrain DA neurons. Diversity in DA neurons is observed in their intrinsic electrophysiological properties, which depends on their location, in the synaptic inputs they receive and the target regions they project to, ultimately dictating the specific behaviors that DA neurons are reported to mediate (Roeper, 2013). This "multi-chotomous" nature of DA neurons arises right from their development, with studies showing differences in the expression of transcription factors (Kadkhodaei et al., 2009)associated with the development of neurons based on the sub-region of the midbrain, or the expression pattern of DA neuron-associated genes, such as GIRK2 or DAT (Lammel et al., 2008). The diverse expression of transcription factors is quite reliable in predicting not only the location of populations of DA neurons in the midbrain, but also their projection targets (i.e. striatum vs. PFC) (Andressoo and Saarma, 2008). Beginning with the identification of the nigrostriatal DA pathway and its implication in PD, DA neurons were linked to distinct projections that enervate different regions in the cortex and sub-cortex, depending on their location in the midbrain (Chuhma et al., 2017). Our understanding of the complexity of these projections, however, has evolved beyond the existence of the nigrostriatal and mesocorticolimbic pathways, for example that medial DA neurons project predominantly to the VS, and the more lateral neurons in the SNPc to the DS (Yetnikoff et al., 2014). Additionally, there are DA neuron populations along the VTA/SNPc border that do not confirm to this topology (Bjorklund

and Dunnett, 2007). Furthermore, medium spiny neurons project back to the midbrain, targeting GABA interneurons or DA neurons in a similar pattern, with neurons in the DS projecting back to the ventral midbrain and those in the VS to the dorsal midbrain (Parent, 1990).

As described in a previous section, DA neurons exhibit tonic and phasic states of activity and whereas tonic activity arises from intrinsic channel activity, phasic activity is efferent excitation-dependent (Grace et al., 2007). Differences exist also in how tonic activity in DA neurons is maintained, with DA neurons in the SNPc and VTA employing different channels and mechanisms to establish basal tone (Wolfart et al., 2001; Khaliq and Bean, 2010). Interestingly, phasic or burst firing of DA neurons also exhibits a gradient, with the activity being the greatest in the medial VTA, and the least in the lateral SNPc (Schiemann et al., 2012). Surprisingly, an opposite gradient exists in NMDA receptor expression, suggested to be essential in the genesis and maintenance of burst firing (Zweifel et al., 2009), with studies suggesting that the gradient in burst firing is also modulated by intrinsic channel activity, in addition to AMPA and NMDA receptors (Krabbe et al., 2015). Furthermore, although studies suggesting differential expression of DAT and TH exist, it is still to be determined if this reflects changes in expression patterns of these key DA-linked genes, or differences in DA neuron population.

Diversity has also been observed in DA terminal density, release, reuptake and the mechanisms controlling release. Studies have reported the highest density of DA terminals in the DS, with the lowest density in the VS, specifically the NAcc shell

(Doucet et al., 1986). A similar gradient has also been observed in DA release and reuptake using slices from mice, rats and monkeys, with the highest magnitude of release and reuptake in the DS, and the lowest in the VS (Calipari et al., 2012). With regards to the release of GABA and GLU in addition to DA, a similar dichotomy has been found, wherein GLU release has been shown to occur only in the DA terminals in the VS, but not the DS (Stuber et al., 2010). Studies to date suggest that GABA release is a property of subpopulations of all DA projections. Differences in DA terminals also extend to the mechanisms by which they are regulated. As discussed earlier, DA release, in addition to action potential propagation from the soma, can also be induced by cholinergic interneuron activity, via the activation of nicotinic ACh receptors on DA terminals. Interestingly, studies have observed marked differences in specifically the cholinergic modulation of DA release in NAcc shell, compared to NAcc core and the DS (Threlfell and Cragg, 2011). Finally, modulation of MSNs and cholinergic interneurons by DA, GLU and GABA release from DA terminals also markedly varies across the DS and VS (Chuhma et al., 2017; Mingote et al., 2017).

In discussing inherent diversity in DA neuron anatomy and physiology, it is important to consider if this diversity translates to the behaviors supported by DA signaling. Studies prior, employing lesion-based approaches to eliminate VTA or SNPc have not supported a simple, dichotomous contribution to behavior. Furthermore, transgenic approaches resulting in the complete elimination of TH following genetic supplementation in the VTA or SNPc or L-DOPA treatment to boost DA synthesis, have also yielded results that do not fully support the control of specific behaviors by any one population (Palmiter, 2008). However, elegant studies in monkeys using single-unit

recordings from DA neurons (Matsumoto and Hikosaka, 2009) and, subsequently, optogenetically-identified DA neurons in mice (Cohen et al., 2012), and the advent of imaging techniques to assay DA terminal and soma activity (Parker et al., 2016) have corroborated the possibility of different facets of behaviors normally controlled by DA, to be supported by specific DA subpopulations. Indeed, utilizing these approaches, studies have shown that DA terminals in the VTA control response to reward and motivation, whereas those in the DS are responsible for the control of movement (Howe and Dombeck, 2016). Furthermore, studies have also shown that DA terminal populations in the dorsomedial (DMS) vs. the dorsolateral striatum (DLS) control facets of behavior in response to valence of stimuli (Lerner et al., 2015).

When considering the impact of DA neuron diversity, it is also important to consider that further microheterogeneity exists within DA neurons in the VTA and SNPc. A subpopulation of VTA DA neurons projects to the PFC, with electrophysiological properties completely distinct from those that impinge on the VS, and that behaviorally, mediate the effects of stress (Lammel et al., 2011; Lammel et al., 2014). In the striatum, studies have shown the existence of patch and matrix components, depending on the expression of different transcription factors and the inputs these sub-regions receive (Crittenden and Graybiel, 2011). Studies have recently reported differences in the properties of DA terminal release and its regulation by psychostimulants in the patch vs. matrix compartments (Salinas et al., 2016). Additionally, a medial to lateral gradient in the SNPc also exists, that project to the DMS vs the DLS and which display different terminal activation properties in response to stimuli (Lerner et al., 2015). Finally, the striatum itself is compartmentalized in many ways, i.e. patch vs. matrix (Crittenden and

Graybiel, 2011), or associative vs. sensorimotor (Gremel and Lovinger, 2017), based on the projections it receives and the behaviors each of these sub-regions control (Chuhma et al., 2017). Thus, the assessment of if and how diversity in DA populations projecting to these sub-structures contributes to different aspects of behavior remains an active area of investigation.

1.9 Dopamine Transporter in Neurotransmission

Reuptake of DA through DAT, in addition to diffusion, is the primary mechanism for the termination of DA signaling at the synapse. DAT is a member of the solute carrier 6 (SLC6) family of transporter proteins, 12 transmembrane domain-containing proteins that use the cotransport of Na^+ and Cl^- across the membrane for the transport of their substrates against the concentration gradient. Like D2AR, DAT is expressed on both somatodendritic and presynaptic compartments of DA neurons (Nirenberg et al., 1996; Nirenberg et al., 1997b; Nirenberg et al., 1997a). D2ARs can be isolated in a physical complex with DAT (Lee et al., 2007), though existing data do not rule out the possibility of populations of uncomplexed D2AR and DAT; nor do we understand if these complexes are static/constitutive or transient/regulated. Furthermore, the bulk of data related to DAT/D2AR interactions, structural or functional, has been found in cell systems transfected with D2AR and DAT, or through the use of striatal synaptosomes, that investigators often deem appropriate to maintain a connection to the actual presynaptic components of DA neurons. D2AR stimulation has been shown to elevate surface density of DAT proteins in transfected cells (Bolan et al., 2007), striatal synaptosomes (Chen et al., 2013) and as will be demonstrated later, in striatal slices,

with the result being a parallel elevation in DA transport capacity. Conversely, D2AR antagonism in the striatum can inhibit DA clearance (Cass and Gerhardt, 1994) suggesting that D2AR signaling establishes a dynamic, ongoing control of DAT activity at DAergic synapses. D2AR has been shown to regulate DAT via the kinases PKCβ and ERK1/2 (Bolan et al., 2007; Chen et al., 2013); however the details of this signaling cascade as to what (if any) post-translational modifications to DAT, or changes in interacting partners induced through the actions of these kinases are yet to be determined. In the soma and dendrites, proximity of D2AR and DAT or potential D2AR-DAT signaling mechanisms are as yet unknown.

DAT is the target of a variety of psychostimulant drugs of abuse all of which act to antagonize the transporter and elevate extracellular DA levels, but with different mechanisms of action. Cocaine and MPH are conventional DAT antagonists that block DA reuptake, thereby elevating DA levels during ongoing vesicular DA release (Gether et al., 2006). In contrast AMPH, in addition to blocking DAT activity as an antagonist, also acts as a substrate that is transported into the cell through DAT. AMPH, once inside the cell, acts as a weak base substrate for VMAT2, sequestering protons and blocking neurotransmitter packaging (Sulzer et al., 2005). Besides being a substrate for DAT, AMPH alters transporter conformation, placing DAT in an "efflux-prone state", a process that involves a cascade of steps that involve changes in DAT phosphorylation (Khoshbouei et al., 2004) and protein associations (Eriksen et al., 2010). These changes lead ultimately to the extrusion of cytoplasmic DA from the cell, a process known as non-vesicular DA release, DA efflux or DAT-mediated DA release (Figure 5). Hence, an important distinction in the actions of AMPH vs. conventional DAT
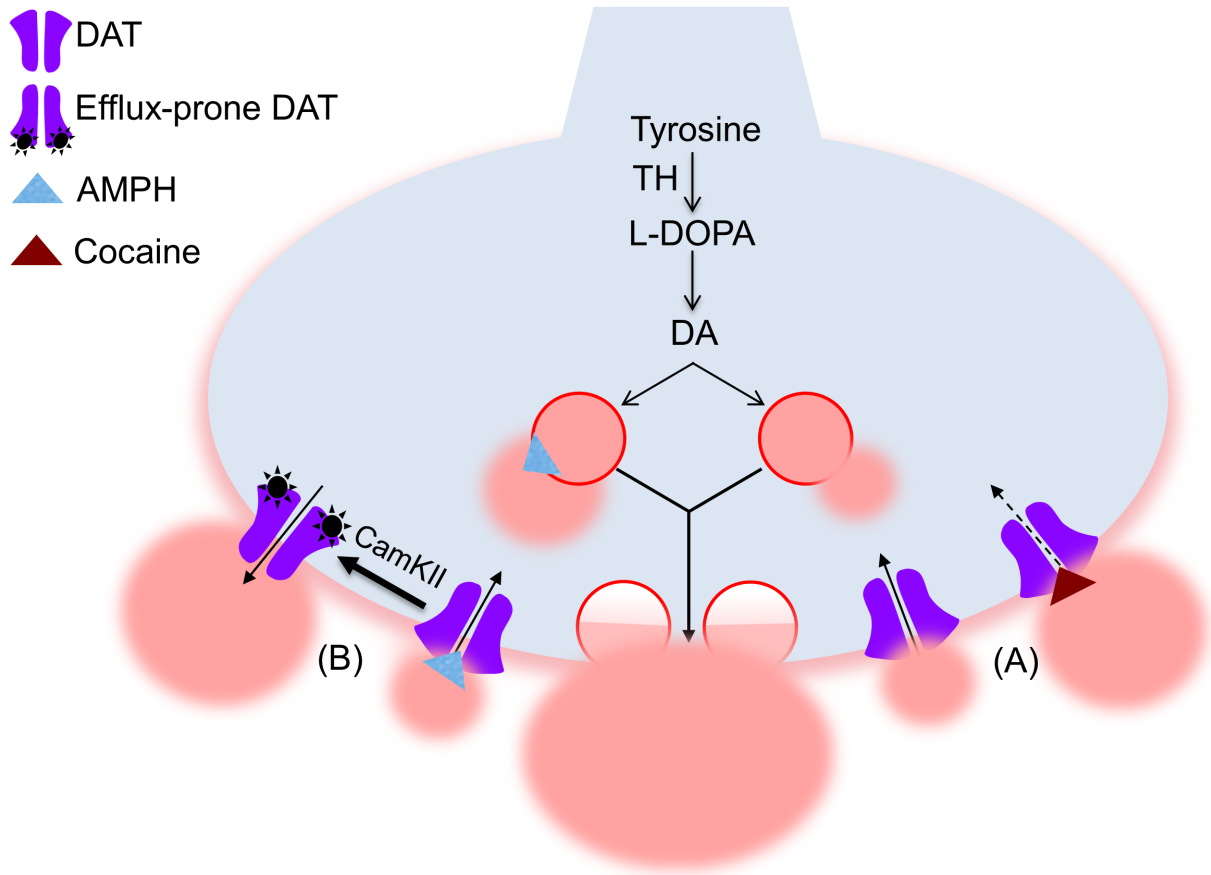
Figure 5. Differential actions of psychostimulants on DAT. At DA terminals, (A) cocaine acts as a conventional antagonist, blocking DA reuptake and elevating DA levels at the synapse. (B) AMPH acts as a substrate, blocking reuptake and vesicular packaging of DA by blockade of VMAT2, and changing the conformation of DAT from "uptake willing" to "efflux prone", resulting in DAT-mediated DA release.

antagonists, one that will become important soon, is that AMPH-induced, transporter-mediated non-vesicular DA efflux is not contingent upon activity-dependent vesicular DA release. Indeed, the action of AMPH can be seen as moving synaptic DA release away from, rather than amplifying, activity-dependent vesicular DA release. Evidence also suggests that DAT is localized to cholesterol-rich membrane microdomains, sometimes referred to as "lipid-rafts", with studies by Cremona and colleagues indicating that DAT interactions with the raft-associated protein flotillin-1 is necessary for AMPH-induced DA efflux (Cremona et al., 2011). However, the importance of this interaction in driving efflux-prone conformations, in mediating mobility across the membrane, or how it might influence perturbations induced by rare variation in DAT remains to be established. Prolonged AMPH application has also been shown, in heterologous cells, to promote DAT internalization (Saunders et al., 2000). Owing to evidence for a physical interaction between D2AR and DAT, and the D2AR-mediated regulation of transporter function, further efforts are needed to explore if and how AMPH-induced DAT trafficking relies on receptor/transporter interactions. In the soma and dendrites, inhibition of DAT can elevate the amplitude and prolong the decay of D2AR-mediated IPSCs,and inhibit burst firing of DA neurons, presumably through the elevation of extracellular DA levels and concurrent D2AR activation (Branch and Beckstead, 2012).

Several labs over the years have carefully considered the importance of DA clearance and DAT's ability to maintain DA homeostasis in DA neurotransmission. For example, studies have found that DA clearance by DAT contributes differentially to tonic vs. phasic DA signaling. Burst firing of DA axons in the NAcc produces transient

increases in high concentrations of synaptic DA and unless DAT is blocked, extracellular DA elevations are not detected (Floresco et al., 2003). These findings indicate that DAT activity constrains DA availability to specific synapses and ensures that synaptic DA matches the demands of presynaptic activation. Conversely, DA released via tonic DA neuron firing is thought to not be dependent on DAT, as extracellular DA levels generated by disinhibition of DA neurons in the NAcc remain unaffected by DAT blockade. However, *in vivo* studies using FSCV show that an increase in extracellular DA levels after exposure to a DAT antagonist alters the frequency and amplitude of transient firing. More recently, Richardson and workers demonstrated that membrane excitability shapes DAT surface trafficking dynamically, with depolarization reducing and hyperpolarization enhancing DAT levels at the surface rapidly (Richardson et al., 2016). Furthermore, studies providing for induced DAT knock down result in markedly decreased DA reuptake and enhanced tonic DA neuron firing without a change in phasic activity (Cagniard et al., 2006a). Moreover, these mice demonstrate greater performance in goal directed-behavior following learning and acquisition of the task (Cagniard et al., 2006b). Additionally, significant elevations in basal extracellular DA levels achieved via the transgenic elimination of the transporter in DAT knockout (DAT KO) mice show alterations in DA neuron activity as well (Jones et al., 1998b), though to what extent these changes reflect compensatory adjustments owing to the complete loss of DAT remain unclear.

As opposed to how DAT activity refines DA signaling following excitation of DA neurons, it is also important to consider the possibility that DAT itself, either owing to its electrogenic nature, or via its ability to support non-vesicular DA release, contributes

directly to DA signaling. DAT has traditionally been considered only to limit DA availability to postsynaptic DA receptors, thereby sculpting signaling at its targets. However, due to being an electrogenic transporter (Kilty et al., 1991), DAT also displays properties of a "mini-neurotransmitter-gated" channel. For example, heterologous expression studies have shown that DAT possesses channel-like states, supporting DA- or AMPH-gated, non-stoichiometric ion flow (Sonders et al., 1997). Furthermore, the Amara lab also provided evidence that DAT-mediated currents produced by DA influx can induce membrane depolarization of cultured mesencephalic DA neurons (Ingram et al., 2002). Along the same lines, Carvelli et al have demonstrated, in cultured *C. elegans* DA neurons, the existence of transporter-mediated channel states and that alterations in the interaction between DAT and the SNARE protein syntaxin 1A enhance the frequency of these channels (Carvelli et al., 2008). Changes in the frequency of transporter currents correlate with changes in DA-dependent motor behavior (Carvelli et al., 2004). It has also been shown (as alluded to previously) that AMPH can also contribute to channel-like states in DAT (Kahlig et al., 2005). Using patch clamp recordings of cells transfected with DAT, Kahlig and colleagues demonstrated that AMPH is capable of inducing a "channel-like" mode in DAT, causing DA release in millisecond bursts, a time-scale similar that of burst firing-induced, vesicular DA release. The authors suggest that this channel-like outward movement of DA happens in parallel to a slower, transporter-mediated efflux of DA that is expected to involve a full reversal of influx-supporting DAT conformations. Finally, recent studies using *in vivo* FSCV have suggested that AMPH can trigger vesicular release of DA (Avelar et al., 2013; Covey et al., 2013; Daberkow et al., 2013); however whether this is through an increase in

membrane excitability as suggested from studies in transfected cells and cultured neurons is unknown. Moreover, it is also unclear as to whether sufficient channel-like ion-flux to change membrane activity and/or induce DA release exist in neurons, and if postsynaptic targets respond differently to these modes of DA efflux compared to activity-dependent, vesicular DA release.

Regardless of mechanism, the ability of AMPH to induce DAT-mediated, non-vesicular DA release raises the question as to whether DAT-dependent DA efflux occurs under normal physiological conditions. Oliver and colleagues first proposed the existence of DAT-mediated, non-vesicular DA efflux when they detected DA release from a fraction of SN DA neurons that could not be explained by vesicular release mechanisms (Olivier et al., 1995). Subsequently, Falkenburger and coworkers raised the possibility of physiological DAT reversal, derived from mGLUR stimulation, as responsible for dendtritic DA release and signaling in the SN (Falkenburger et al., 2001). Somatodendritic DA release was still a novel concept when this study was conducted, and although small quantities of non-localized synaptic vesicles and vesicular machinery was reported to exist in the soma and dendrites of DA neurons, the authors explored further the nature of DA release from the DA soma and dendrites in the SN in relation with the stimulation of afferents onto the DA neurons. Using *ex vivo* slices, the authors observed that stimulation of the subthalamic nucleus, previously shown to provide excitatory control to SN DA neurons, resulted in DA release from dendrites. They also showed that DAT antagonism abolished this dendritic DA release (as explained above, blockade of DAT normally results in elevations in extracellular DA), suggesting that it was DAT-dependent. Further support for the non-vesicular

nature of this release was obtained via the observation that the DA release the authors found was Ca^{2+} independent. Interestingly, although DAT-dependent DA release could be evoked by GLU, it was independent of the actions of ionotropic GLURs that could enhance trigger vesicular release or via membrane depolarization, trigger DAT reversal. Notably, the group also showed that dendritic DA release causes inhibition of DA neuron excitability via activation of somatodendritic D2 autoreceptors.

Other efforts subsequent to the Falkenberger studies provide data supportive of vesicular mechanisms of somatodendritic DA release, and that this mode of release can also support D2AR inhibition. Importantly, Opazo et al showed that DAT-mediated, somatodendritic DA release is supported by mGLUR coupling to PKC via Gq protein-coupled signaling mechanisms (Opazo et al., 2010). More recently, Garcia-Olivares and colleagues provided evidence using transfected cells for a Gβγ subunit-dependent mode of DA release that is DAT-dependent (Garcia-Olivares et al., 2017). The Torres group had shown prior that Gβγ binding to DAT can inhibit DA reuptake through DAT, and notably observed through this study that stimulation of M5 muscarinic receptors, a G_q-coupled GPCR, induced DA efflux through the transporter. Evidence for physiological mechanisms of DAT-mediated DA efflux have also been found *in vivo,* through voltammetric studies measuring levels of endogenous DA in anesthetized rats (Moquin and Michael, 2011). The authors provided evidence of two distinct states of DA neurotransmission in the dorsal striatum – a fast component, arising from phasic DA release and a slow component, arising from putative reverse transport of DA through DAT, as the latter is sensitive to DAT blockade via cocaine. The authors also suggested that this slow mode of DA release results in tonic autoinhibition of DA terminals

mediated by presynaptic D2ARs. Although thought-provoking and challenging, the dogma that DAT is merely a sink for neurotransmitters persists, and it is apparent that the field would benefit from understanding the importance of DAT function using techniques that study the control of activity-evoked DA release and reuptake/efflux on the time scale of synaptic transmission. In this effort, studies that utilize transgenic mice expressing engineered mutations that can promote or limit efflux through the transporter without affecting DA reuptake would enabling a better understanding of the physiological roles for DAT-mediated DA efflux and its impact at the dopaminergic synapse.

1.10 Dysfunction in Dopamine Homeostasis and Disease

Aberrant DA signaling has been implicated in multiple neuropsychiatric disorders. However, despite DAT being a well-known target for drugs of abuse and the most pharmacological therapies for certain disorders, only recently has a clearer picture begun to emerge regarding a definitive link between DAT dysfunction, disruptions in DA homeostasis and disease. Below, after a brief overview on the well-studied impact of abnormal DA neurotransmission in disease, I will consider the importance of DAT dysfunction in disease, notably in ADHD, and focus on work from the Blakely lab in identifying rare DAT variants in patients with ADHD.

Parkinson's Disease

Of all the diseases with clear links to disrupted DA signaling, PD is arguably the best studied and understood. PD, as determined by seminal studies from Hornykiewicz

and colleagues, and many other groups starting from the 1960s, involves the age-dependent degeneration of DA neurons originating from the SNpc. PD patients present with multiple symptoms relating to locomotor deficits that result from the loss in SNpc DAergic tone such as involuntary movements, rigidity and bradykinesia (Hornykiewicz, 2002). Many unanswered questions remain pertaining to the role of DA dysfunction in PD – why are DA neurons selectively targeted (pontine cholinergic neurons are also found to decline in PD patients, but it is as yet unknown if this is causal to the disease), and why are nigral DA neurons specifically degenerated? As described briefly above, cytotoxicity associated with enhanced cytosolic DA and resulting oxidative stress induction has been implicated in PD (Lohr et al., 2017). Indeed, the most commonly used animal models for PD employ the use of dopaminergic toxicants such as 6-hydroxydopamine (6-OHDA) and MPTP that cause oxidative stress and mitochondrial dysfunction, resulting in cell degeneration. Interestingly, both 6-OHDA and MPP+ (the metabolite of MPTP) are substrates of DAT, and carry out their actions upon uptake into the cell through the transporter (Javitch et al., 1985). The genetic mouse models isolated in familial forms of PD have been extensively studied; however, most of these do not involve variation in DA-linked genes, but still lead to neurochemical and behavioral deficits associated with PD (Dauer and Przedborski, 2003). Recently, the Gether group identified DAT variants from patients with PD and found markedly reduced DAT uptake and anomalous, transporter-mediated DA release in transfected cells (Hansen et al., 2014); however how these mutants impact mammalian DA neuron signaling has yet to be determined.

Substance Abuse Disorders

      The link between the DA system and substance abuse has been extensively studied. All drugs of abuse have direct or indirect effects on DA neurotransmission – psychostimulants such as cocaine, AMPH and methamphetamine directly target DAT and elevate extracellular DA (albeit with different mechanisms; see above for explanation). Alcohol results in the disinhibition of DA neurotransmission (Grace, 2000), whereas heroin and prescription opioids, the abuse of which is progressively reaching epidemic proportions, also target DA neuron activity either directly through opioid receptors on DA neurons (some of which, like kappa-opioid receptors, can also directly target DAT), or on projections that target DA neurons (Sulzer, 2011). Consistent with the complexity of DA neuronal projections and physiological properties, drugs of abuse have differential effects on DA neurons projecting to the VS vs. DS (Bamford et al., 2018). Regardless of mechanism, however, substance abuse disorders are highly complex in how they affect DAergic signaling and co-opt reward systems, resulting in the development of addictive states and relapse. Many non-contingent (for e.g. conditioned place preference, ethanol vapor chamber) and contingent (for e.g. self administration) behavioral paradigms have been established and utilized to model drug addiction and relapse in rodents, following which the effects of these paradigms at different stages (i.e. acquisition vs. withdrawal vs. relapse) on the DAergic system and target regions have been studied extensively (Nutt et al., 2015). Although associations between DAT genetic variation and susceptibility to addiction have been reported, the evidence so far remains circumstantial.

Schizophrenia And Bipolar Disorder

The first effective antipsychotics, used in the treatment of schizophrenia and some affective disorders such as bipolar disorder (BPD), were discovered by serendipity. Their success in alleviating symptoms led many groups to determine their modes of action, with Carlsson and colleagues first postulating that antipsychotics acted by antagonizing brain DA receptors, specifically the D2-type DA receptors (Carlsson and Lindqvist, 1963), and subsequently for groups to posit a DA hypothesis for schizophrenia (Snyder et al., 1974). Subsequently, schizophrenia has been associated with enhanced DA neurotransmission in the striatum, with studies showing elevated DA and DA receptor expression post-mortem (Abi-Dargham et al., 2000) and enhanced DA release following AMPH in patients compared to controls (Iversen and Iversen, 2007). Unanswered questions remain, however, as these studies were conducted in patients with prior exposure to antipsychotics, making it harder to interpret the above studies as central to the disease versus a combination of schizophrenia and long-term exposure to antipsychotics. Additionally, schizophrenia has also been linked to hypodopaminergia in the frontal cortex (Slifstein et al., 2015). Rodent studies that model schizophrenia-like symptoms have relied on AMPH-induced stereotypy, as this behavior can be alleviated by treatment with anti-psychotics (Byun et al., 2014), and more recently by 6-OHDA injections into the PFC to mimic reduced DA signaling associated with the disease (Crofts et al., 2001). Multiple genetic association studies have linked DA-related genes to schizophrenia, notably the COMT variant Val158Met (Winterer et al., 2003). Interestingly, the focus of this thesis, the DAT Ala559Val variant was first found in a female subject with bipolar disorder (Grunhage et al., 2000). The above examples,

supporting DAergic dysfunctions in schizophrenia and BPD notwithstanding, several well-studied, non DA-related hypotheses including disruptions in glutamate homeostasis (Moghaddam and Javitt, 2012), excitation/inhibition imbalance (Sullivan and O'Donnell, 2012) and a loss in early-life developmental synaptic pruning (Ross et al., 2006) are also actively being tested by many research groups.

Dopamine Transporter Deficiency Syndrome

Missense mutations leading to a near complete loss of DAT function have also been reported in human patients. These patients exhibit a severe phenotype of infantile parkinsonian dystonia with the mutations resulting in a reduced affinity for DA by the transporter, decreased DAT expression and an inability for the transporter to be trafficked to the membrane when studied in transfected cell systems. Although rare, these mutations provide insight into the importance of DAT in controlling DA homeostasis and DA-related behaviors and highlight how rodent models that lack DAT altogether, or have markedly reduced expression of DAT, diverge in the phenotypes they present with, as will become more apparent below (Kurian et al., 2009; Kurian et al., 2011).

Attention-Deficit/Hyperactivity Disorder

ADHD is the most common neurodevelopmental disorder, affecting as many as 12% of school-aged children and 5% of adults. ADHD is characterized by hyperactivity/impulsivity, inattention or a combined subtype. There is a longstanding recognition that disruptions in DA signaling contribute to the symptomology of ADHD;

however there has been much debate over what the disruptions are and how they are causally related to the symptoms. Human structural imaging studies have reported reduced sizes of regions comprising of high DA density, such as the caudate nucleus and the globus pallidus, in children with ADHD, compared to typically developing children, a deficit that in some cases is normalized by pharmacological therapy and/or over age/development (Dougherty et al., 1999). Functional imaging studies have also reported reduced activity of frontal-sub cortical projections in ADHD patients compared to healthy controls. The most common pharmacological therapies for ADHD, Adderall (a combination of AMPHs) and Ritalin (or MPH) target the DAergic system via DAT, albeit with different mechanisms as explained above. Their modes of action to elevate DA levels extrasynaptically have led many groups to hypothesize that ADHD is characterized by a deficit in DAergic neurotransmission in frontal and subcortical brain regions (Spencer et al., 2007). However, other studies in human patients and rodent models, as will be elaborated below, in addition to common symptoms such as hyperactivity, that can be borne out of elevated extracellular DA levels, have given rise to the hypothesis that ADHD may be characterized as a case of "hyperdopaminergia", wherein elevated tonic levels of DA can in turn inhibit phasic DA, which is then alleviated by psychostimulant treatment. Several genetic studies also implicate DA-related genes in the genesis of ADHD. Associations with polymorphisms in the D4 DA receptor gene (DRD4) and ADHD have been widely reported (Swanson et al., 1998), in addition to polymorphisms in the D1 (Bobb et al., 2005) and D2 receptor genes (Teicher et al., 2000), COMT (Bellgrove et al., 2005), and as I will expand upon below, in the DAT gene. Finally, there is also support for noradrenergic imbalance in relation to

ADHD, especially in the frontal regions linked with inattention and ADHD, with NE thought to play a prominent role in shaping responses to DA (for a comprehensive review, see (Arnsten, 2006).

DAT Dysfunction In Disease

As described above, a connection to dysfunctions in DA signaling and brain disorders dates back over five decades, and DAT is a target for multiple drugs used in the pharmacological therapy of disease, or of drugs of abuse. However, only recently has direct evidence been obtained that changes in DAT structure or function can contribute to neuropsychiatric disease. Despite correlational evidence linking DAT to other neuropsychiatric disorders, ADHD is particularly compelling in relation to a direct contribution of DAT perturbation to disease risk. Again, whereas the DA system's role in ADHD has been well accepted and appreciated, only recently has a dysfunction in DAT been of significant focus. Furthermore, the most common pharmacological therapies used in the treatment of ADHD symptoms, Adderall and Ritalin directly target DAT and bring about their actions through DAT antagonism. This consideration led Cook and workers to examine putative genetic links between DAT and ADHD, with findings of a significant association between ADHD and a 10 copy VNTR (variable number tandem repeat), in the non-coding region at the 3' end of the DAT-1 gene (Cook et al., 1995). Additionally, owing to DAT being the target for psychostimulant-based treatments, studies have also explored potential differences in DAT levels in regions known to have high DA density (i.e. regions targeted by DA neurons), in ADHD patients. Using positron emission tomography (PET), multiple groups have looked at DAT binding to a DAT

antagonist analogue in ADHD populations relative to controls. Some of these studies, however, have produced mixed and controversial results, reporting enhanced DAT binding in the basal ganglia of children with ADHD or no change (Spencer et al., 2007), likely due to them being conducted in subjects with prior exposure to psychostimulant-based therapies. Following these studies, Volkow and colleagues observed decreased DAT binding in the caudate nucleus of drug-naïve ADHD patients compared to controls (Volkow et al., 2007). This reduction could reflect either a decrease in DAT density, or an increase in extracellular DA that could be competing with the radiotracer to bind to DAT. These investigators also showed that whereas there were no changes in DAT binding in the putamen, there was a significant correlation with enhanced DAT binding to increasing scores of inattention within the ADHD population (Volkow et al., 2007). Although lacking in circuit and cellular resolution, these studies, conducted in drug-naïve patients, highlight the importance of altered DAT function in ADHD.

As briefly mentioned above, transgenic elimination of the *Slc6a3* gene-encoding DAT in mice can produce key features of ADHD such as hyperactivity, inattention and impulsivity. In the first study characterizing the DAT knockout (DAT KO) mice, Giros and coworkers observed striking hyperactivity in DAT KO mice, especially in novel environments, in accordance with the apparent face validity required for a mouse model of ADHD (Giros et al., 1996). The authors also showed that DAT KO mice displayed significant elevations in extracellular DA levels and concurrently, reduced tissue DA levels. Consistent with a reduction in intracellular DA stores, DAT KO mice exhibited a significant reduction in the amplitude of electrically evoked endogenous DA release, measured using *ex vivo* FSCV in slices containing the DS(Jones et al., 1998b).

Subsequently, Jones and workers showed that the lack of DAT-mediated reuptake in the DAT KO mice restricted contributions of DA levels to DA synthesis (Jones et al., 1998b), versus DA recycling, a very important conclusion at the time, highlighting the importance of DAT in DAergic neurotransmission. Thus, in considering the "hyperdopaminergia" theory of ADHD, DAT KO mice reliably mimic the neurochemical expectations of elevated tonic, yet reduced phasic DA. In addition to face validity in the form of spontaneous hyperactivity, DAT KO mice also lacked elevations in locomotor activity produced by AMPH. In cognitive tests, DAT KO mice show deficits in the Morris Water Maze, a test for the learning of spatial memory, impaired behavioral inhibition measured in the 8-arm maze test, enhanced reward and motivation as observed by a bias towards a positive tastant, and deficits in the extinction of habit memory, displayed by the enhanced resistance to extinguish learning in a food reinforcement task (Gainetdinov et al., 1999; Hironaka et al., 2004; Costa et al., 2007; Morice et al., 2007).

Since the generation of the DAT KO, several strains with varying degrees of DAT expression have been developed. DAT siRNA mice (where DAT knock down was achieved using small interfering RNA to 60% expression) also recapitulate multiple biochemical and behavioral abnormalities of the DAT KO, albeit less pronounced, similar to the effects observed in DAT heterozygous mice (Salahpour et al., 2007). DAT knockdown mice (DAT KD), engineered to have a 90% reduction in DAT expression, exhibit milder hyperactivity than DAT KO mice, also display blunted locomotion in response to AMPH (Zhuang et al., 2001), and show enhanced motivation to reward (Cagniard et al., 2006a). Further studies demonstrate that DAT KD mice exhibit deficits in the ability to exploit learning, albeit not being impaired in reward learning (Cagniard et

al., 2006b).

Although the behavioral and biochemical features of the DAT KO mouse, align well with expectations from an ADHD mouse model, and suggest that a near to complete loss in DAT function could underlie ADHD symptoms, humans that are homozygous for null alleles in the DAT gene do not exhibit ADHD, but rather, infantile parkinsonian dystonia, as described above (Kurian et al., 2009; Kurian et al., 2011). Infants harboring these loss of function DAT alleles exhibit hyperkinesia immediately after birth, but eventually display rigidity and immobility characteristic of PD and do not survive very long after birth. Interestingly, DAT KO mice also do not live as long as WT mice. Regardless, these findings call into question whether basal hyperactivity, a widely accepted trait often sought after in an ADHD mouse model, is actually supportive of disease model relevance. Furthermore, given the support for DA imbalances in ADHD, several mouse models in addition to the DAT KO or KD have been developed with genetic/environmental insults resulting in perturbations in DA signaling (for a comprehensive review, see (Mergy et al., 2014a). Although many of these models are good at reproducing face and predictive validity, they still lack construct validity and cannot fully recapitulate the complexities of ADHD symptomology, or help in understanding the physiological mechanisms impacted by ADHD.

DAT Variants and Disease

The lack of construct validity in many DA-linked neuropsychiatric disorders, specifically ADHD, motivated the Blakely lab to screen subjects with ADHD (and other disorders) for potential coding variation in the DAT gene (Mazei-Robison and Blakely,

2005). This effort yielded multiple rare, yet functional and penetrant coding variants that are either absent or found at frequencies << 1% in population studies (Mazei-Robinson and Blakely, 2006). The variants identified are highlighted in Figure 6 relative to their location on DAT. Of all the variants identified, one of these variants, the Ala559Val (DAT Val559) was particularly compelling to study in relation to ADHD, owing to its location on DAT, and the alterations it induced to DAT function (Mazei-Robison et al., 2008). The mutation is located in transmembrane 12 and there is very little information on the importance of TM 12 with regards DAT structure and function. Modeling studies have suggested that TM 12 is important in homo-oligomerization of the transporter. Interestingly, transporter oligomerization has been reported to play a role in AMPH-induced, DAT-mediated DA efflux (Chen and Reith, 2008; Siciliano et al., 2018).

Upon analysis in transfected cells, DAT Val559 showed normal total and surface expression, unchanged reuptake of DA and affinity for DA, relative to cells expressing WT hDAT. Interestingly, when loaded with DA using a patch pipette and analyzed for DA efflux using carbon fiber amperometry, cells expressing DAT Val559 exhibited basal anomalous DA efflux (ADE), compared to hDAT-expressing cells, that could be shown to be transporter-dependent as it could be blocked by MPH, a conventional DAT antagonist. DAT Val559-dependent ADE was also found to be voltage-sensitive and was magnified when transfected cells expressing the variant compared to those expressing hDAT were held at depolarizing potentials to mimic neuronal firing. Notably, whereas AMPH caused DAT-mediated DA efflux in cells transfected with the WT transporter, DAT Val559-mediated ADE was actually blocked by AMPH, failing to further increase DAT-mediated DA efflux. Subsequently, Bowton and colleagues showed that
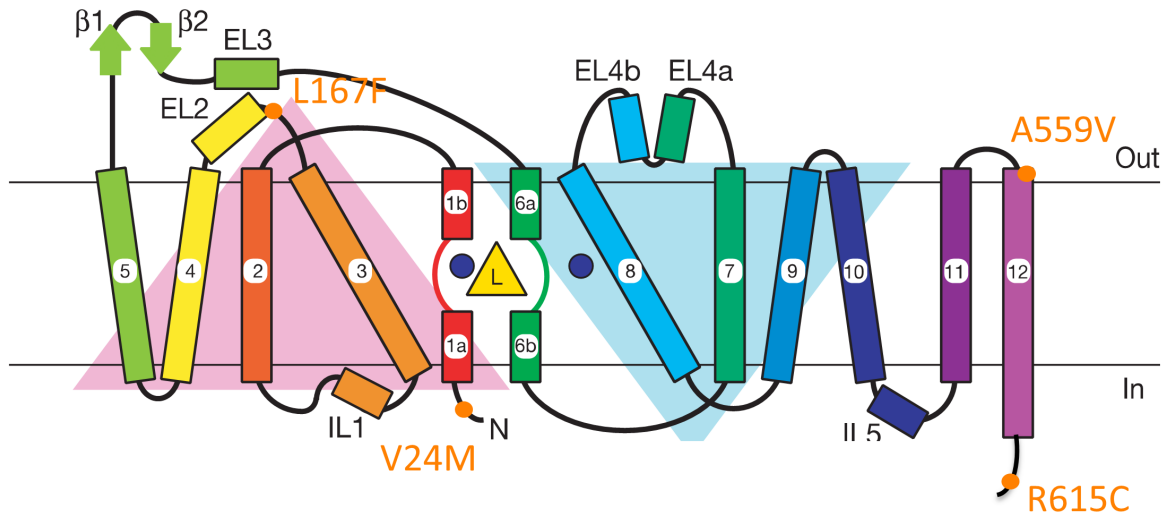
Figure 6. Human DAT variants associated with ADHD. Highlighted in the figure are the hDAT variants isolated from patients with ADHD at different locations on the transporter structure, overlayed onto the structure of LeuT. Image modified from Yamashita et al, 2005.

ADE through DAT Val559 in transfected cells is sustained by the D2-type receptor, expressed in small amounts in these cells, as demonstrated by ADE blockade upon D2R antagonism with raclopride (Bowton et al., 2010). This study also found that D2R signals through a non-canonical pathway involving Ca^{2+}/calmodulin-dependent protein kinase II (CamKII). This finding is of particular importance with regards to DAT Val559 and ADE, as Fog and workers have previously shown that CamKII can directly phosphorylate DAT at a cluster of serine residues in the N-terminus, and that its actions at the N-terminus are necessary for AMPH-induced DAT-dependent DA efflux (Fog et al., 2006). Consistent with this idea, when assayed for Ser phosphorylation at the N-terminus, DAT Val559 was found to be hyperphosphorylated, and ADE was abolished when serines were mutated to alanines in the DAT Val559.

More recently, the Blakely lab elucidated the properties of another DAT coding variant derived from a screen for subjects with the inattention subtype of ADHD, DAT Cys615 (Sakrikar et al., 2012). Sakrikar et al showed that the DAT Cys615 variant does not present with ADE, unlike the DAT Val559, and responds normally to AMPH, with comparable levels of DA efflux to WT hDAT induced by AMPH. However, DAT Cys615 displayed a reduction in DA reuptake, attributable to a disruption in the surface localization of the transporter, resulting in its reduced association with GM1 ganglioside-associated membrane microdomains. As a result, it was found that DAT Cys615 lacks the ability to respond properly to PKC activation or AMPH via changes in surface expression. Additionally, the DAT Cys615 variant was found to exhibit reduced interactions with flotillin-1. Flotillin-1 binding to DAT was previously shown to sequester DAT in membrane microdomains and to be important for AMPH's actions on the

transporter (Cremona et al., 2011). Interestingly, DAT Cys615 was found to have enhanced associations with CamKII, also essential for AMPH actions on DAT, resulting in hyperphosphorylation, like DAT Val559. These studies added strength to the conclusion that changes in DAT structure can impose an increased risk for ADHD through alterations in distinct features of DAT function and regulation.

ADHD is also found in association with a number of other disorders, notably Autism Spectrum Disorder (ASD). Indeed many groups have begun to suggest a role for DA in reward deficits associated with ASD. After the *in vitro* characterization of DAT Val559 and Cys615, Hamilton and colleagues characterized a *de novo* DAT coding variant, DAT Met356, identified in an ASD patient who was part of a prior large scale ASD sequencing effort (Hamilton et al., 2013). DAT Met356, when expressed in transfected cells, also displayed ADE, but also presented with deficits distinct from other coding variants identified in the Blakely lab. Notably, although DAT Met356 exhibited ADE reminiscent of the DAT Val559, AMPH failed to attenuate it. Furthermore, DAT Met356 also displayed reduced DA reuptake despite normal surface and total transporter expression, whereas DA uptake of the Val559 variant is equivalent to that seen with WT DAT. The authors recently reported, using a transgenic *Drosophila* model expressing the DAT Met356 variant, basal hyperactivity compared to animals expressing WT hDAT. An important issue to resolve, which will become apparent in the subsequent chapters, is whether the cause of hyperactivity in this model derives from a loss of DAT function, or an elevation in extracellular DA derived from ADE, or a combination of both. Nonetheless, the studies by Hamilton et al provided direct evidence that perturbations in DAT function, modeled from individuals with mental illness, may contribute to the risk for

neuropsychiatric disorders.

Following the studies on DAT Met356, Bowton and colleagues reported the presence of the DAT Val559 variant in two unrelated subjects with ASD, which, as noted above is a disorder where a majority of subjects also meet clinical criteria for ADHD (Bowton et al., 2014). Bowton et al showed, in addition to ADE, a lack of a trafficking response to AMPH treatments in transfected cells, owing to hyperactivity of the PKCβ isoform, a kinase previously shown to be involved in DAT trafficking to the surface in response to D2AR, as explained above. Together, the studies from Bowton et al on DAT Val559 in transfected cells emphasize the possibility of targeting regulators of DAT, such as PKCβ and CamKII, to diminish dopaminergic alterations in subjects with ADHD and ASD. Finally, the identification of DAT Val559 in BPD, ADHD and ASD makes it a particularly compelling variant to study in animal models with regards to a broader translational impact of findings.

SPECIFIC AIMS

The work described in this dissertation stems from the need to delineate more precisely the underlying molecular and physiological adaptations that arise in DA signaling as a consequence of the enduring expression of DAT Val559. To achieve this goal, I sought to pursue the following aims

1. Elucidate presynaptic and somatodendritic perturbations associated with DAT Val559 expression in DAT Val559 mice (discussed in Chapter 2 and Appendix I).

    a. Measure basal and AMPH-evoked alterations in extracellular DA levels induced by DAT Val559-ADE *in vivo*.

    b. Interrogate changes in evoked DA release from presynaptic DA terminals *ex vivo* in the striatum owing to DAT Val559 expression.

    c. Determine putative changes in DA neuron physiology in the DAT Val559 midbrain.

2. Examine the region dependence of DAT regulation and delineate how these differences may shape the physiological impact of DAT Val559 (discussed in Chapter 3 and Appendix II)

    a. Determine potential changes in surface expression and phosphorylation in D2AR-DAT regulation *ex vivo* using DAT Val559 DS and VS slices.

    b. Elucidate impact of altered D2AR-DAT regulation by DAT Val559 expression on DAT function *ex vivo*, and on DA clearance *in vivo*

    c. Examine putative region and DAT Val559-dependent differences in D2AR regulation of DA synthesis *ex vivo* on DA terminals in DS and VS slices.

Chapter 2 – DAT Val559-Induced Alterations
at the Dopaminergic Synapse

(The work described in this chapter is part of, and adapted from the manuscript "Mergy MA, Gowrishankar R, Gresch PJ, Gantz SC, Williams J, Davis GL, Wheeler CA, Stanwood GD, Hahn MK, Blakely RD (2014) The rare DAT coding variant Val559 perturbs DA neuron function, changes behavior, and alters *in vivo* responses to psychostimulants. Proc Natl Acad Sci U S A 111:E4779-4788.")

2.1 Introduction

As discussed in the previous chapter, there is a longstanding recognition that perturbations in DA signaling contribute to neuropsychiatric disease. In the past, these disease models have relied on environmental insults, such as local 6-OHDA injections (Deumens et al., 2002) or the transgenic elimination of genes related to DA synthesis, release or inactivation (Zhou and Palmiter, 1995; Giros et al., 1996; Wang et al., 1997). Although studies using these rodent models have provided valuable information on the control of DAergic neurotransmission and the impact of gross genetic and environmental insults, they lack much needed construct validity and translational relevance when trying to model complex disease. Motivated by this need, the Blakely lab conducted a screen for functionally penetrant mutations in DA-related genes, specifically the DAT and found multiple rare, functional and heritable variants in the ADHD population, including the DAT Val559 (Mazei-Robison et al., 2005). As described above, DAT Val559 expressed in transfected cells results in a basal, transporter-dependent DA leak or ADE, that can be blocked following treatment with AMPH or MPH (Mazei-Robison et al., 2008). Rodent models harboring gene variants that could confer risk to mental illness, such as the DAT Val559, isolated in ADHD, BPD and ASD would be a better approach to model dysfunction associated with neuropsychiatric disorders.

Furthermore, disruptions in DAT function conferred by the variant, as opposed to a frank loss in transporter function altogether would enable a better understanding of the impact of disrupted DA homeostasis on disease. Hence, the Blakely lab created a rodent model transgenically engineered to express DAT Val559.

Following successful generation of the transgenic mice, DAT Val559 animals exhibited normal growth, size and survival (Mergy et al., 2014a). Measurements of total DAT expression from whole brain extracts revealed no changes in mice heterozygous or homozygous for DAT Val559, compared to WT littermates. DAT Val559 heterozygous and homozygous mice also had normal DA tissue content and concentration of metabolites, when compared to WT littermates, in whole striatum and PFC extracts. Furthermore, DAT Val559 heterozygous and homozygous mice showed no change in DA reuptake capacity, as assayed using [^{3}H]DA accumulation in striatal synaptosomes. Although the above results are unsurprising, since DAT Val559 expressed in transfected cells also showed no changes in the above parameters compared to WT DAT (Mazei-Robison et al., 2008), the propensity of DAT Val559 to confer basal, DAT-mediated ADE and eliminate AMPH's ability to induce DA efflux suggested further evaluations would reveal relevant phenotypes. In particular, whereas transfected cell systems are useful in testing the impact of genetic variation such as the DAT Val559 on transporter function, we may not be able to understand the physiological influence of ADE unless studied in the context of a DAergic synapse. Hence, in this chapter, after a description of the methods used, I will discuss key findings pertaining to how lifelong expression of DAT Val559 in mice, as found with the subjects from which the mutation was isolated, engenders molecular and physiological alterations in DAergic neurotransmission.

2.2 Methods

Animals. All experiments adhered to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Vanderbilt University and Oregon Health Science University. For the experiments performed in this chapter, mice homozygous for DAT Val559 or WT were bred from homozygous dams and sires, obtained from heterozygous breeders, no more than two generations removed. Genetic background for all animals used was 75% 129:25% C57. 4 week old, age-matched males were used for the electrophysiological studies, 6-8 week old age-matched males for the [³H]DA superfusion studies and 8-10 week old female mice for the *in vivo* striatal microdialysis studies. All animals were bred in a 12:12 light cycle, and upon weaning (~3 weeks old), were transferred to a reverse light cycle and acclimated for a week, such that all experiments could be performed in their active phase.

Whole-cell patch clamp electrophysiology. Mice were anesthetized using isofluorane and killed by rapid decapitation. Brains were excised into an ice-cold, physiologically equivalent saline solution (modified Krebs' buffer) containing in mM, 126 NaCl, 2.5 KCl, 1.2 MgCl2, 2.4 CaCl2, 1.4 NaH2PO4, 25 NaHCO3 and 11 D-glucose with 10 μM MK-801, an NMDA receptor antagonist. Horizontal midbrain slices (220 μm) containing the SNPc or VTA were made using a vibratome (Leica) in the solution as above and incubated at 30 °C in the above solution with 10 μM MK-801 under constant oxygenation (95%/5% O2/CO2) for at least 30 min. Once mounted on a recording chamber attached to an upright microscope (Olympus), slices were perfused at a rate of 3–4 mL/min at 35 °C with the above solution. Whole-cell patch-clamp recordings were

obtained using glass electrodes (1.3–2.0 MΩ) filled with an internal solution containing 115 mM K-methanesulfonate, 20 mM NaCl, 1.5 mM MgCl2, 2 mM ATP, 0.2 mM GTP, 10 mM phosphocreatine, and 10 mM BAPTA, (pH 7.33–7.43) 275–288 mOsm. DA neurons in the SNPc were identified visually by their morphology, location in relation to the medial terminal nucleus of the accessory optic tract, the presence of a large hyperpolarization-induced I_h current, the presence of spontaneous pacemaker firing of wide (~2 ms) action potentials at 1–5 Hz, and the presence of a D2R-mediated IPSC or sensitivity to exogenously applied DA. Cells were voltage clamped at −60 mV with an Axopatch 200B amplifier (Molecular Devices). Immediately after cell access, membrane capacitance, series resistance, and input resistance were measured with the application of three pulses (+2 mV for 50 ms) averaged before computation by using AxoGraph (sampled at 50 kHz, filtered at 10 kHz). Resting membrane potential and firing rate were determined in current-clamp mode within 2 min of break-in. Current was injected when necessary to ensure that cells were resting between −60 and −65 mV before testing excitability. All drugs were applied through perfusion with the exception of DA, applied via iontophoresis. Electrical stimulation of DA release was evoked by a single (0.5 ms) or train of electrical stimuli (5 × 0.5-ms pulses at 40 Hz), and GABA release was evoked by a train of electrical stimuli (5 × 0.5-ms pulses at 60 Hz). D2AR-mediated IPSCs and GABAB-mediated IPSCs were pharmacologically isolated by using the receptor blockers in the external bath solution as follows: picrotoxin (100 μM), hexamethonium (50 μM), DNQX (10 μM), and CGP 55845 (200 nM) or sulpiride (600 nM), respectively. Data acquisition was achieved using AxoGraph software (sampled at 10 kHz, filtered at 5 kHz) and Chart 5 (AD Instruments). Recordings were post hoc filtered at 1 kHz. Peak

amplitudes IPSCs were determined by averaging the current ±20 ms from the greatest upward deflection. To measure the kinetics of sIPSCs, spontaneous events with a single peak were selected. Duration of IPSCs was determined by measuring the width at 20% of the peak amplitude. Values are given as means ± SEM. Statistical significance was determined in two group comparisons by unpaired Student's two-tailed t tests or two-tailed Mann– Whitney u tests and in multiple comparisons by one-way ANOVAs, when appropriate with uncorrected Fisher's LSD or Bonferroni's post hoc tests or two-way repeated measure ANOVA with Sidak's post hoc tests (GraphPad Prism 6 and AxoGraph X).

Ex vivo **striatal slice DA release studies.** Brains were excised from mice, killed via rapid decapitation. Brains were immediately placed on an ice-cold metal platform and the striatum sections containing both DS and VS were quickly was dissected. Striatal slices were incubated for 30 min with 150 nM [^{3}H]-DA at 37 °C in modified Krebs'-Ringer Bicarbonate (KRB) buffer (in mM - 126 NaCl, 2.5 KCl, 2.4 CaCl2, 1.2 MgCl20.6H2O, 1.2 NaH2PO4, 10 D-glucose, and 21.4 NaHCO3, pH 7.4) supplemented with 50 µM pargyline, 50 µM tropolone, and 50 µM ascorbic Acid (Sigma Aldrich). Slices were then loaded into the perfusion chambers of a Brandel SF-12 superfusion system (Brandel), sandwiched between GF/B glass fiber filter discs (Whatman). The chambers were perfused at a flow rate of 0.7 mL/min with oxygenated KRB buffer at 37 °C for 45 min before drug application to achieve a steady baseline of [^{3}H]-DA release. Following baseline, 4-aminopyridine (Sigma Aldrich) (4-AP) or AMPH (Sigma Aldrich) were perfused in KRB buffer through the chambers at a flow rate of 0.7 mL/min and samples were collected every 2 min. After collection of two baseline samples, perfusate was

switched to KRB buffer supplemented with 50 μM 4-AP to evoke vesicular $[^3H]$-DA release or 1 μM AMPH to evoke nonvesicular $[^3H]$-DA release for 6 min and then returned to perfusion with KRB buffer for an additional 8 min. For experiments involving AMPH–evoked [3H]-DA release, 5 μM GBR12909 was added 2 min before and during stimulation by AMPH, followed by subtraction from values obtained with AMPH-only treatment to define DAT-dependent AMPH-evoked $[^3H]$-DA release. For studies of quinpirole (Tocris Biosciences) or raclopride (Tocris Biosciences) modulation of 4- AP–evoked vesicular $[^3H]$-DA release, one 2-min baseline perfusate was collected and then the perfusion buffer was switched to that supplemented with 250 nM quinpirole or 500 nM raclopride for 2 min. Then the perfusate was switched to KRB containing either 250 nM quinpirole or 500 nM raclopride, each combined with 50 μM 4-AP, and samples were collected for the next 6 min, followed by a return to perfusion for 8 min with KRB buffer. Five mLs of EcoScint XR scintillation fluid (National Diagnostics) was added to all samples, and radioactivity was counted by using a TriCarb 2900TR scintillation counter (Perkin-Elmer). Data for $[^3H]$-DA released are presented as the fraction of the total [3H]-DA loaded into each sample (amount released + amount remaining in the tissue), normalized as a percent of baseline. Data were analyzed by using two-way repeated measures analysis of variance (RMANOVA) with Sidak's post hoc test for multiple comparisons (GraphPad Prism 6.0).

In vivo **microdialysis studies.** Female mice were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments). A guide cannula (CMA7) was placed 1 mm above the dorsal striatum (−0.86 AP from Bregma, ±1.6 mL and −2.0 DV from dura) and secured to the skull by using glass ionomer cement (Instech Solomon).

After accounting for a sufficient recovery period from surgery (18–24 h), animals were placed in individual dialysis chambers (clear cylindrical enclosure, 14 cm diameter, 22 cm high; Instech Solomon). A microdialysis probe (CMA Microdialysis) with an active length of 2 mm was inserted into the guide cannula. One end of a tether was attached to the headpiece and the other end was attached to a liquid swivel (Instech Solomon) that was mounted on a counterbalanced arm above the dialysis chamber. Mergy et al. www.pnas.org/cgi/content/short/1417294111 2 of 3 The probe was perfused with artificial cerebral spinal fluid (aCSF; 149 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, and 5.4 mM D-glucose, pH 7.2) at a flow rate of 1.0 µL/min overnight. After the fourth baseline sample, the aCSF was switched to aCSF containing 0.1 µM AMPH for the remaining nine samples. Dialysate samples were stored at −80 °C and analyzed by HPLC-EC for DA and serotonin levels, as described above. For evaluation of changes in extracellular neurotransmitter levels in response to systemic AMPH, animals were cannulated and dialysates collected as above before and after 3 mg/kg i.p. AMPH. After each dialysis session, animals were overdosed with sodium pentobarbital, brains were removed and postfixed in 4% paraformaldehyde in 100 mM phosphate buffer, sectioned, stained for Nissl substance, and then inspected for acceptable probe placement. Data were analyzed by using two way RM-ANOVA tests with Tukey's or Sidak's post hoc test for multiple comparisons, or Student t test statistic.

2.3 Results

DAT Val559 mice display elevated extracellular DA levels and blunted AMPH-induced DA efflux in the striatum *in vivo*. Given our observations that DAT Val559 expression in transfected cells engenders basal ADE through the transporter, and that AMPH blocks ADE without inducing further DAT-mediated DA efflux (Mazei-Robison et al., 2008), we first sought to determine potential changes in basal and AMPH-evoked elevations extracellular DA levels in the striatum in freely moving mice. We employed *in vivo* microdialysis, as it affords the opportunity to determine spatially precise, absolute values of extracellular DA and drug-induced changes in endogenous DA levels in the striatum. We found significantly elevated basal extracellular DA levels in the striatum of DAT Val559 mice, compared to WT (Figure 7A). Additionally, in order to assay the effects of AMPH in elevating DA in the striatum, we first locally applied AMPH (0.1µM) into the striatum and observed a profound reduction in AMPH-induced elevations in endogenous DA in the DAT Val559 mice (Figure 7B). To determine if this loss was true for systemic AMPH injections as well, we i.p injected WT and DAT Val559 mice with a low, therapeutically valid dose of AMPH (3 mg/kg), and once again found a reduction in DA elevations in the DAT Val559 mice, compared to WT (Figure 7E). In both cases, we observed small increases in extracellular DA in response to AMPH, suggesting that the capacity for AMPH to block DA reuptake is indeed intact. In contrast, basal and AMPH-evoked elevations in 5-HT were unchanged across genotypes (Figure 7C-D). These results corroborate our *in vitro* data, suggesting DAT Val559-mediated ADE causes an elevation in extracellular DA levels, and compromises the ability of AMPH to induce non-vesicular DA efflux through DAT reversal.
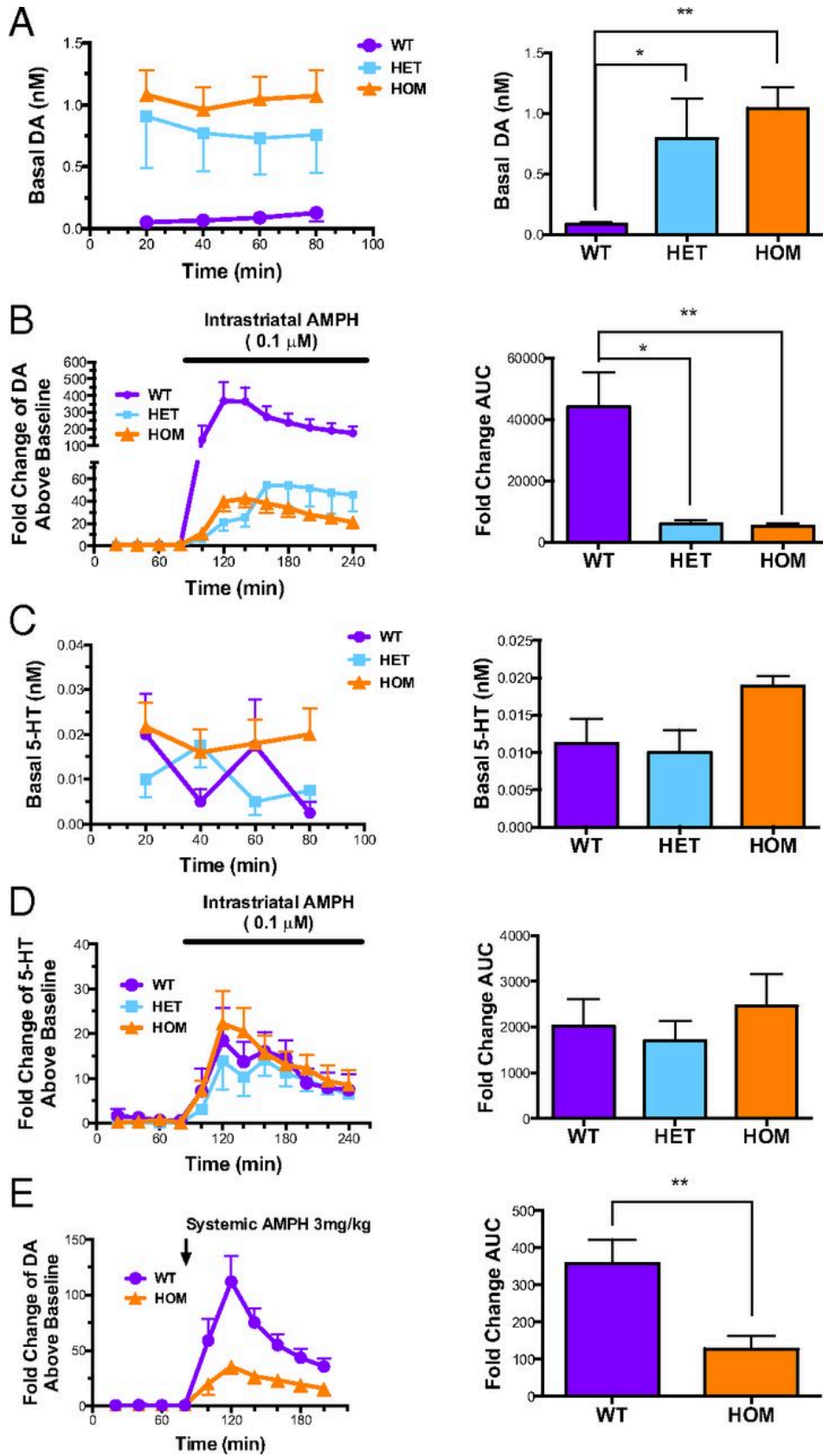
Figure 7. DAT Val559 mice display elevated extracellular DA levels and blunted AMPH-induced DA efflux in the striatum *in vivo*. (A) Basal extracellular DA levels are elevated in DAT Val559 mice (n = 7 WT, 4 HET, 8 HOM; Left, time course of basal DA release, P(genotype)<0.01, post hoc tests reveal P < 0.05 at 20 and 40min for WT vs. HET, and P<0.001 at 20, 60 and 80 min and P<0.01 at 40 min for WT vs. HOM; Right, mean ± SEM extracellular DA concentration before AMPH stimulation, P<0.05 one-way ANOVA, post hoc testing reveals P<0.05 (*) for WT vs. HET and P<0.01 (**) WT vs. HOM). **(B)** With intrastriatal AMPH, AMPH-evoked DA is reduced ~10-fold in DAT Val559 mice (n = 7 WT, 4 HET, 8 HOM; Left, time course of evoked DA release, fold change above baseline ± SEM, P(genotype)<0.01, post hoc tests reveal P < 0.05 at 200 and 220 min, P < 0.01 at 180 min, P < 0.001 at 160 min, P < 0.0001 at 120 and 140 min for WT vs. HET, P < 0.05 at 100 min, P < 0.01 at 220 and 240 min, P<0.001 at 180 and 200 min and P<0.0001 at 120, 140 and 160 min for WT vs. HOM; Right, mean ± SEM, integrated fold change of DA relative to baseline, P<0.01 one-way ANOVA, post hoc testing reveals P<0.05 (*) for WT vs. HET and P<0.01 WT vs. HOM (**)). **(C)** Basal extracellular 5-HT levels do not differ between genotypes (n = 4 WT, 4 HET, 6 HOM; Left, time course of basal 5-HT release; Right, mean ± SEM extracellular 5-HT concentration before AMPH stimulation). **(D)** Equivalent AMPH-induced increases in extracellular 5-HT (n = 4 per genotype; Left, time course of evoked 5-HT release, fold change above baseline ± SEM; Right, mean ± SEM integrated fold change of 5-HT relative to baseline). **(E)** Systemic AMPH-evoked DA is reduced ~3-fold in DAT Val559 mice (n = 7 each genotype; Left, time course of evoked DA release, fold change above baseline ± SEM, P(genotype)<0.05, post hoc tests reveal P<0.05 at 100 min, P<0.001 at 140 min, and P<0.0001 at 120 min for WT vs. HOM; Right, mean ± SEM integrated fold change of DA relative to baseline, P<0.01 (**), WT vs. HOM).

DAT Val559 expression affects AMPH and 4-AP-evoked presynaptic DA release accompanied by tonic D2AR activation. Our observations of elevated extracellular DA, and the blunted ability for AMPH to enhance DA levels were *in vivo*, which precludes our ability to understand the adaptations induced by DAT Val559 at the level of DA neurons and terminals. Hence, in order to determine putative changes in the control of DA release from terminals in the striatum, we utilized [^{3}H]DA superfusion studies in striatal slices from WT and DAT Val559 animals. We first determined a suitable concentration of [^{3}H]DA (150 nM) that would ensure reuptake through DAT in the linear range. We then determined a concentration of AMPH (1μM) that would evoke non-vesicular, DAT-mediated [^{3}H]DA release that would preclude floor or ceiling effects of AMPH. Furthermore, our superfusion setup maintains slices under constant perfusion, thereby preventing the detection of DA elevations induced by conventional DAT blockers. Therefore, in order to ensure that we ascertain specifically transporter-dependent, non-vesicular [^{3}H]DA release, as studies prior have reported that AMPH can also influence vesicular DA release, we subtracted out the component of [^{3}H]DA sampled from striatal slices obtained from the same mouse, treated with a potent DAT blocker, GBR12909 (10μM), in addition to AMPH. Upon doing so, we found that DAT-mediated, non-vesicular [^{3}H]DA release from DA terminals in DAT Val559 striatal slices was significantly reduced compared WT (Figure 8A).

The data presented above mirror our results obtained from *in vitro* studies (Mazei-Robison et al., 2008), wherein AMPH's ability to evoke DA efflux is compromised. In order to determine if a loss in non-vesicular DA release is accompanied by a concurrent alteration in vesicular DA release, we treated striatal

slices, preloaded with [³H]DA, with 4-Aminopyridine (4-AP, 50μM), a K⁺ channel blocker, to induce nerve terminal depolarization and evoke vesicular DA release. Surprisingly, we found that 4-AP-induced [³H]DA release was also reduced in DAT Val559 striatal slices (Figure 8B). As described in the introduction of this chapter, tissue content of DA is comparable across genotypes, suggesting that this decrease is not due to a reduction in vesicular DA stores, but adaptations in the control of DA release machinery. Presynaptic D2ARs have been shown to inhibit evoked, vesicular DA release by inhibiting vesicular fusion mechanisms, leading us to posit that DAT Val559 DA terminals might be under tonic inhibition due elevated D2AR activity. To test this hypothesis, we pre-treated slices with quinpirole (250 nM), a D2/D3 receptor agonist. We found, replicating results found using other techniques (such as *ex vivo* FSCV) in multiple studies that quinpirole caused a reduction in vesicular DA release (Figure 8C). However, quinpirole failed to inhibit 4-AP-evoked [³H]DA release in DAT Val559 striatal slices (Figure 8D). This loss of D2AR modulation could arise from ongoing D2AR activity due to ADE, or could result from receptor desensitization. To test this idea, we performed the converse of the prior experiment and treated slices with raclopride (500 nM), a D2/D3 antagonist. As antagonist actions are largely dependent upon agonist (i.e. DA) tone, raclopride caused no change to vesicular [³H]DA release in WT slices (Figure 8E). However, D2AR antagonism elevated vesicular release in DAT Val559, comparable to those induced by 4-AP in WT striatal slices, strongly suggesting that DAT Val559 ADE causes tonic D2AR activation, resulting in a D2AR-dependent inhibition of vesicular release (Figure 8F).
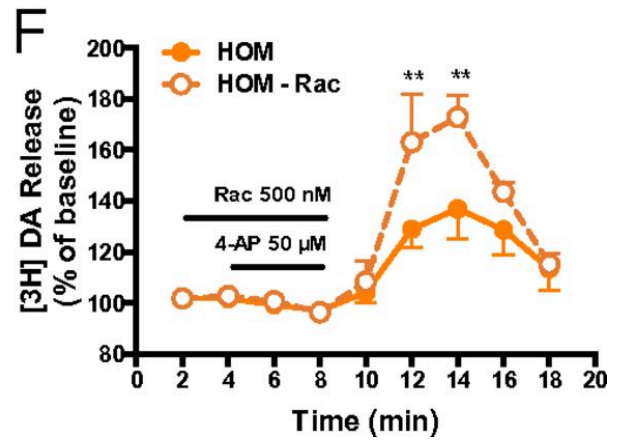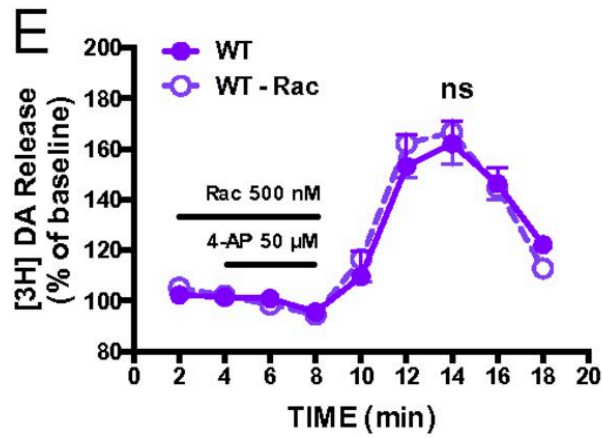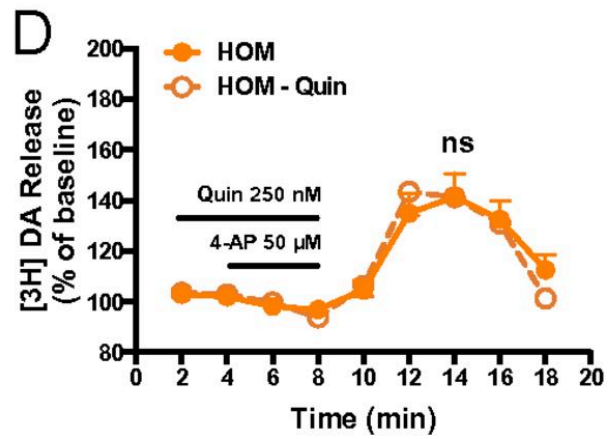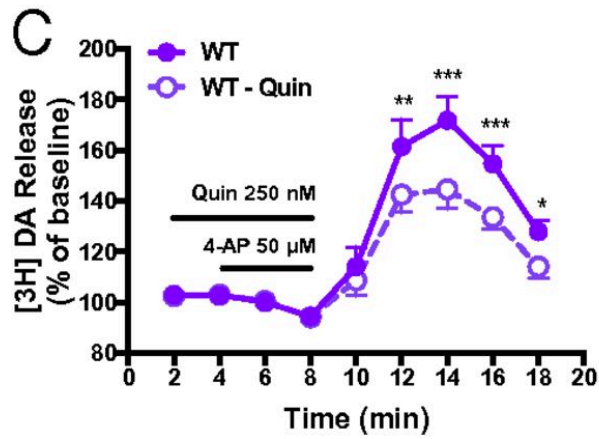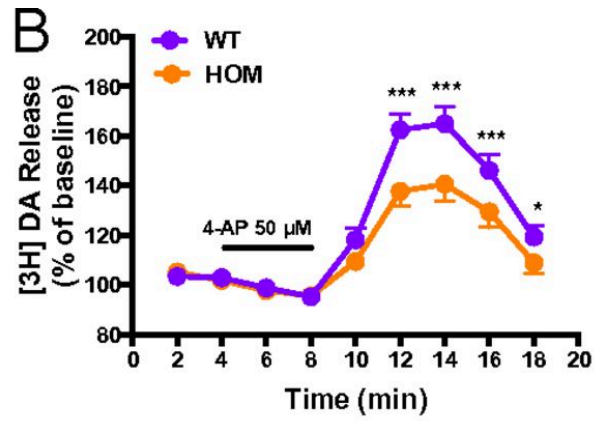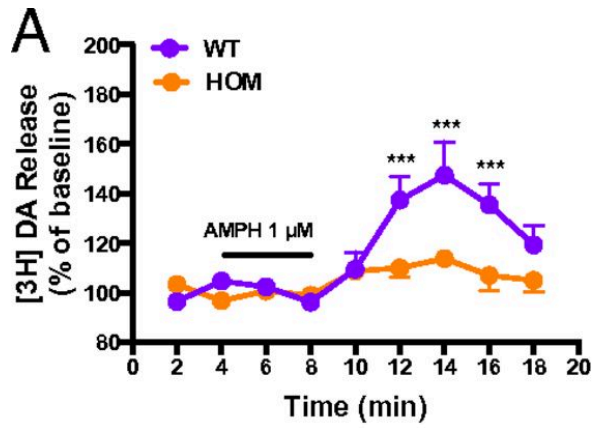
Figure 8. DAT Val559 expression affects AMPH and 4-AP-evoked presynaptic DA release accompanied by tonic D2AR activation. (A) Striatal slices from WT mice exhibit significant elevation in [^{3}H]DA release above baseline upon application of 1 μM AMPH, whereas DAT Val559 tissue releases significantly less [^{3}H]DA than WT tissue (n = 5 WT, 5 HOM; P(genotype)<0.05, P(time)<0.0001, P(interaction)<0.0001, post hoc Sidak's multiple comparisons test reveal P<0.001 at 12 and 16 min and P<0.0001 at 14 min. **(B)** Following application of 50 μM 4-AP, WT striatal slices exhibit significant [^{3}H]DA release above baseline (n = 12; P<0.0001), but DAT Val559 slices show significantly diminished [^{3}H]DA release compared with WT (n = 12 WT, 12 HOM; P(genotype)<0.0001, P(time)<0.0001, P(interaction)<0.0001, post hoc Sidak's multiple comparisons test for time-dependent genotype differences reveal P<0.001 at 12, 14 and 16 min and P<0.05 at 18 min). **(C)** Quinpirole (250 nM) significantly decreases 4-AP–evoked [^{3}H]DA release in WT striatal slices (n = 7 WT; shows P(quinpirole)<0.05, P(time)<0.0001, P(interaction)<0.0001, post hoc Sidak's multiple comparisons test for time-dependent genotype differences reveal P< 0.01 at 12 min, P<0.0001 at 14 min, P<0.001 at 16 min and P<0.05 at 18 min). **(D)** Quinpirole-mediated suppression of 4-AP–evoked, striatal [^{3}H]DA release is absent in DAT Val559 (n = 7 HOM; P(quinpirole)>0.05, P(time)<0.0001, P(interaction)>0.05). **(E)** After 500 nM raclopride, WT striatal tissue showed no changes in 4-AP–evoked [^{3}H]DA release (n = 5 WT; P(raclopride)>0.05, P(time)<0.0001, P(interaction)>0.05). **(F)** Raclopride application enhanced 4-AP–evoked [^{3}H]DA release in DAT Val559 striatal slices (n = 5 HOM; P(raclopride)<0.05, P(time)<0.0001, P(interaction)<0.05, post hoc Sidak's multiple comparisons test for time-dependent genotype differences reveal P<0.01 at 12 and 14 min).

DAT Val559 expression induces alterations in basal and AMPH-augmented D2AR-mediated IPSCs in midbrain DA neurons. As described in the previous chapter, DAT is expressed both pre/perisynaptically and in somatodendritic compartments, along with D2ARs, DA vesicular and release machinery (Sesack et al., 1994a). Somatodendritic DAT has been shown to limit the ability of D2ARs in dampening DA neuron firing, since D2R activation in the midbrain is tightly controlled by DA reuptake via DAT (Ford et al., 2010). Vesicular somatodendritic release of DA in both SNPc and VTA DA neurons produces an inhibitory postsynaptic current (IPSC) via D2AR activation of GIRK channels, which can inhibit DA neuron firing through the flux of ions out of the cell (Beckstead et al., 2004; Gantz et al., 2013). In order to ascertain the influence of DAT Val559 on the physiological properties of DA neurons, and D2AR–mediated IPSCs, we performed whole cell recordings of DA neurons in horizontal midbrain slices containing the SNPc in collaboration with John Williams and Stephanie Gantz at the Vollum Institute in Oregon Health Science University. We found whole cell capacitance of SNPc DA neurons in slices prepared from DAT Val559 mice to be reduced (WT: 35.8±0.67 pF, n = 139; DAT Val559: 32.7±0.56 pF, n = 153; P = 0.001, Mann Whitney test) and resistance to be increased (WT: 315.7±14.1 MΩ, n = 138; DAT Val559: 347.3±12.6 MΩ, n = 149, P = 0.012, Mann Whitney test). Other basic electrophysiological parameters such as resting membrane potential, percentage of quiescent cells and firing rates in response to current injection exhibited no genotype effects. D2AR-mediated IPSCs were evoked by electrical stimulation by a single or five stimuli, with glutamatergic, GABAergic, and cholinergic inputs into the SNPc silenced with receptor blockers. We observed a significant difference in the time-to-peak of the

D2AR-mediated IPSC evoked by a single stimulus and the duration of the IPSC evoked by a single or five stimuli (Figure 9A). Additionally, we also found that spontaneous D2AR-mediated IPSCs (D2AR-sIPSC) were prolonged (Figure 9A), albeit with no difference in amplitude. In contrast to D2AR-mediated IPSCs, we found no differences in the time-to-peak or duration of electrically evoked $GABA_B$-mediated IPSCs, in the presence of glutamatergic, cholinergic and DAergic receptor blockers (Figure 9B).

It has also been shown previously in the Williams lab that DAT antagonists such as cocaine and AMPH prolong the duration and amplitude of D2AR-mediated IPSCs (Beckstead et al., 2007; Branch and Beckstead, 2012). In order to determine the effects of AMPH on SNPc DA neurons, we bath-applied AMPH onto slices, following which we recorded D2AR-mediated IPSCs. As expected, the amplitude of electrically evoked D2AR-mediated IPSCs following AMPH treatment increased significantly relative to vehicle application, reaching a plateau. However, AMPH-induced elevations in D2AR-mediated IPSC amplitude were blunted in DA neurons recorded from DAT Val559 slices (Figure 9C), with some cells showing a brief enhancement followed by a decrease to baseline amplitude. Since this augmentation in D2AR-mediated IPSCs is found in all DAT blockers, we repeated these studies using MPH, a conventional DAT blocker. Interestingly, we found no genotype differences in MPH-induced increases in D2AR mediated IPSC amplitude (Figure 9D), suggesting that the changes we observed in DAT Val559 SNPc DA neurons were specific to AMPH.
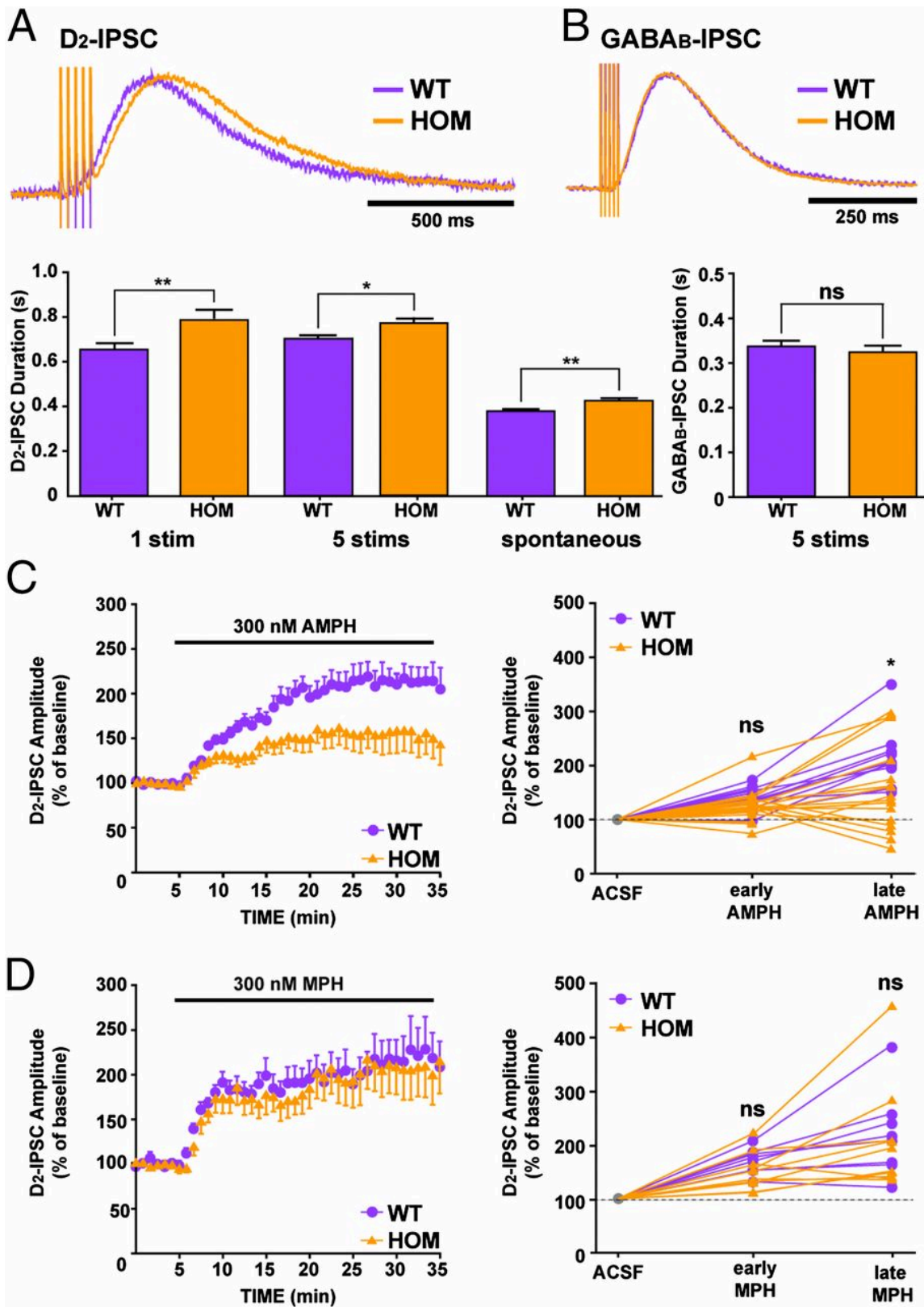
Figure 9. DAT Val559 expression induces alterations in basal and AMPH-augmented D2AR-mediated IPSCs in midbrain DA neurons. Scaled representative synaptic currents (IPSCs) mediated by D2R (A) or GABAB receptors (B) were evoked by electrical stimulation in brain slices containing substantia nigra dopamine neurons. D2R-mediated IPSCs recorded in DAT Val559 slices are significantly slower than in WT slices, whether from a single electrical stimulation or using a train of stimuli to evoke DA release (1 stim: n = 32 cells WT, 35 cells HOM; P=0.003; 5 stims: n = 64 cells WT, 67 cells HOM; *P<0.05, one-way ANOVA), or those occurring spontaneously (n = 135 sIPSCs WT, 150 sIPSCs HOM; **P=0.01, Mann–Whitney). No genotype differences were detected in the kinetics of GABAB-mediated IPSCs (n = 18 cells WT, 17 cells HOM). Upon AMPH (C) or MPH (D) application, the amplitude of evoked D2R-mediated IPSCs increases significantly. (C) The AMPH-induced increase in D2R-mediated IPSC amplitude is blunted in DAT Val559 slices Left, time course of AMPH response; Right, averaged increase during the period 2.5–5 min after AMPH application (early) and averaged increase during 20–30 mins (late); normalized to pre-AMPH amplitude; n = 18 cells WT, 19 cells HOM, *P<0.05, two-way RMANOVA]. (D) No genotype differences were detected in the MPH-induced increase in D2R-mediated IPSC (Left, time course of MPH response; Right, averaged increase during the period 2.5–5 min following MPH application (early) and averaged increase during 20–30 min (late); normalized to pre- MPH amplitude; n = 10 cells WT, 11 cells HOM).

It has also been previously shown by the Williams lab that the DA release evoked following electrical stimulation that produces the D2AR-mediated IPSC is vesicular in nature, as evidenced by a run-down of the amplitude following blockade of VMAT2 via reserpine (Beckstead et al., 2004). Following reserpine application, D2AR-mediated IPSCs in DA neurons from both WT and DAT Val559 slices showed comparable reductions in amplitude, suggesting that DA neurons in both genotypes employ vesicular mechanisms for DA release to generate D2AR-mediated IPSCs (observations by Gantz and Williams, data not shown). The Williams lab also reported that the ratio of electrically evoked D2AR-mediated IPSCs using five stimuli over a single stimulus is dependent on the probability of somatodendritic DA release, and is unaltered by changes in neurotransmitter content, or blockade of DA reuptake (Beckstead et al., 2007). Notably, mirroring our results of reduced presynaptic DA release, this ratio was reduced in DAT Val559 DA neurons (WT: 298±22.5%, n=16, DAT Val559: 223±16%, n=17; P=0.03, unpaired t test), indicative of a reduction in somatodendritic DA release as well in SNPc DA neurons from DAT Val559 compared to WT.

2.3 Discussion

Rare genetic variation is now increasingly recognized as a potential source to understand the complex etiology of neurological disorders (Horschitz et al., 2005b, a; Kauwe et al., 2008; Quast et al., 2012; Takata et al., 2013; Zech et al., 2014). When confined to a single gene, found in populations with neuropsychiatric disorders offers the opportunity to be able to model facets of disorders with translational relevance, delineate how such variation might contribute to mental illness. The widely accepted

transgenic rodent model for ADHD, the DAT KO mouse, recapitulates multiple phenotypes associated with the ADHD population, accompanied by neurochemical observations that align well with what has been reported in patients (Giros et al., 1996; Fumagalli et al., 1998). However, as explained above, human subjects with a complete loss of DAT function do not present with ADHD, but instead infantile parkinsonian dystonia (Kurian et al., 2009; Kurian et al., 2011). The screen for rare DAT variants conducted by the Blakely lab, and from it, the identification of the DAT Val559 (Mazei-Robison and Blakely, 2005; Mazei-Robison et al., 2005; Mazei-Robinson and Blakely, 2006; Mazei-Robison et al., 2008), is the first example of an assessment of the functional impact of a disease-associated genetic DAT variant *in vivo* (Mergy et al., 2014a; Mergy et al., 2014b).

In order to assess the impact of DAT Val559 expression on DAT in the striatum, we first quantified the amount of total DAT protein from striatal extracts, finding no changes compared to WT littermates. In accordance also with our *in vitro* data in transfected cells (Mazei-Robison et al., 2008), we found no change for the capacity of DA reuptake, as measured via [^{3}H]DA uptake using striatal synaptosomes. An important point for consideration is the use of synaptosomes, essentially reduced nerve terminals with the capacity for DA storage, release and reuptake. Indeed as we will find out in the next chapter, the use of synaptosomes is a good measure of the intrinsic capacity for DAT function, but is removed from the influence of the extracellular milieu, such as the morphology of the DA terminals and the influence of extracellular DA. Furthermore, we also found no changes in tissue DA content, suggesting that intracellular stores of DA are not depleted in lieu of the capacity for DAT Val559-ADE.

Our assessment of extracellular DA levels also yielded results essentially corroborating our findings in transfected cells. That extracellular DA levels are elevated in mice heterozygous and homozygous for DAT Val559 is indicative of the existence of DAT Val559-dependent ADE. Furthermore, we also observed a profound reduction in AMPH's ability to enhance extracellular DA in the striatum, mirroring our *in vitro* data, suggesting that AMPH blockade of DAT function is unchanged across genotypes. Concurrently, we also performed [^{3}H]DA release experiments from striatal slices containing DA terminals and after accounting for transporter-dependent [^{3}H]DA release in response to AMPH, by subtracting out the component of [^{3}H]DA release in the presence of GBR12909, a potent and specific DAT blocker, once again found a near complete loss of [^{3}H]DA release. In our studies related to AMPH, however, we did not assay the levels of AMPH taken up by DAT Val559 in striatal tissue. Interestingly, Bowton and colleagues showed that DAT Val559 is compromised in its ability to transport AMPH into the cell and thereby initiate signaling mechanisms culminating in DA reverse transport and subsequent DAT (Bowton et al., 2014). AMPH, however, is very labile and can penetrate a cell through the membrane devoid of DAT (Jones et al., 1998a), making measurements regarding its uptake specifically through the transporter problematic. Thus, these issues make it all the more imperative for us to assay specifically, the DAT-dependent component of AMPH-stimulated DA release, which we found to be severely compromised in the DAT Val559 striatum.

Surprisingly, we also found a significant reduction in vesicular DA release, upon stimulation of DA terminals from the DAT Val559 striatum via 4-AP. An interesting parallel can be drawn to observations made in DAT KO mice that also have elevated

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extracellular DA and reduced stimulation-evoked vesicular DA release, although the deficits are much more exaggerated (Jones et al., 1998b). Intracellular striatal DA levels are significantly reduced in the DAT KO mice as well, given the complete transgenic elimination of DAT in these mice and the resulting reliance only on DA synthesis for the replenishment of vesicular stores (Gainetdinov et al., 1999). However, this is not the case with the DAT Val559 mice, as evidenced by normal DA reuptake through DAT and unchanged intracellular striatal DA levels when compared to WT. Notably, we demonstrated that the reduction in vesicular release observed in DAT Val559 striatum was due to tonic activation of presynaptic D2ARs, owing to ongoing DAT Val559-dependent ADE. Jones and colleagues, observed a similar lack of response to D2AR activation on evoked DA release in the DAT KO as our results and deemed this to be due to D2AR desensitization (Jones et al., 1999). However, the authors did not assay the effects of D2AR antagonism on vesicular release in the DAT KO mice, which we found to reverse the blunted release in the DAT Val559, strongly suggesting that the D2ARs are tonically active owing to ADE, and not desensitized. One caveat to our experiments measuring DA release in striatal slices is that our studies were conducted using [^{3}H]DA loading to monitor DA release capacity versus measurements of the release of endogenous DA. Although we were able to demonstrate that [^{3}H]DA uptake in this preparation was unchanged across genotypes and prior studies have suggested that the [^{3}H]DA is packaged into readily releasable pools of vesicles, potential changes in vesicular storage of DA that may be engaged to sustain DAT Val559's capacity for ADE are yet to be determined.

Our results demonstrating ADE-dependent tonic activation of D2ARs comports

with the *in vitro* studies performed by Bowton and colleagues (Bowton et al., 2010). The authors showed that DAT Val559-mediated ADE is sustained by D2R (found endogenously in small amounts in heterologous cells), as evidenced by the fact that D2R antagonism abolished ADE in these cells. Furthermore, this study showed that DAT Val559 is hyperphosphorylated, which could be driven by D2R tonic activity signaling via CamKII, although evidence supporting this was circumstantial at best. This nonetheless raises an important question of how D2AR can regulate DAT through post-translational modifications and signaling cascades, and how these mechanisms may be disrupted in DAT Val559, in the context of the DAergic synapse. Whereas it is already known that D2AR activity can enhance membrane DAT trafficking (Bolan et al., 2007; Chen et al., 2013), the mechanisms through which D2AR asserts its action on DAT and if DAT populations are selectively trafficked to membrane microdomains are as yet unclear. Moreover, in addition to regulating DAT and inhibiting vesicular fusion mechanisms and DA neuron firing, D2ARs also control DA synthesis by inhibiting the activity of TH, via its coupling to regulatory G protein subunits and kinases (Dunkley et al., 2004). Hence, it will also be important to determine if other aspects of how D2AR regulates DA signaling are also affected due to DAT Val559 expression.

We also observed alterations in electrophysiological properties of DAT Val559 DA neurons in the SNPc. Studies prior have demonstrated that DA is also released from somatodendritic compartments (Beckstead et al., 2004), under tight control by DAT (Ford et al., 2010), although there may very well be a component to this release that is non-vesicular and DAT–dependent in nature (Falkenburger et al., 2001). Given this knowledge and our data regarding DA terminal alterations in the DAT Val559 striatum, it

is unsurprising that we observed a delay in the decay of the electrically evoked and spontaneous D2AR-IPSC in DAT Val559. Paralleling our presynaptic data on AMPH-stimulated release, we also observed deficits in AMPH's ability to increase the amplitude of evoked D2AR-IPSCs, but MPH's ability to induce this effect was unchanged across genotypes, suggesting once again that DAT blockade by AMPH (And MPH) is intact, but not AMPH's ability to cause DAT reversal, and hence non-vesicular DA release. Furthermore, we also found a marked reduction in the 5-stimulus to 1-stimulus ratio of evoked D2AR-IPSCs, an indication of a decrease in stimulated vesicular release in the somatodendritic compartment, in addition to that in presynaptic terminals.

In the above study, behavioral characterizations of the DAT Val559 mice were also carried out. DAT Val559 mice, surprisingly, do not display heightened basal locomotor activity in the home cage or in a novel environment, despite showing elevated extracellular DA levels. However, under imminent handling, mice heterozygous and homozygous for DAT Val559 show a conditional hyper-reactivity, which we termed "darting", and we observed elevated frequency and speed of darting in the DAT Val559 mice, compared to WT littermates. Furthermore, DAT Val559 mice also showed a reduced locomotor response to AMPH, owing to the loss in AMPH-stimulated DA release and to a conventional DAT blocker, MPH, presumably due to the reduction in vesicular DA release, as the effects of MPH are contingent upon excitation-evoked elevations in extracellular DA. As we will find in subsequent chapters and from recently published evidence, DAT Val559 mice also display alterations in motivation and impulsivity (Davis et al., 2018), traits that align well with what one would expect from a

mouse model of ADHD.

How might elevations in tonic DA caused by DAT Val559 lead to behavioral deficits in mice akin to what is observed in patients? One possibility, for which I will provide more evidence in the next chapter, is altered signal to noise. Just like in the DAT KO, DAT Val559-mediated ADE results in "functional hyperdopaminergia", while the DA neurons are still compromised in the ability to release DA in response to excitability. Hence, this change in dynamic range could disrupt the ability of DA neurons to appropriately shift from tonic to phasic firing, thereby compromising the behaviors reliant on patterned neuronal activity. Furthermore, DAT itself is regulated by several complex mechanisms (Bermingham and Blakely, 2016), each contributing an additional level of control to DA signaling and the behaviors it controls and ADE is found not only in the DAT Val559, but also in ASD-associated DAT variants (Hamilton et al., 2013) (with the DAT Val559 also found in ASD) and in patients with PD (Hansen et al., 2014). Therefore, understanding physiological mechanisms of DA efflux through DAT would help us better understand the functional consequences of non-vesicular DA release in disease states.

Chapter 3 – Region-Specific DAT Regulation Shapes Impact Of DAT Val559

3.1 Introduction

In the pursuit of a mouse model for neuropsychiatric disorders, with the expectation of being able to recapitulate behavioral features with translational relevance, one must be sensitive of the complex etiologies contributing to the risk for mental illness. The DSM V recognizes the existence of across-patient variability in symptoms for multiple neuropsychiatric disorders; however studies identifying driving factors for this variability, which would ultimately enable the development of better treatment strategies are few, and difficult to pursue in humans. Indeed, rare genetic variation in multiple genes that ultimately impinge on similar downstream pathways arising in different people with the same disorder could definitely contribute to this variability in symptomology. However, given the heterogeneity in seemingly uniform populations of neurons and in mechanisms that regulate their signaling, similar to what has been reported in the DA system (Roeper, 2013), one could argue that selective disruptions in divergent molecular- or circuit-level features could drive variability in symptomology. Hence the identification of such differences in the brain and discoveries that the differential regulation of molecular pathways sculpts the impact of disease-associated environmental or genetic factors, ultimately shaping the presentation of behavioral symptoms could be extremely valuable.

As discussed in the previous chapter, the DAT Val559 model presents with behavioral phenotypes similar to the symptoms found in the patient populations it was identified from, thereby providing a tractable model for the determination of molecular disruptions in DA signaling that may lead to disease. Our studies showed an ADE-driven tonic activity of the D2ARs, resulting in alterations in the electrophysiological properties of DA soma, but also deficits in evoked vesicular DA release (Mergy et al., 2014b). A lesser understood and underappreciated aspect of the regulation of DA signaling by D2ARs is that in addition to inhibiting release (Benoit-Marand et al., 2001), the autoreceptors also physically couple with (Lee et al., 2007), and regulate DAT, by trafficking more transporter to the surface and enhancing DA reuptake upon activation (Bolan et al., 2007; Chen et al., 2013), presumably to match the demands of vesicular release; however the details of this interaction are as yet unclear. Additionally, D2ARs also negatively regulate DA synthesis by inhibiting TH activity, thereby constraining cytosolic DA levels (Bello et al., 2011). Our *in vitro* (Mazei-Robison et al., 2008)and striatal synaptosome (Mergy et al., 2014b) data discussed in prior chapters show no changes in DA reuptake; however heterologous cell systems have very low amounts of D2-like receptor mRNA and synaptosomes are dispersed in large volumes and lack much of the cytoarchitecture found in intact preparations. Hence, the preparations used to assay DAT activity and surface expression by us thus far may preclude potential effects the extracellular milieu, or ADE-driven D2AR activity may have on DA reuptake and DAT regulation. Therefore, this chapter, in part, seeks to determine the impact of the DAT Val559 variant on D2AR-mediated DAT regulation and DA synthesis employing *ex vivo* slice preparations.

In considering how ADE impacts DA signaling, it is also important to recognize that DA neurons, as discussed in Chapter 1, despite sharing similar mechanisms to synthesize, release and inactivate DA, express diversity in their anatomy and physiology depending on their anatomical location, target projections, inputs they receive, control of release mechanisms and the behaviors they regulate. The majority of brain DA neurons are located in the midbrain, with those in the SNpc projecting to the DS and in the VTA projecting to the VS, in addition to a small VTA population sending terminals to the PFC (Bjorklund and Dunnett, 2007). Recent studies have suggested diversity in DA neuron signaling by location of origin (i.e. SNPc vs. VTA) and target (i.e. DS vs. VS) (Chuhma et al., 2017), and that this heterogeneity supports different behaviors (Howe and Dombeck, 2016). However, whether expression of genetic variation in all DA neurons, such as the DAT Val559 can result in pathway-specific or circuit-biased molecular and/or behavioral phenotypes is unclear. Here, after a description of the methods used, I will provide evidence for previously unknown, region-specific D2AR-dependent regulation of DA homeostasis in distinct DA terminals that contributes to unexpected, region-specific bias in penetrance of the DAT Val559. Broadly, these studies provide the first example of how molecular pathway-specific differences in the regulation of neurotransmitter signaling can sculpt the impact of genetic variation, found in genes expressed homogenously, and may explain the prevalence of distinct clinical features of neuropsychiatric disorders.

3.2 Methods

Animals. All experiments were performed under protocols approved by IACUC of Florida Atlantic University and Vanderbilt University. Unless noted otherwise, 4-6 week old mice homozygous for DAT Val559 or WT were bred from homozygous dams and sires derived from heterozygous breeders, no more than 2 generations removed. Genetic background for all animals was 75% 129/6: 25% C57 as with prior studies (Mergy et al., 2014b; Davis et al., 2018). Only age-matched juvenile males were used owing to the male bias reported for ADHD diagnoses and the reported age of symptom onset. Animals were housed on a 12:12 reverse light cycle with water and food available *ad libitum*. All experiments were performed during the animal's active cycle or dark phase.

Brain slice preparation, drug treatments and immunoblotting. All chemical reagents used in experiments were obtained from Sigma-Aldrich unless otherwise specified. Brain slice preparation and experiments were carried out under constant oxygenation ($95\%O_2:5\%CO_2$). Animals were killed by rapid decapitation and excised brains were moved to ice-cold sucrose-artificial cerebrospinal fluid (S-ACSF = in mM - sucrose 250, KCl 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 26, D-glucose 11, $MgCl_2.6H_2O$ 1.2, $CaCl_2.2H_2O$ 2.4, pH – 7.4, 300-310 mOsm). A vibratome (Leica VT100 S) was used to prepare 250 μm slices that contained the dorsal striatum (DS) and/or ventral striatum (VS) (matching coordinates of 0.2-0.5 mm A/P for DS, and 1.0-1.5 mm A/P for VS in the Allen Mouse Brain Atlas) in S-ACSF following which, slices were allowed to recover at 30-32°C for 1 hr in ACSF, substituting 92 mM NaCl for sucrose (pH – 7.4, 300-310

mOsm). Prior to drug treatments, slices were washed with ACSF at 37°C and then treated with vehicle, 1μM (-)-quinpirole hydrochloride (quinpirole), a D2/D3 agonist for 5 min (biotinylation) or 10min (immunoprecipitation), 10μM (±)-sulpiride hydrochloride (sulpiride), a D2/D3 antagonist for 20min (biotinylation and immunoprecipitation) or 100μM 3-hydroxybenzylhydrazine dihydrochloride (NSD1015), an amino acid decarboxylase inhibitor for 10min (L-DOPA accumulation). With the exception of biotinylation and L-DOPA accumulation (see below), slices were washed in ice cold ACSF, followed by rapid dissection of DS and VS, and tissue solubilization in lysis buffer (in mM - NaCl 150, KCl 2.5 m, Tris 50, and 1% Triton X-100). Protein concentrations were determined using the BCA protein assay (ThermoFisher, Waltham, MA, USA) with bovine serum albumin (BSA) as standard, with a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Following SDS-PAGE (10% gel), proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) at 4°C overnight and then blocked with 5% dry milk (for DAT) or 5% BSA (for tyrosine hydroxylase, TH) in room temp wash buffer (in mM - NaCl 150, 2.5 KCl, 50 Tris and 0.1% Tween-20). Blocked membranes were incubated with rat anti-DAT (MAB369, RRID: AB_2190413, Millipore, Billerica, MA, USA,) and rabbit anti-TH (2025-THRAB, RRID: AB_2492276 PhosphoSolutions, Aurora, CO, USA,) at a dilution of 1:1000 in wash buffer overnight at 4°C. Membranes were then rinsed 4X in wash buffer prior to incubation with goat anti-rat or goat anti-rabbit HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) at a 1:10000 dilution for 1hr. Following multiple washes using wash buffer, membranes were subjected to chemiluminescent visualization (BioRad Clarity ECL, Hecules, CA, USA) and bands

were visualized using an ImageQuant LAS 4000 imager (GE Healthcare Life Sciences, Chicago, IL, USA). Blot images were minimally processed during quantitation and cropped for the presentation of representative figures.

Slice biotinylation. Methods to assess DAT surface expression in acute brain slices were adapted from those described previously(Gabriel et al., 2014). Briefly, following washes in ice cold ACSF, slices were treated with sulfo-NHS-SS-biotin (1mg/mL, ThermoFisher, Waltham, MA, USA) on ice for 30min. Reactions were quenched by treatment with 0.1M glycine 2X for 10 min, followed by rapid washes with ice cold ACSF. DS and VS were rapidly dissected, solubilized in lysis buffer as noted above, and protein concentration determined. Detergent lysates were added to streptavidin agarose beads (ThermoFisher, Waltham, MA, USA) at a ratio of 20µg protein per 50µL bead slurry and mixed overnight at 4^oC. Following washes with lysis buffer, protein was eluted/denatured at room temp for 30min, subjected to SDS-PAGE, PVDF transfer and immunoblotting for DAT and TH as described above.

[^{3}H]DA slice uptake. [^{3}H]DA uptake in acute slices was performed as described previously(Wu et al., 2015). In brief, brain slices containing DS and VS were washed in 37^oC were incubated in 50nM [^{3}H]DA (Dihdroxyphenylethylamine, 3, 4-[Ring-2, 5, 6-^{3}H], Perkin-Elmer, Waltham, MA, USA) for 10min, which lies within the linear portion of the time dependence of uptake in our assays (r^2=.86), in the presence of GBR12909, a DAT inhibitor. Slices were then rapidly washed in ice-cold ACSF, DS and VS dissected and proteins extracted using lysis buffer. Lysates were then added to Ecoscint H (National Diagnostics, Atlanta, GA, USA), followed by gentle shaking overnight. Accumulated

radioactivity was determined using a TriCarb 2900TR scintillation counter (Perkin-Elmer, Waltham, MA, USA). Counts were normalized to DAT total levels in each condition.

High-speed chronoamperometry. For *in vivo* high-speed chronoamperometry (HSC) assessment of DA clearance, 8-10 week old mice were used. HSC for DA was performed as described previously (Owens et al., 2012). Animals were anesthetized by i.p injection of α-chlorolose (400mg/kg) and urethane (4mg/kg), fitted with an endotracheal tube to ensure steady breathing, and placed on a heating pad at 37°C with head mounted in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Electrode-micropipette assemblies were made prior to the experiment using a single, 20µm diameter carbon fiber (Specialty Materials) and 7-barrel micropipettes (FHC, Bowdoin, ME, USA), ensuring a distance of 200µm between the electrode tip and micropipette. The electrode was coated with 5% nafion to enhance selectivity for DA and calibrated *in vitro* using 2µM serial additions of DA to Phosphate Buffer Saline (PBS). Each barrel of the micropipette was filled with either 200µM DA (in PBS) or 400µM sulpiride (in ACSF+10% DMSO). The electrode-micropipette assembly was lowered into the DS (1.5 mm M/L, 0.5 mm A/P, 2.5 mm D/V) or VS (1.0 mm M/L, 1.5 mm A/P, 3.5 mm D/V). DA pressure ejection was achieved using a Picrospritzer (Parker Hannifin, Hollis, NH, USA) with an ejection volume of 8-125 nL (10-30 psi, 0.05-5 sec). Drugs were applied at a volume of 100-125 nL (0.5 psi). Recordings of DA clearance were conducted using the FAST12 system (Quanteon, Nicholasville, KY, USA), following 100ms of 550mV step potentials to the carbon fiber, separated by a 0.9ms return to 0mV, relative to a reference electrode mounted under the neck. DA was identified using its current

oxidation/reduction ratio – 0.5 to 0.8. Signal parameters used in quantitation were the T_{80} (s), the time for amperometric signal to decay to 80% signal amplitude, and the clearance rate (T_C in nM/s), estimated as the slope of the linear portion of current decay from 20-60% signal amplitude.

Phospho-Thr53 DAT immunoprecipitation. Quantification of DAT phosphorylation at Thr53 was assessed as previously described(Foster et al., 2012). Briefly, rabbit Thr53 DAT antibody (Roxanne A. Vaughan, RRID: AB_2492078) was cross-linked to Protein A magnetic beads (Dynabeads, ThermoFisher, Waltham, MA, USA) at a ratio of 1mg Thr53 antibody or rabbit IgG (Antibodies Inc. Davis, CA, USA) to 10μL bead slurry using 25mM dimethyl pimelimidate in 0.2M triethylamine 3X for 30 min each, quenched with 50mM ethanolamine then treated with 5% BSA in PBS + 0.1% Triton X-100 at 4°C for 1hr to reduce non-specific binding. Detergent lysates of brain slices were added to cross-linked DAT Thr53 antibody-conjugated beads at a ratio of 250μg protein to 25μL of bead slurry, rocked at 4°C for 4hr, washed with lysis buffer, treated with 2X Laemmli sample buffer (BioRad, Hecules, CA, USA) to elute/denature protein prior to SDS-PAGE and immunoblotting for DAT as described above.

Slice L-DOPA accumulation. Following slice preparation and recovery, slices were treated with NSD1015 (100μM) alone, or quinpirole (1μM) and NSD1015 (100μM) for 10min at 37°C, then rapidly washed in ice-cold ACSF and sonicated in 250 μL of 0.2 M $HClO_4$. Isoproterenol (10ng) was added as an internal standard, the protein denatured and samples centrifuged at 20,000xg for 15min at 4°C, with 1M sodium acetate addition to modify the supernatant pH to 3.0. The supernatant was then filtered

with a spin column and analyzed by HPLC. The HPLC system consisted of an Eicom Insight Autosampler (AS-700), Eicom Stand-Alone HPLC-Electrochemical Detection System (HTEC-510), Eicompak SC-30DS (ID 3.0 x 100 mm) reverse-phase column with a graphite working electrode (WE-3G) (Eicom, San Diego, CA, USA). Assays used an applied potential of +750 mV vs. Ag/AgCl. A mobile phase consisting of 85% 0.1M citrate-acetate buffer (pH 3.5), 15% methanol, 220mg/L sodium octane sulfonate and 5mg/L EDTA-2Na was used to separate L-DOPA and its metabolites. Signals were quantitated relative to known concentrations of standards using Envision Data System software. The final oxidation current values were converted to ng and adjusted to mg protein amounts determined by BCA protein assay as described above.

3.3 Results

DAT Val559-driven tonic, region-specific presynaptic D2AR activation induces elevated DAT surface expression in DS, but not VS. Our previous studies in transfected cells and cultured neurons demonstrated that DAT Val559-driven ADE is sustained by DA stimulation of endogenous D2Rs (Bowton et al., 2010). However, this D2R engagement did not enhance DAT surface trafficking (Mazei-Robison and Blakely, 2005), although studies in transfected cells expressing hDAT and D2R constructs utilizing quinpirole for D2R stimulation, and D2AR activation with quinpirole in striatal synaptosomes has been reported to increases DAT surface expression (Chen et al., 2013). Morevoer, we also did not observe any D2R-driven changes in DAT activity in transfected cells expressing DAT Val559, or in striatal synaptosomes prepared from DAT Val559 animals, suggesting that differences between heterologous, culture and native preparations may limit detection of DAT Val559 phenotypes. Therefore, we determined the effects of D2AR stimulation on transporter surface levels using quinpirole (1μM, 5min) via *ex vivo* biotinylation approaches (Gabriel et al., 2014). Also sensitive to the aforementioned molecular and functional heterogeneity in DA projections, we assessed transporter trafficking in WT and Val559 mice using slices containing either the DS or VS. Upon such a region-selective analysis, we found that total levels of DAT protein were not affected by DAT Val559 in either DS or VS (Figure 10A-B). However, surface DAT density was significantly elevated in the DAT Val559 DS relative to WT animals (Figure 10C). Surprisingly, this effect of DAT Val559 expression was absent in slices prepared from the VS (Figure 10D) Additionally,
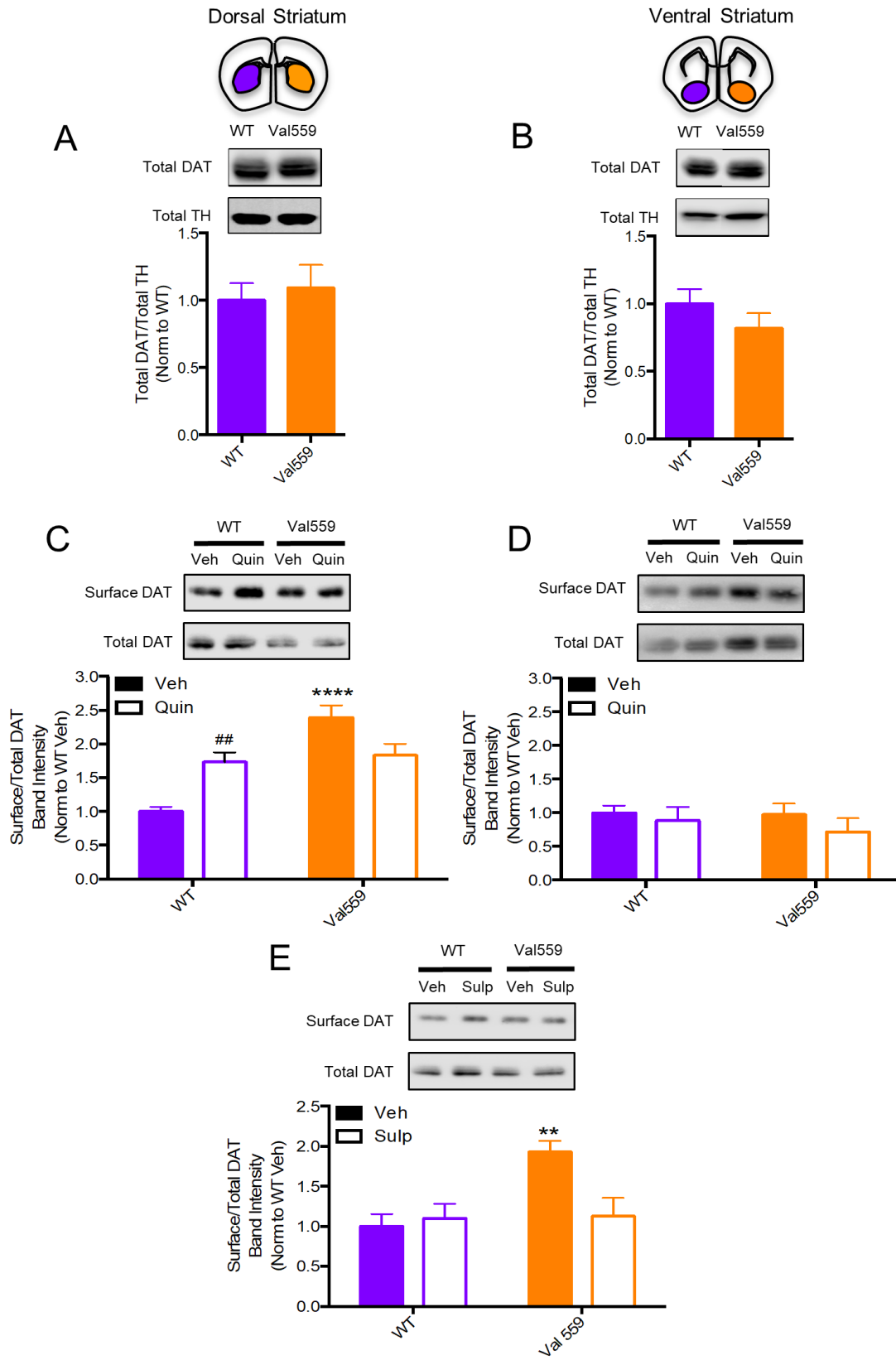
Figure 10. DAT Val559-driven tonic, region-specific presynaptic D2AR activation induces elevated DAT surface expression in DS, but not VS. Total DAT levels, normalized to total TH, are unchanged across genotypes in both **(A)** DS (two-tailed Student's t-test, t(14)=0.4344, P=0.671, N=8) and **(B)** VS (two-tailed Student's t-test, t(10)=1.164 P=0.271, N=6). In the DS **(C)** surface DAT in DAT Val559 is higher relative to WT and Quin (1μM, 5min) elevates WT surface DAT, not DAT Val559 (two-way ANOVA, genotype F(1,24)=25.64, P<0.0001, Quin F(1,24)=0.3471, P=0.561, interaction F(1,24)=19.23, P=0.0001. Bonferroni's multiple comparisons test – P<0.0001* for WT Veh vs. Val559 Veh, P=0.009 for WT Veh vs. WT Quin[#], P=0.072 for DAT Val559 Veh vs. DAT Val559 Quin. N=8). In the VS, **(D)** membrane DAT is unchanged across genotypes and surface levels are unaffected in response to Quin (1μM, 5min) (two-way ANOVA, genotype F(1,16)=0.306, P=0.5873, Quin F(1,16)=1.145, P=0.300, interaction F(1,16)=0.159, P=0.696. N=5). **(E)** Sulp (10μM, 20min) treatment abolishes genotype effects in DAT Val559 (two-way ANOVA, genotype F(1,20)=7.117, P=0.015, Sulp F(1,20)=3.855, P=0.064, interaction F(1,20)=6.402, P=0.0199. Bonferroni's multiple comparisons test – P=0.009 for WT Veh vs. DAT Val559 Veh*, P>0.999 for WT Veh vs. DAT Val559 Sulp. N=6).

quinpirole treatment elevated cell surface transporter levels in WT DS slices, shown for the first time in a slice preparation, lending credence to the validity of D2AR-stimulated increases in membrane DAT. However, no effect of quinpirole was detected in DAT Val559 DS slices (Figure 10C). Remarkably, quinpirole was without effect on surface DAT levels in VS slices regardless of genotype (Figure 10D). We hypothesized that ongoing, tonic activation of D2ARs by ADE-driven extracellular DA might be responsible for elevated basal surface DAT in DAT Val559 DS slices, as well as the lack of quinpirole-induced trafficking to the membrane. This was corroborated by the elimination of the DAT Val559 genotype effect on basal surface DAT levels with the D2-type receptor antagonist sulpiride (10μM, 20min)(Figure 10E).

DAT Val559-mediated ADE impacts *in vivo* basal and D2AR-mediated clearance of exogenous DA, highlighting impact of ADE in the DS, not VS. Our findings showing enhanced surface DAT in the DAT Val559 DS were surprising, as we previously observed no changes in DAT-mediated [^{3}H]DA uptake using transfected cells (Mazei-Robison and Blakely, 2005) or striatal synaptosomes (Mergy et al., 2014b). We speculated that an inability to detect changes in DA uptake in DAT Val559 striatal synaptosomes could arise from either the disruptive and reduced nature of synaptosomal preparations or due to the diffusion of DA owing to synaptosomal incubations in large volumes that could both contribute to the dampening of D2AR-dependent alterations in DAT surface expression and activity. So we next attempted to ascertain putative genotype effects on [^{3}H]DA uptake assays in brain slices, which, however, revealed further complexities. Here, surprisingly, we detected a significant reduction in [^{3}H]DA uptake in DAT Val559 versus WT DS slices (Figure 11A-B). One
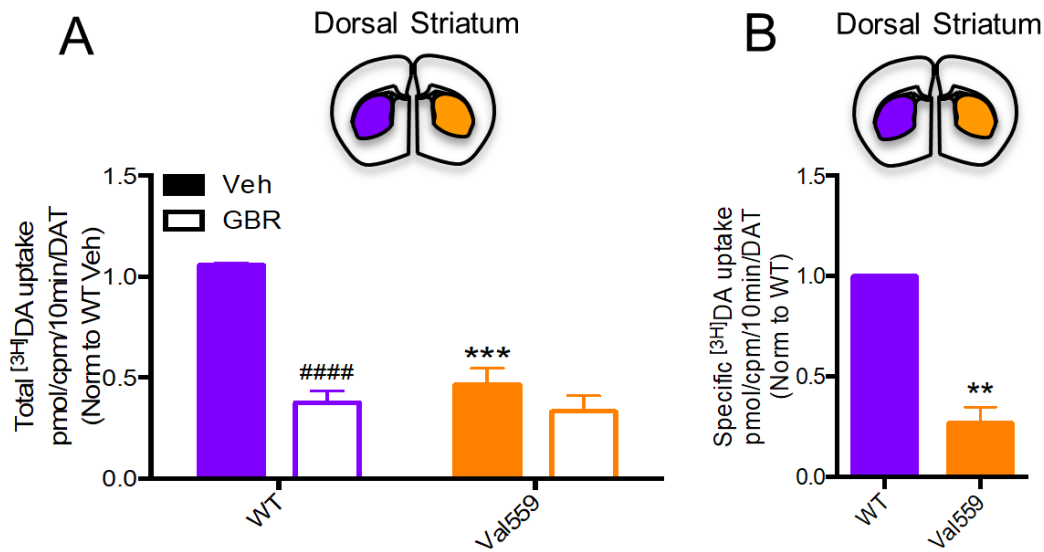
Figure 11. DAT Val559 causes a reduction in [^{3}H]DA uptake in DS slices. DAT Val559 DS slices show a profound reduction in **(A)** total (two-way ANOVA, genotype $F_{(1,12)}=25.56$, $P=0.0003$, GBR $F_{(1,12)}=42.11$, $P<0.0001$, interaction $F_{(1,12)}=18.98$, $P=0.0009$. Bonferroni's multiple comparisons test shows $P=0.0001$* for WT Veh vs. DAT Val559 Veh, $P<0.0001$# for WT Veh vs. WT GBR and $P=0.944$ for Val559 Veh vs. DAT Val559 GBR. N=4) and **(B)** specific [^{3}H]DA (50nM, 10min) uptake (two-tailed Student's t-test, $t_{(6)}=9.188$, $P<0.003$*, N=4).

possible explanation is that, in the DS slices, DA released via ADE could remain trapped in the tissue, causing a reduction in the reuptake and/or detection of [^{3}H]DA specific activity, thereby appearing to reduce [^{3}H]DA uptake. Moreover, a slice preparation, as studies prior have shown has very minimal amounts of extracellular DA (Ferris et al., 2013), thereby resulting in a pronounced effect of elevated extracellular substrate, such as in the case of slices from DAT Val559 mice. Should this be a possibility, we would be able to measure a reduction in apparent DA affinity, or an increase in K_M in dose-response studies for DA. To explore this idea, and to move our functional evaluations *in vivo*, we determined clearance of exogenously applied DA in anesthetized WT and Val559 mice using high-speed chronoamperometry (HSC). Exogenous application of DA, as opposed assaying the clearance of DA derived from the stimulation of endogenous DA release, would help us determine the effects of DAT Val559 on DA clearance alone, removed from potential alterations in DA release that might affect our estimation of reuptake. Pressure ejection of DA resulting in a signal amplitude of 1μM supported our findings *ex vivo*, showing a greater time to clear 80% of the DA signal (T_{80}) and reduction in DA clearance rate (T_C) in the DAT Val559 DS (Figure 12A). Once again, remarkably, DA clearance in the VS was unchanged across genotypes (Figure 12B). Moreover, DA clearance in VS versus the DS was slower, similar to studies showing the same results in slices via *ex vivo* FSCV (Calipari et al., 2012). Subsequently, we performed dose-response studies, where we pressure-ejected increasing concentrations of DA and ascertained DA clearance rates (T_C) at each concentration. As seen in Figure 12C-D, T_C profiles conformed well to Michaelis-Menten kinetics in a curvilinear fashion, regardless of genotype or region (see legend for r^2

Dorsal Striatum
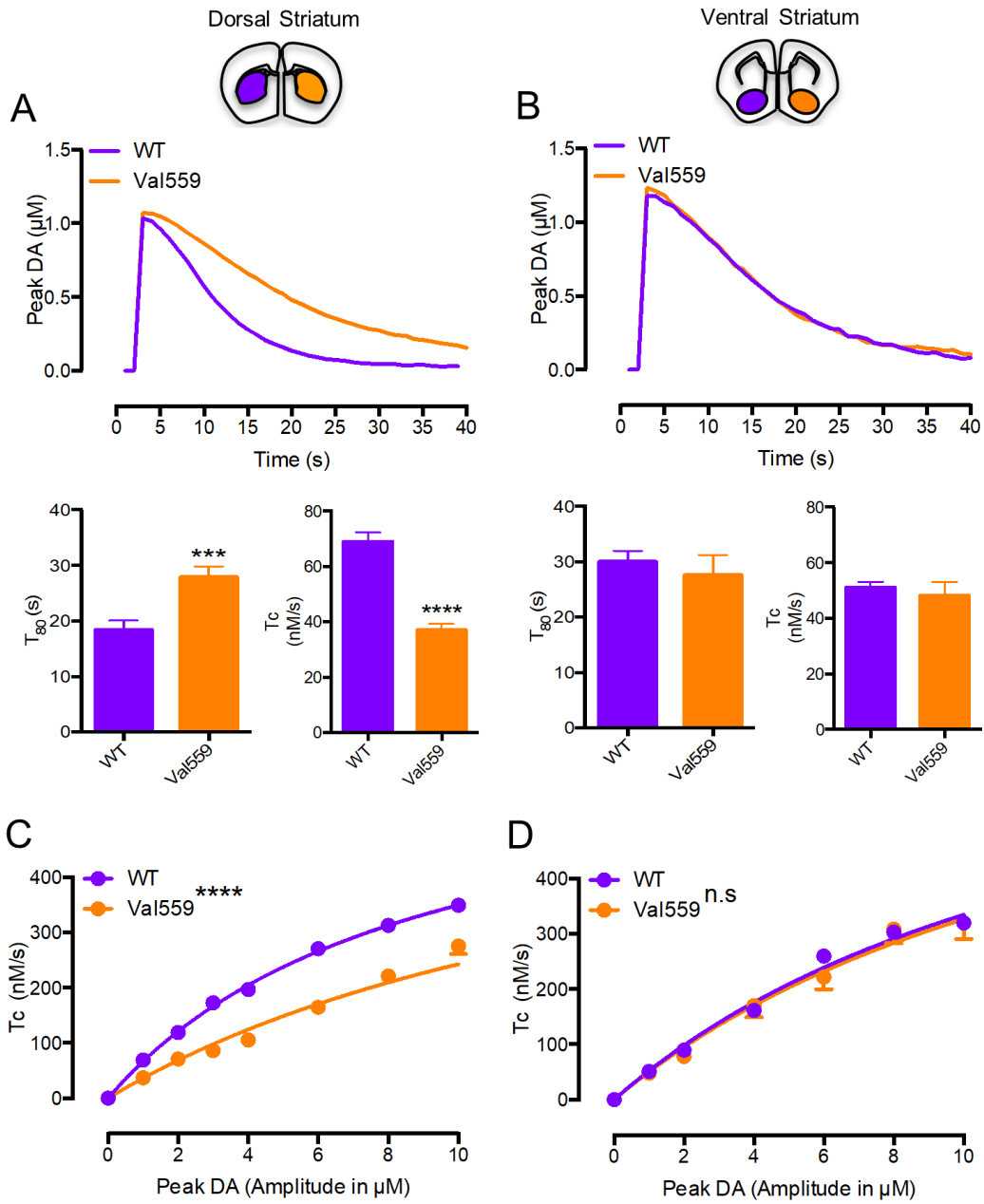
A

Ventral Striatum

B

C

D

Figure 12. DAT Val559 reduces *in vivo* clearance of exogenous DA, highlighting impact of ADE in DS, not VS. In the DS, (A) DAT Val559 expression results in decreased T_C (two-tailed Student's t-test, t(30)=8.499, *P*<0.0001*, N=5-6) and a delay in T_{80} (two-tailed Student's t-test, t(30)=3.661, *P*=0.001*, N=5-6), also apparent in the representative clearance trace at 1 µM, but in the VS (B) causes no change in T_C (two-tailed Student's t-test, t(22)=0.549, *P*=0.568, N=4) or T_{80} (two-tailed Student's t-test, t(22)=0.400, *P*=0.693, N=4). In dose-dependent studies in the DS, (C) clearance rates (T_C) are attenuated in the DAT Val559 compared to the WT (two-way ANOVA, genotype F(1,218)=401.8, *P*<0.0001, peak DA F(7,218)=506.7, *P*<0.0001, interaction F(7,218)=18.33, *P*<0.0001. Bonferroni's multiple comparisons test shows *P*=0.002 for WT vs. DAT Val559 at 1µM and *P*<0.0001 at 2, 3, 4, 6, 8 and 10µM peak DA. N=5-6). T_C profiles conformed to Michaelis-Menten kinetics in both WT and DAT Val559 (WT-r^2=0.969, Val559-r^2=0.873), but show apparent K_M for DA being significantly higher in Val559 (WT K_M=8.9±0.14µM, DAT Val559 K_M=17.3±0.50µM, two-tailed Student's *t*-test, t(9)=14.49, *P*<0.0001). In the VS, (D) DAT Val559 T_C is comparable to WT over all DA signal amplitudes (two-way ANOVA, genotype F(1,154)=0.561, P=0.478, peak DA F(6,154)=162.2, *P*<0.0001, interaction F(6,154)=0.619, *P*=0.715. N=4) and conformed well to Michaelis-Menten kinetics for WT and DAT Val559 (WT-r^2=0.971, DAT Val559-r^2=0.861), with no change in apparent K_M (WT K_M=14.99±0.24µM, DAT Val559 K_M=15.59±0.82µM, two-tailed Student's *t*-test, t(6)=0.7, *P*=0.528).

values). Consistent with our hypothesis, we found a significant increase in the apparent K_M for exogenous DA from fits of DS recordings in Val559 vs. WT mice (Fig. 12C, see legend for K_M values). In contrast, apparent DA K_M values were unchanged across genotypes in recordings from the VS (Figure 12D).

Previous studies using HSC have also shown that local injection of D2AR antagonists can enhance the time to clear exogenous DA, and result in increases in the DA signal amplitude, possibly due to the inhibition of D2AR-induced elevations in DAT surface levels brought about by the dynamic regulation of DAT by the exogenous DA pulse (Cass and Gerhardt, 1994; Dickinson et al., 1999). We posited that DAT Val559-induced ADE in the DS would prevent D2AR antagonist effects, owing to the greater amount of DA needing to be blocked to antagonize D2AR activation. In WT DS, sulpiride (50pmol, 20min) pressure-ejections following a stable 1µM DA signal amplitude, significantly elevated DA signal amplitude and increased the time to clear 80% of the DA signal (T_{80}), compared to vehicle (Figure 13A, 1µM DA signal amplitude presented). Under the same conditions, in the DS of Val559 mice, sulpiride failed to increase DA signal amplitude (actually a small but significant decrease) or to delay T_{80} (Figure 13B). Together with our slice DAT trafficking and [^{3}H]DA uptake studies, our HSC dose-response and D2AR antagonist studies support a model where the DAT Val559 DS exhibits a tonic D2AR activity-driven elevation in surface levels of DAT protein in an "efflux-prone" conformation. In the VS, however, DAT Val559 ADE fails to modify transporter membrane levels and lacks the ability to amplify ADE, due to a lack of D2AR-DAT coupling.
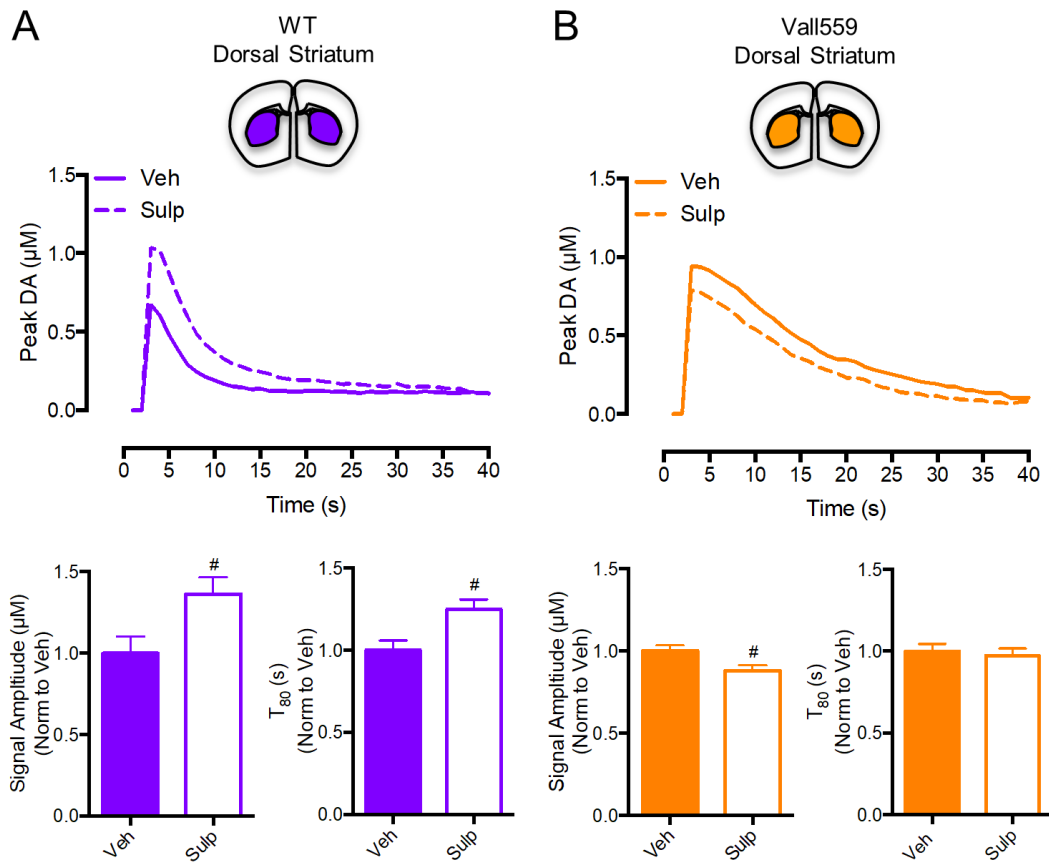
Figure 13. DAT Val559 attenuates the effects of *in vivo* D2AR antagonism on clearance of exogenous DA in the DS. In the WT DS, **(A)** Sulp injection (50pmol, 20min) enhanced signal amplitude (two-tailed Student's *t*-test, t(12)=2.519, *P*=0.027[#]. N=7) and delayed T_{80} (two-tailed Student's *t*-test, t(12)=2.998, *P*=0.011[#]. N=7), but (B) reduced signal amplitude (two-tailed Student's *t*-test, t(6)=2.608, *P*<0.04[#]. N=4) and had no effect on T_{80} (two-tailed Student's *t*-test, t(6)=0.431, *P*=0.681. N=4) in the DAT Val559 DS.

DAT Val559-triggered tonic, region-specific presynaptic D2AR activity causes enhanced DAT phosphorylation in DS, but not VS. Multiple DAT N-terminal Ser/Thr residues are phosphorylated under basal conditions (Foster et al., 2002; Foster et al., 2012), and elevations in DAT phosphorylation at these sites has been demonstrated following drug treatments (Foster et al., 2006; Challasivakanaka et al., 2017). However, the role of endogenous receptors (such as D2AR) in these phosphorylation mechanisms as a way of controlling native DAT trafficking and function is as yet unclear. Furthermore, how transporter phosphorylation is impacted by disease-associated mutations, remains an active area of investigation (Ramamoorthy et al., 2011; Bermingham and Blakely, 2016; Foster and Vaughan, 2017). Bowton and colleagues demonstrated enhanced phosphorylation of DAT at the distal N-terminus Ser residues, but how this relates to DAT regulation and contributes to ADE is still unknown. Additionally, the broader impact of transporter phosphorylation mechanisms on disease-associated mutations still remains an active area of investigation. Recently, Foster and colleagues detected basal phosphorylation of DAT at Thr53 (p-Thr53) in both transfected cells and striatal synaptosomes (Foster et al., 2012). Thr53 phosphorylation was also found to be necessary for AMPH-induced DA efflux, important in the context of ADE, induced spontaneously by DAT Val559. Moreover, Thr53 lies within a MAPK consensus phosphorylation site, of relevance to our studies becuase D2AR regulation of DAT trafficking has been found to be dependent on signaling via MAPK ERK1/2 (Bolan et al., 2007). Upon p-Thr53 estimation in WT and DAT Val559 slices, we once again found region-dependent changes. Our studies showed a significant elevation in p-Thr53 DAT in DS slices, but not the VS slices prepared from DAT Val559 mice (Figure

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14A-B). To determine whether enhanced DAT phosphorylation at Thr53 might be a consequence of ADE-driven D2AR stimulation, we treated slices with quinpirole (1μM, 10min), and detected an induction in phosphorylation at Thr53 in the WT DS but not in DAT Val559 DS slices (Figure 14A). Consistent with the hypothesis that basal increases in DAT Val559 p-THr53 were dependent on tonic D2AR activity, p-Thr53 DAT levels in DAT Val559 DS slices were normalized by treatment of slices with sulpiride (10μM, 20min) (Figure 14C). Paralleling our observations supporting a lack of D2AR-DAT coupling in the VS, quinpirole treatment failed to elevate p-Thr53 levels in VS slices regardless of genotype (Figure 14B).

DAT Val559 expression compromises the ability of D2AR to inhibit tyrosine hydroxylase (TH) activity in the DS, but not VS. Our studies so far regarding DAT trafficking, DA clearance and DAT phosphorylation at Thr53 strongly suggest the existence of a region-dependent perturbation by DAT Val559, driven by the functional coupling of D2AR and DAT in the DS, but not the VS. However, VS DA projections express functional D2ARs (Sesack et al., 1994a), regulating DA release and DA synthesis in both the DS and the VS. Hence, we explored whether the region-specific impact of DAT Val559 extends beyond the control of presynaptic regulation, specifically to that of DA synthesis and the activity of TH, the rate-limiting enzyme for DA synthesis. Previous studies have shown that D2AR stimulation in the striatum via quinpirole can inhibit TH activity, as measured by L-3,4-Dihydroxyphenylalanine (L-DOPA) accumulation in the presence of the amino acid decarboxylase inhibitor, NSD1015 (Lindgren et al., 2001; Bello et al., 2011). We determined the modulation of NSD-stimulated L-DOPA accumulation by quinpirole in DS and VS slices prepared from WT

A **Dorsal Striatum**

B **Ventral Striatum**

C
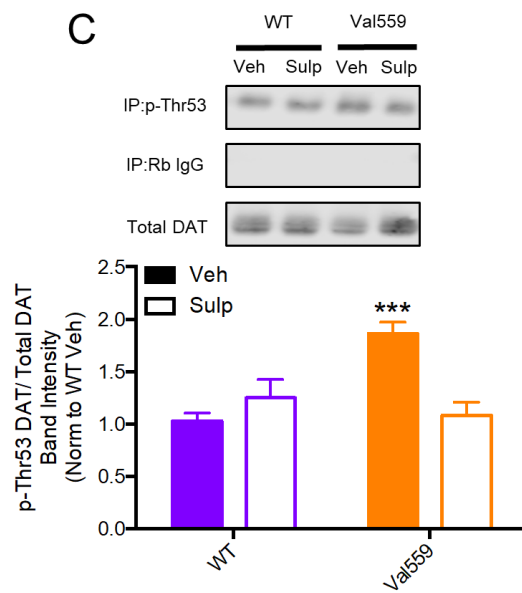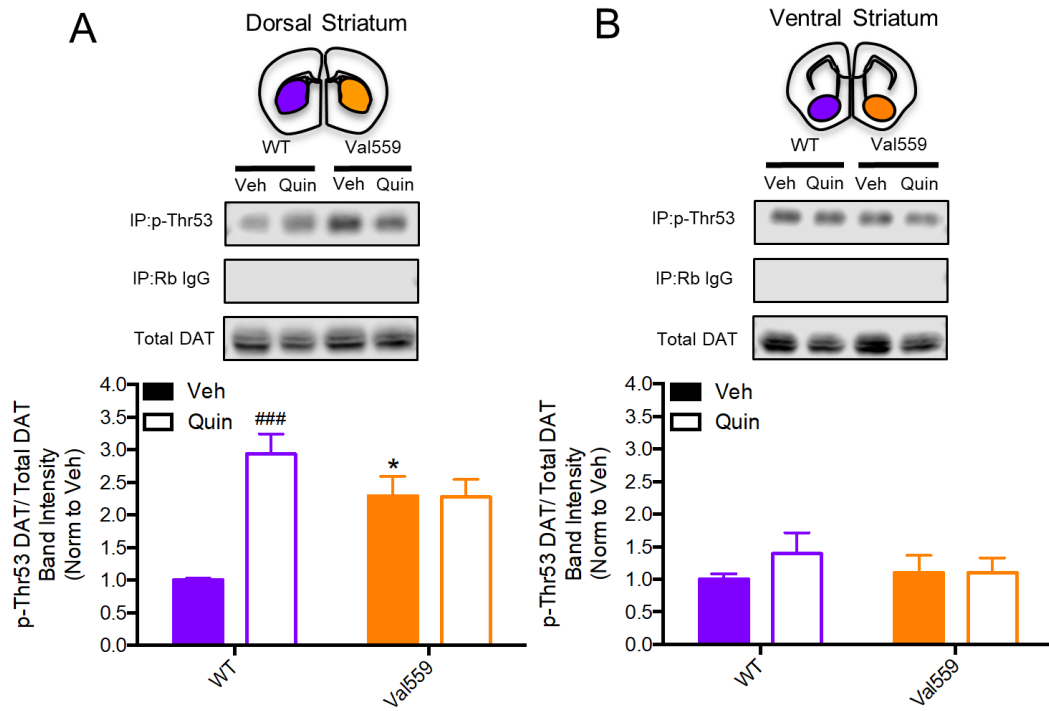
Figure 14. DAT Val559-triggered tonic, region-specific presynaptic D2AR activity causes enhanced DAT phosphorylation in DS, but not VS. In the DS **(A)** basal DAT Val559 p-Thr53 is higher relative to WT and Quin (1μM, 10min) increases WT p-Thr53, whereas DAT Val559 p-Thr53 is unchanged (two-way ANOVA, genotype $F_{(1,16)}=1.553$, $P=0.231$, Quin $F_{(1,16)}=14.47$, $P=0.001$, interaction $F_{(1,16)}=14.89$, $P=0.002$. Bonferroni's multiple comparisons test – $P=0.014$* for WT Veh vs. DAT Val559 Veh, $P=0.0003$[#] for WT Veh vs. WT Quin, $P>0.9999$ for DAT Val559 Veh vs. DAT Val559 Quin. N=5). In the VS **(B)** DAT Val559 and WT p-Thr53 are comparable and Quin (1μM, 10min) has no effect on WT or DAT Val559 p-Thr53 (two-way ANOVA, genotype $F_{(1,16)}=0.566$, $P=0.699$, Quin $F_{(1,16)}=0.155$, $P=0.430$, interaction $F_{(1,16)}=0.670$, $P=0.425$. N=5). **(C)** Sulp (10μM, 20min) normalized DAT Val559 p-Thr53 to WT levels (two-way ANOVA, genotype $F_{(1,24)}=7.132$, $P=0.013$, Sulp effect $F_{(1,24)}=4.998$, $P=0.035$, interaction $F_{(1,24)}=16.05$, $P=0.0005$. Bonferroni's multiple comparisons test – $P=0.0003$* for WT Veh vs. DAT Val559 Veh, $P>0.999$ for WT Veh vs. DAT Val559 Sulp. N=7).

and Val559 mice. We found no genotype effects on TH activity in either DS or VS following NSD treatment (Figure 15A-B), or total TH protein levels (Figure 15C-D). However, we observed an inability for quinpirole application to attenuate NSD-stimulated TH activity in the DAT Val559 DS, whereas WT DS demonstrated quinpirole sensitivity by showing a reduction in L-DOPA accumulation. Additionally, D2AR stimulation also reduced L-DOPA accumulation, and thus TH activity in the VS of both WT and Val559 slices, similar to our findings with D2AR regulation of DAT (Figure 15E-F). These effects expand the region-dependent effects of DAT Val559 to include disruptions in the D2AR regulation of DA synthesis as well as DAT surface trafficking and phosphorylation.

3.4 Discussion

Genetic, pharmacological, and brain imaging studies have suggested that synaptic DA homeostasis and signaling is affected in neuropsychiatric disorders, including addiction (Volkow and Morales, 2015), ADHD (Cook et al., 1995; Volkow et al., 2007), schizophrenia (Slifstein et al., 2015; Howes et al., 2017) and ASD (Jeste and Geschwind, 2014). Past efforts to model DAergic perturbations in these disorders, albeit important, have used lesion and pharmacological approaches, or knockout models that disrupt DA synthesis, release or inactivation (Zhou and Palmiter, 1995; Giros et al., 1996; Wang et al., 1997). Thus, we pursued the identification of functionally-penetrant, disease-associated variants in hDAT (Mazei-Robinson and Blakely, 2006) with which animal models with improved construct validity could be constructed. The current report is borne out of the effort to model the impact of DAT Val559, which we identified in two
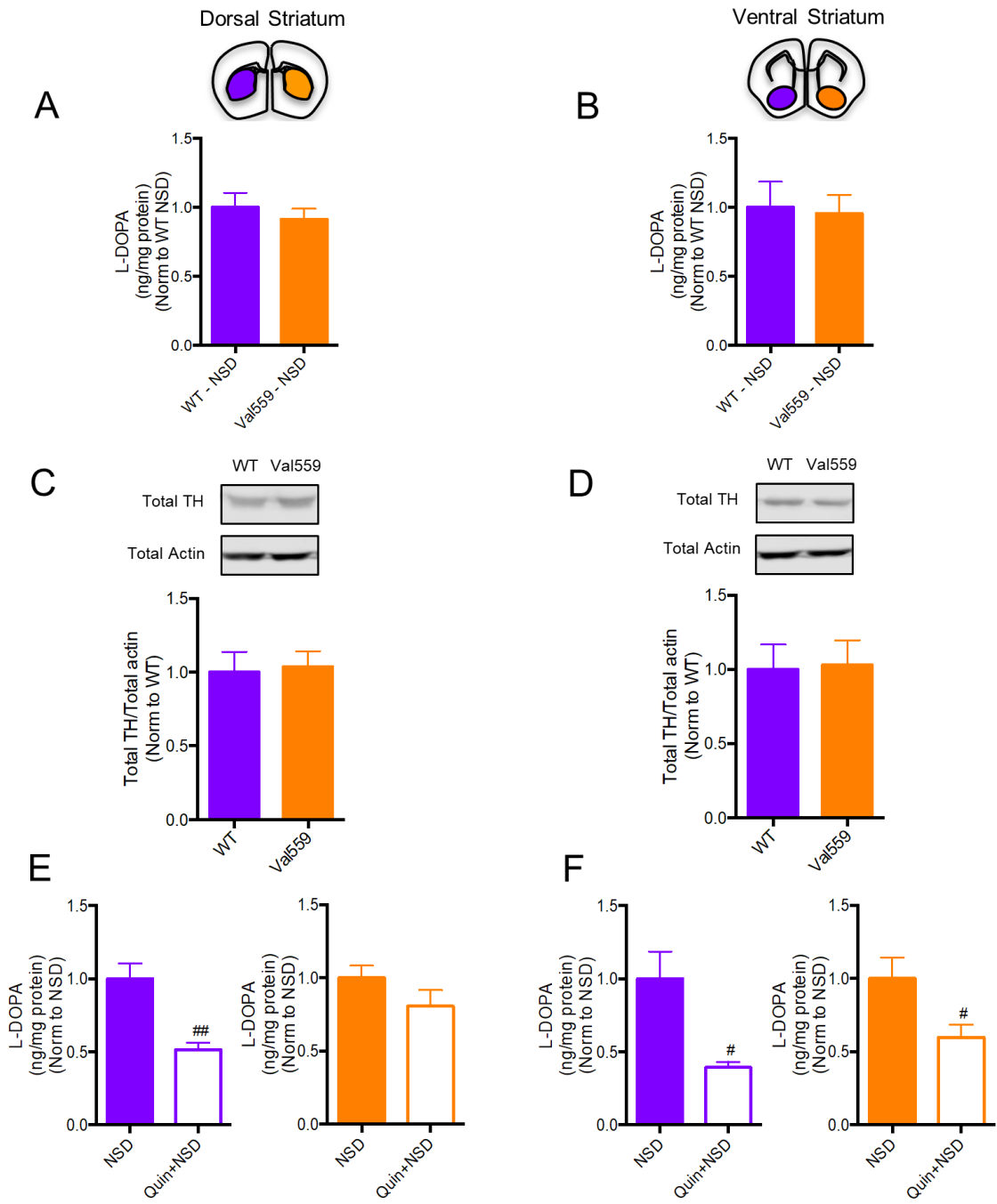
Figure 15. DAT Val559 expression compromises the ability of D2AR to inhibit tyrosine hydroxylase (TH) activity in the DS, but not VS. (A-B) L-DOPA levels post NSD1015 only across genotypes was unchanged in both DS (two-tailed Student's t-test, t(12)=0.659, P=0.524. N=7) and VS (two-tailed Student's t-test, t(8)=0.191, P=0.854. N=5). **(C-D)** Total TH levels are unchanged across genotypes in both DS (two-tailed Student's t-test, t(10)=0.216, P=0.834, N=6) and VS (two-tailed Student's t-test, t(14)=0.126, P=0.904, N=8). In the DS, **(E)** Quin (1µM, 10min) along with NSD (100µM, 10min) treatment reduced WT L-DOPA accumulation compared to NSD (100µM, 10min) alone (two-tailed Student's t-test, t(12)=4.155, P=0.001[#]. N=7), with no effect in Val559 (two-tailed Student's t-test, t(12)=1.406, P=0.187[#]. N=7). In the VS **(F)** Quin (1µM, 10min) decreased L-DOPA levels comparably in both WT (two-tailed Student's t-test, t(8)=3.18, P=0.013[#]. N=5) and DAT Val559 (two-tailed Student's t-test, t(8)=2.411, P=0.041[#]. N=5).

male siblings with ADHD (Mazei-Robison and Blakely, 2005), and that others identified in a girl with bipolar disorder (Grunhage et al., 2000) and two unrelated boys with ASD (Bowton et al., 2014).

Following our initial demonstration that DAT Val559 induced ADE in transfected cells (Mazei-Robison et al., 2008), Bowton and colleagues showed that endogenous D2 receptors expressed in cells and cultured DA neurons were required to sustain ADE (Bowton et al., 2010). Although D2ARs had been reported to elevate DAT surface expression and function (Bolan et al., 2007), presumably to match the demands arising from phasic vesicular DA release, we detected no effects of DAT Val559 on surface expression and DA reuptake in transfected cells or DAT Val559 synaptosomes. We reasoned that the lack of an effect of ADE on trafficking might be sue to the use of *in vitro* assays, where its impact might be minimized through rapid diffusion of extracellular DA, or the apparent lack of the cytoarchitecture found in intact or *ex vivo* slice preparations.

To pursue this idea, we utilized striatal slices, using which we previously demonstrated D2AR-dependent, tonic inhibition of 4-AP-evoked, DA vesicular release as a consequence of DAT Val559 ADE (Mergy et al., 2014b). Also acknowledging that DS and VS receive distinct DA inputs, we assessed DAT trafficking in DS and VS slices separately. In WT DS slices, we found that D2AR activation elevates DAT surface expression, similar to what has been reported in transfected cells, and striatal synaptosomes. Moreover, in DAT Val559 slices, we observed a basal elevation in surface DAT that is normalized by D2AR antagonism. Together, these results suggest

that DAT Val559 ADE in the DS drives tonic elevations in surface DAT via the aberrant, ADE-dependent tonic activation of D2AR. In the VS, where D2AR regulation of DAT is absent, DAT Val559 ADE and subsequent D2AR effects are greatly diminished or absent.

To accompany our trafficking studies and understand its impact on function, we performed [^{3}H]DA uptake in DS slices, and observed, surprisingly, a striking reduction in DAT Val559-dependent [^{3}H]DA uptake compared to WT. To assess the impact of DAT Val559 on *in vivo* DA clearance, removed from potential effects of endogenous DA neuron stimulation owing to studies indicating rapid DAT trafficking following electrical stimulation (Richardson et al., 2016), we chose HSC, where DA is applied exogenously, over other approaches (e.g. fast scan cyclic voltammetry). Our recordings demonstrated a dependence on Michaelis-Menten kinetics for DA clearance over a range of concentrations previously used (Owens et al., 2012), reflecting that these amounts of DA are in the physiological range of DAT function. Consistent with slice uptake, we found decreased DA clearance in the DAT Val559 DS, compared to WT DS. We could reconcile these seemingly contradictory findings if these results are impacted by competition of exogenous DA with endogenous, ADE-generated DA. We detected, as expected, an increase in apparent DA K_M, as well as the inability of D2AR inhibition by sulpiride to delay DA clearance in DS, which supports this hypothesis. As with our membrane DAT studies, we detected no genotype effects on DA clearance in the VS. Together, these findings suggest that a lack of D2AR-DAT coupling in the VS precludes the ability of DAT Val559 to establish ADE and impact VS DA clearance. Additionally, they suggest that any released DA in the DS is inefficiently cleared by DAT, despite

elevated surface DAT.

In vitro studies indicate that the conformational changes required for AMPH to induce DAT reversal, and possibly to bias DAT Val559 to ADE, rely on N-terminal phosphorylation at a cluster of distal Ser residues (Khoshbouei et al., 2004) and Thr53, located proximal within a MAPK consensus site (Foster and Vaughan, 2017). As ERK1/2 signaling is essential for D2AR-mediated DAT trafficking (Bolan et al., 2007), and p-Thr53 is important for DA efflux, we assessed whether changes in p-Thr53 are linked to D2AR-DAT regulation. Similar to our earlier observations, D2AR activation in WT DS slices elevated p-Thr53 levels. Moreover, p-Thr53 was already significantly elevated in DAT Val559 DS relative to WT, insensitive to D2AR stimulation and could be reversed by D2AR inhibition, whereas the VS displayed no genotype differences. These findings motivate future studies to assess the requirement for elevated p-Thr53 in DAT Val559-ADE and/or D2AR-modulation of DAT trafficking. They also suggest that a lack of regulation of DAT by D2AR in the VS, compared to the DS, may arise from differences in D2AR signaling to DAT via ERK1/2. Interestingly, a recent study found elevations in p-Thr53 in the VS to be dependent on oestrus cycle, associated with enhanced reward (Calipari et al., 2017), warranting future exploration into sex-dependent mechanisms of p-Thr53 regulation and their convergence with D2AR-linked pathways. In this regard, DAT can also be regulated by other presynaptic G-protein-coupled receptors, with the possibility that D2AR tonic activity in the DS may bias DAT regulation to heteroreceptors vs. autorecpetors.

The loss in D2AR control of DAT regulation in the VS is interesting, as the VS does not lack functional D2ARs. Previous studies have shown that VS DA terminals express D2ARs (Sesack et al., 1994b), with the ability to control DA synthesis and release. Notably, we also found that D2AR inhibits TH activity comparably in WT DS and VS slices, though regional differences emerged across genotypes here as well, with TH activity in the DAT Val559 DS, but not VS, being found insensitive to D2AR stimulation. D2ARs have been shown to downregulate TH activity through a reduction in PKA-dependent TH phosphorylation (Dunkley et al., 2004). Compromised D2AR signaling through this pathway could lead to the desensitization of D2ARs by ADE-derived DA. Interestingly, Jones and colleagues demonstrated a similar lack of TH control by D2AR in the DAT KO, suggesting a loss in D2AR function due to desenstization. We have been able to reverse changes in DAT Val559-mediated DAT phosphorylation, surface trafficking and DA clearance (this study) and DA release via acute D2AR antagonism. However, we have not seen any basal changes in TH activity, nor have we tested the ability of D2AR antagonism to enhance L-DOPA accumulation, suggesting that the D2ARs regulating TH might be different from those controlling release/reuptake. Distinct D2AR populations could arise from differential receptor post-translational modifications, localization with proteins, or pools of D2AR-accessible G-protein subunits, some of which have been shown to support DAT-mediated DA efflux (Garcia-Olivares et al., 2017). Importantly, this evidence that DS D2ARs in the DAT Val559 do not provide negative feedback control of DA synthesis indicates that cytosolic DA continues to sustain ADE without negative feedback inhibition.

Our report adds to a growing body of literature on the inherent diversity of midbrain DA neurons and their projections. Approaches to assess *in vivo* DAT function in DA projections without membrane stimulation have yet to be demonstrated. Nonetheless, striatum-projecting DA neurons that populate the SNpc and VTA, preferentially project to the DS and VS respectively and exhibit differences in afferent inputs, modulation by neurotransmitters and peptides, and intrinsic electrophysiological properties (Chuhma et al., 2017). Further microheterogeneity has emerged from studies showing diverse properties of DA projections to dorsomedial vs. dorsolateral striatum (Lerner et al., 2015), nucleus accumbens core vs. shell (Shin et al., 2017), and striatal striosome vs. patch matrices (Salinas et al., 2016). Here, we demonstrate region-dependent differences in presynaptic D2AR regulation of DAT that, in the DAT Val559 DS, leads to tonic activation of presynaptic D2AR, elevated DAT trafficking, DA availability unabated by feedback inhibition, increased ADE and inefficient DA clearance. Functionally, these changes are predicted to shift DA terminals in the DS away from reliance on tightly controlled excitation-secretion mechanisms (Figure 16).

A desire to understand underlying mechanisms at molecular and circuit level, potentially driving specific behavioral traits has inspired creation of the Research Domain Criteria framework (RDOC) (Insel et al., 2010). To pursue these goals, systematic studies aimed at defining potentially different pathways that could drive behavioral heterogeneity in disease are needed. At the circuit level, DA projections to the DS mediate movement and habit formation whereas those to the VS control motivation, incentive salience and response to reward (Howe and Dombeck, 2016). We have observed that DAT Val559 mice display altered spontaneous and
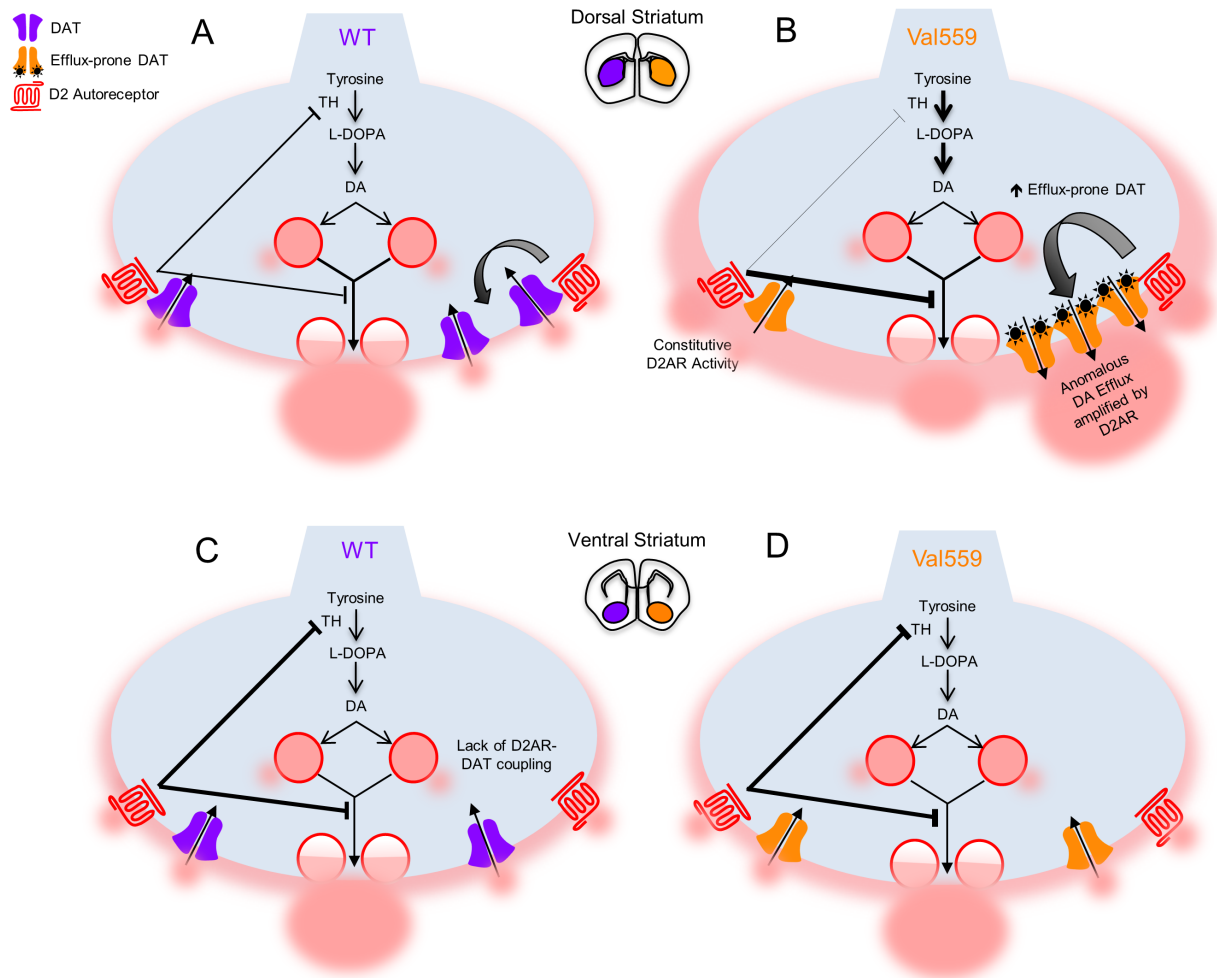
Figure 16. D2AR and DAT are functionally coupled selectively in the DS, biasing DAT Val559 phenotypic penetrance. (A) In WT DS, D2AR control over DAT plays an important role in the termination of DA signaling, in addition to D2AR-induced inhibition of DA synthesis and release. **(B)** Functional D2AR-DAT coupling selectively in the DS results in the upregulation of DAT Val559 regulation, biasing the enhancement of efflux-prone transporters that amplify ADE and compromise presynaptic DA signaling. However, at **(C)** VS DA terminals, DAT lacks D2AR regulation. Hence, **(D)** despite DAT Val559 expression, the lack of D2AR-DAT coupling in the VS leaves DAT regulation, DA clearance and TH activity unperturbed.

psychostimulant-induced locomotion (Mergy et al., 2014b) and impulsivity (Davis et al., 2018), behavioral abnormalities that could arise through DS-specific ADE, though we have not analyzed the contribution of DAT Val559 in the cortex. We have also found changes in motivation for reward (Davis et al., 2018), which could normally be attributed to perturbations in VS control. However, we are as yet to assess VS-specific behaviors and speculate that our behavioral defects may derive from an inability to shift behavioral responses from goal-directed actions, dependent on reward, to habit-based behaviors.

Chapter 4 – Conclusions and Future Directions

Dysfunction in DA signaling has long been recognized as a major contributing factor to neuropsychiatric disorders. All drugs of abuse directly or indirectly target the DAergic system and genetic variation in DA-linked genes have been associated with mental illness. Moreover, pharmacological therapies for many disorders directly or indirectly target the DA system. Attempts to model imbalances in DA neurotransmission have relied on lesion or pharmacological animal models, or transgenic eliminations of DA-related genes that have been found associated with mental illness (Giros et al., 1996; Wang et al., 1997). However, correlations do not necessarily imply causation, and complete elimination of genes associated with disease do not provide information on how these genes could confer risk or if the phenotypes observed are due to prevailing compensatory mechanisms. For example, the DAT KO mouse is the most widely accepted model for ADHD; however people with loss of function mutations in DAT completely incapacitating DAT function do not have ADHD, but infantile parkinsonian dystonia (Kurian et al., 2009; Kurian et al., 2011). Additionally, the complete elimination of a key protein involved in DA signaling could lead to compensatory mechanisms that may in turn contribute to the phenotypes observed. Hence, in a targeted approach to identify genetic variation associated with ADHD, and with the potential to develop construct-valid models for neuropsychiatric disease, the Blakely lab screened patients with ADHD for rare variants associated with DAT and found among others, the DAT Val559 variant, in two male siblings with the combined subtype of ADHD, successfully treated with Adderall. DAT Val559 was previously identified in a female subject with BPD (Grunhage et al., 2000), but lacked any functional characterization. When

expressed in transfected cells, DAT Val559 engendered transporter-mediated DA leak or ADE, while maintaining normal surface and total expression, and DA reuptake capacity (Mazei-Robison et al., 2008). Studies also showed that the variant's capacity for ADE is blocked by D2R antagonism (Bowton et al., 2010), and treatment with AMPH, which in cells expressing the WT transporter, induced DAT-mediated DA efflux.

The existence of ADE, despite normal DAT function, from a mutation in DAT TM12 poses an interesting question related to DAT structure-function. Studies prior using computational modeling or heterologous cell systems suggest that TM12 is involved in transporter oligomerization, and that the existence of oligomers promotes transporter-mediated DA efflux (Siciliano et al., 2018). Indeed our results showing ADE in the DAT Val559 support the above suggestions, although targeted studies assaying specifically, a role for TM12 in DA efflux or oligomerization in transfected cells or a determination of the structure of DAT mutants with mutations in TM12 need to be conducted. Furthermore, our results support the existence of an "efflux-prone" mode of reverse transport in DAT, one previously shown to exist only in response to AMPH. Whether such a mode is static, with the existence of "efflux-prone" or "uptake-willing" populations of DAT, with variants such as DAT Val559 causing an imbalance, or is dynamic, dealing with the actual cycling of the transporter based on intrinsic structure, ionic and neurotransmitter gradients, association partners and membrane microdomains, with DAT Val559 disrupting the cycling between these states is an interesting question that warrants further interest. Moreover, studies have also shown lateral mobility within the membrane in addition trafficking to and from the surface, also contributing to transporter function (Chang et al., 2012). How mobility across the

membrane contributes to transporter states, the factors regulating it and how this lateral movement is disrupted in the case of DAT Val559 are also important questions that would contribute to how the variant impacts DAT function, and more importantly, uncover mechanisms on how to reverse it.

Following our studies, the DAT Val559 was also identified in two unrelated subjects with ASD (Bowton et al., 2014). The identification of this variant in multiple disorders, coupled with the discovery of other DAT variants that also induce transporter-mediated ADE when expressed in transfected cells, make a strong case for ADE and non-vesicular DA leak through DAT as a molecular endophenotype conferring risk for mental illness. The demonstration of ADE *in vitro*, and the ability of AMPH to block DA leak led the Blakely lab to pursue the development of genetically engineered mice expressing DAT Val559, the first, construct-valid mouse model for neuropsychiatric disorders (Mergy et al., 2014a), wherein the enduring expression of DAT Val559 can lead to behavioral phenotypes associated with disease, along with the potential of elucidating disruptions in physiological mechanisms relevant to neuropsychiatric disorders. Using synaptosomes made from DAT Val559 mice, we found no changes in DAT function, total DAT expression or tissue content of DA. Notably, corroborating our data in transfected cells, we found elevated extracellular DA levels *in vivo* in the striatum of freely moving mice, indicative of the presence of ADE, without a change in DA reuptake. We also found a small but significant elevation in tissue 5-HT levels, but no changes in AMPH-evoked 5-HT release in the striatum. Interactions between 5-HT and DA have long been suggested to be important in how animals respond to reward, in mediating drug abuse and in the response to stress. Indeed, in animals lacking TH, and

effectively a functional DAergic system, the 5-HT system takes over as the brain's primary modulator of reward and motivation. Moreover, responses to AMPH and cocaine in DAT KO mice are mediated by the 5-HT system as well. However, the molecular and circuit-level mechanisms regarding the interplay between the 5-HT and DA systems in the brain, and specifically in the striatum are as yet unclear. Hence, how DAT Val559 induced ADE leads to adaptations in the 5-HT system would not only provide information on how 5-HT systems are impacted when modeling disruptions in DA neurotransmission, but also inform on the physiological mechanisms by which 5-HT neurons are regulated by DA.

We also observed, as a consequence of ADE and increased extracellular DA, a tonic D2AR activity-driven reduction in evoked vesicular release from DA terminals in the striatal slices. The technique used to assay DA release involved the reuptake of [^{3}H]DA into DA terminals via DAT, followed by 4-AP-evoked release using a superfusion setup, which enabled us to determine DA release capacity removed from potential changes in DA synthesis and/or vesicular DA packaging (although intracellular DA levels were unchanged). Hence, an important future direction would be to determine endogenous DA release from DA terminals, evoked using optogenetic stimulation of DA axons specifically. Furthermore, studies have also shown the ability of cholinergic interneurons to enhance DA terminal activity and result in DA release (Threlfell et al., 2012), which is regulated by D2Rs on these neurons. Hence, potential differences in DA release properties (when evoked by DA neuron versus cholinergic interneuron stimulation), and tonic activity of D2ARs vs. D2Rs on cholinergic interneurons are also important questions, giving us more information about whether DAT Val559-induced

adaptations are specific to DA neurons or extend beyond them. Additionally, the existence of ADE also raises the question of where the DA needed to sustain leak is obtained from. As we discussed before, intracellular/cytosolic DA levels are tightly regulated to avoid neurotoxicity (Lohr et al., 2017) and it will be interesting to measure the capacity for vesicular packaging of DA, and the relative composition of DA in readily-releasable vs. reserve pools of vesicles, although these are difficult to determine. We also found, in addition to difference in DA terminal properties, adaptations in SNPc DA cell bodies, with DAT Val559 expression resulting in altered D2-IPSCs and in the burst firing evoked somatodendritic DA release. Several questions remain regarding the potential adaptations in electrophysiological properties of DA neurons, such as how an elevation in effectively tonic DA, leads to changes in activation patterns of DA neurons to efferent stimulation, and if ADE leads to changes in tonic vs. phasic DA neuron activity and/or release. Importantly, since the alterations in D2-IPSCs by DAT Val559 are similar to that induced by psychostimulants that directly target DAT, and as psychostimulants induce long-lasting changes in the plasticity of DA neurons such as long-term potentiation (LTP), it would be interesting to assay potential changes DA neuron plasticity in DAT Val559 mice, such as potential changes to the AMPA/NMDA receptor ratio.

DAT, in addition to being regulated by D2ARs, is enmeshed in tightly regulated complexes, many of which are essential for the actions of AMPH and transporter-mediated DA efflux (Eriksen et al., 2010). Among all the reported interacting partners for DAT, syntaxin 1A, which along with regulating vesicular fusion, also complexes with DAT, necessary for reverse transport of DA in response to AMPH (Binda et al., 2008).

Hence, it would be interesting to determine putative changes to DAT and syntaxin 1A interactions owing to DAT Val559 expression, and if disrupting this interaction reverses the molecular disruptions induced by DAT Val559. Additionally, DAT also interacts with flotillin, a membrane microdomain-associated protein, an interaction that is also important for AMPH's actions (Cremona et al., 2011). Thus potential changes in the interaction between DAT Val559 and flotillin, in addition to providing insight into the effects of the variant on DAT function and regulation, would also inform on the microdomain-association changes of DAT Val559 within the DAergic terminal membrane. DAT also interacts with kinases such as PKCβ and CamKII, all shown to be important for AMPH actions on DA efflux and/or DAT trafficking (Eriksen et al., 2010). As we delve deeper into the signaling mechanisms that could be disrupted by DAT Val559 expression, it is important to determine if signaling cascades, modulated by these kinases, essential to the regulation of DAT are changed at DAT Val559 DA terminals.

D2ARs, in addition to inhibiting vesicular release (Benoit-Marand et al., 2011), and restraining DA neuron excitability (Lacey et al., 1987), also physically interact with DAT and control membrane levels of the transporter. Studies prior have shown that D2AR stimulation enhances DAT surface levels and activity (Bolan et al., 2007; Chen et al., 2013), whereas D2AR antagonism delays DA clearance by DAT (Cass and Gerhardt, 1994). Given this knowledge, we were puzzled by the lack of an effect of DAT Val559 on transporter trafficking and activity, in both transfected cells, known to express small amounts of D2R, and in striatal synaptosomes. Whereas heterologous cell systems and synaptosomes are good to determine intrinsic activity and regulation of

DAT, they may not be the best systems to observe the influence of changes in the extracellular milieu (i.e. elevated extracellular DA). Hence, upon using slices containing the DS (or the VS) prepared from DAT Val559 mice, we observed a D2AR-dependent enhancement in DAT trafficking to the surface. Interestingly, we found no effects of D2AR stimulation to DAT in VS slices and a corresponding lack of a genotype effect in the DAT Val559, suggesting that DAT and D2ARs are functionally uncoupled in the VS. When assayed for activity using the same slices, we surprisingly found a reduction in [^{3}H]DA uptake, which could be explained by a potential competition for radioligand substrate by the elevated endogenous DA. To gather further evidence and to measure DA clearance *in vivo* removed from the potential influence DAT Val559 expression may have on endogenous DA release/reuptake mechanisms, we performed chronoamperommetry in the DS and VS using exogenously applied DA. Once again, we found a delay in DA clearance, indicative of competition from endogenous DA, as evidenced by a shift in K_M upon performing Michaelis Menten kinetics on DA clearance. Further evidence for elevated extracellular DA/ADE was observed by lack of an effect of a D2AR antagonist on delaying clearance. Paralleling our findings with surface DAT in slices, we observed these DAT Val559-dependent deficits only in the DS, and not the VS. Our results suggest that even though there are more DATs on the surface, they are predisposed to an "efflux-prone" conformation, resulting in a D2AR-dependent amplification of ADE, selectively in the DS. Further evidence for this was obtained by our observations of elevated phosphorylation of DAT Val559 at Thr53, previously shown to be essential for the action of AMPH in inducing reverse transport of DA (Foster et al., 2012). The fact that D2AR induced phosphorylation at Thr53 in the WT DS, and that

Thr53 is a putative ERK1/2 phosphorylation site raises the important question regarding the post-translational modifications induced in DAT via D2AR. As shown before, D2AR-mediated DAT surface expression is ERK1/2-dependent (Bolan et al., 2007). Hence, an interesting question is if ERK1/2 is hyperactive in the DS DA neurons of DAT Val559 mice. Our findings showing D2AR antagonism-induced reversal of elevated surface and p-Thr53 DAT, however suggest otherwise, pointing to the possibility that the alterations in DAT regulation are limited to the tonic activity of D2ARs, and do not extend into the signaling mechanisms. One approach to better answering this question is determining if there are any differences in heteroreceptor-mediated regulation. Studies previously have shown that DAT surface levels and activity can be regulated by multiple GPCRs, including the kappa-opioid receptor (KOR) (Kivell et al., 2014),m. KOR and DAT are also found in physical complexes in whole-striatal extracts, and KOR stimulation results in the elevation of DAT surface expression, dependent on ERK1/2 activity. Hence, it would be interesting to determine if KOR activation also induces Thr53 phosphorylation in DAT in the WT DS and/or VS, whether this is perturbed in the DAT Val559, and also if KOR-mediated induction of DAT surface expression is intact in the DAT Val559 DS, or present in the VS.

We also observed that the lack of D2AR effects in the VS was not due to a lack of functional D2ARs altogether in the VS, or due to an artifact in our preparations. Using the same slice preparation, we were able to show a D2AR-dependent reduction in L-DOPA accumulation, as a proxy for DA synthesis and TH activity, in both the WT DS and VS. Region-dependent effects in the DAT Val559, however, prevailed in this assay as well, where we saw a lack of D2AR modulation of TH activity selectively in the DAT

Val559 DS, but not in the VS, suggesting that a lack of feedback inhibition provides for the cytosolic DA necessary to sustain ADE in the DS. Our data altogether point to a selective amplification of ADE, owing to functional D2AR-DAT coupling, in the DAT Val559 DS, but not the VS. However, DAT Val559 is broadly expressed in all DA neurons, which raises the important question of whether ADE exists and can be detected in the VS. Our microdialysis studies utilized a probe spanning the entire length of the striatum, and hence an important future direction would be to ascertain extracellular DA levels selectively in the DS and the VS of freely-moving animals. Our data also showed D2AR tonic activity in striatal tissue; although a systematic estimation of endogenous DA release from terminals in the DS vs. the VS is yet to be determined, this would provide insight into whether D2ARs controlling release are similarly affected in the VS and the DS owing to DAT Val559 expression. The discovery of functional uncoupling of D2AR and DAT also raises the question of whether this lack of crosstalk between the 2 proteins is also structural; D2AR and DAT are involved in a structural complex (Lee et al., 2007) and it would be interesting to determine if D2ARs and DAT exist in such a complex only the DS, and if this interaction is dependent on specialized microdomains within the membrane. Should there be differential associations of D2AR and DAT in microdomains, it is important to assay if all D2ARs and DATs exist in functional complexes, or if there exists different populations of D2ARs – one controlling release/synthesis and the other controlling DAT, with that regulating DAT structurally and functionally uncoupled in the VS. Additionally, this uncoupling also points out a putative dependence on purely heteroreceptor-mediated regulation of DAT (for eg. KOR), for the dynamic control of DA clearance. Moreover, if KOR-mediated

mechanisms of DAT modulation exist in the VS, it would also be important to assay if ERK1/2-dependent signaling mechanisms for the regulation of DAT are similar in the DS or VS.

Although the focus of this dissertation is on striatal sub-regions, important findings with behavioral relevance may emerge from future DAT Val559 studies in cortical DAergic projections. These terminals have been reported to express DA release, but not DA synthesis-modulating D2ARs (Galloway et al., 1986; Wolf et al., 1986; Wolf and Roth, 1987). However, DA fibers and overall DAT density is low in the cortex (Lewis et al., 2001), and DAT is thought to play a diminished role in DA clearance, compensated for the ability of norepinephrine transporters on nearby noradrenergic axons to clear DA (Moron et al., 2002). However, functional D2AR effects on DAT, and a consequential maintenance of DAT Val559 ADE may relate more to receptor/transporter proximity and/or physical interactions versus tissue density. Although cortical DATs have not been found to be modulated by D2ARs, indirect evidence for functional coupling between receptor and transporter was provided by Cass and Gerhardt, showing D2AR antagonist-mediated inhibition of DA clearance in the cortex (Cass and Gerhardt, 1994), and Pehek, who detected diminished AMPH evoked DA release in cortex following D2 antagonist (haloperidol) treatment (Pehek, 1999). Thus, cortical DA terminals may differ from both DS and VS in retaining D2AR regulation of DAT in the absence of D2AR regulation of TH.

Behaviorally, the DAT Val559 mice display changes in basal and psychostimulant-induced locomotor activity, altered motivation and impulsivity. Although

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highly simplified, at a circuit-level, studies have shown that DA projections to the DS support movement and to the VS are involved in reward (Howe and Dombeck, 2016). However, our results support the idea that these and other forthcoming behavioral changes might be shaped by DS-dependent ADE, as currently our results support molecular perturbations only with regards to DA terminals in the DS. One might argue, therefore, as to why we see deficits in motivation and impulsivity, not classically associated with DA neurons projecting the DS. Our results also serve to highlight the complexity in considering the behaviors distinct populations of DA neurons control, as studies prior have also shown that lesions to the VTA impact movement (Dunnett et al., 1984), and that local L-DOPA injections into the DS of TH KO mice rescue a loss in motivation in these animals (Robinson et al., 2007). These studies led Richard Palmiter to propose that whereas DA signaling in the VS/NAcc is important in responding to salient stimuli and shapes future behaviors accordingly, DA signaling in the DS acts to modulate the gain of these responses (Palmiter, 2008). Hence, a loss in patterned neural activity in the DS driven by ADE in the DAT Val559 mice may lead to a host of DA-linked behavioral perturbations regardless of pathway-specific molecular disruptions. Moreover, anatomical studies with newer technologies involving tracing with viral vectors also support the idea that whereas parcellation of inputs that lead to divergent behaviors exist, the complexity in this diversity extends beyond just oversimplified anatomical boundaries. Our results also do not discount the possibility of there being DAT Val559-dependent perturbations at DAergic synapses in the VS, leading to perturbations in VS-dependent behaviors such as cue salience or contingent paradigms of drug administration and relapse. Employing state of the art techniques

such as fiber photometry to monitor activity of DA neurons, or the use molecular GPCR-based sensors developed for real-time monitoring of fluctuations in extracellular DA in the DS vs. the VS during animal behavior is an important direction the field should undertake, when trying to determine the enduring impact of rare variants such as the DAT Val559.

In summary, diversity in intrinsic signaling mechanisms in sub-regions sculpts the penetrance of DAT Val559, adaptations that could explain variability in behavioral perturbations in the patient population it was identified from.

Appendix A – Reversal of DAT Val559-Induced Deficits in DA Release

We had previously discussed in Chapter 2 that ADE, and a subsequent elevation in extracellular DA owing to DAT Val559 expression engendered alterations in [^{3}H]DA release from striatal slices. Firstly, we found that striatal slices incubated with [^{3}H]DA prepared from DAT Val559 mice displayed a near complete loss in AMPH-evoked, non vesicular, DAT-mediated [^{3}H]DA release. Additionally, we also found a reduction in 4-AP-stimulated, vesicular [^{3}H]DA release, dependent on tonic D2AR activity, as evidenced by a reversal in blunted release upon treatment of DAT Val559 striatal slices with a D2AR antagonist, raclopride.

Next, we attempted to rescue the loss in AMPH-induced release from DA terminals by D2AR antagonism with raclopride. In striatal slices from WT animals, we observed a reduction in AMPH-evoked [^{3}H]DA release following raclopride treatment (Figure 17A). Our results suggest that under perfusion conditions, the DA release evoked by AMPH could feedback onto the D2ARs, stimulating trafficking of DAT to the surface, sustaining DA release in response to AMPH. This ongoing, dynamic trafficking of DAT to the membrane is prevented by D2AR antagonism, resulting in a reduction in AMPH-evoked [^{3}H]DA release through DAT. A similar result has also been observed *in vivo* using chronoamperometry, where Owens and colleagues observed the inhibition of AMPH-stimulated endogenous DA release in the DS following pressure ejection of raclopride (Owens et al., 2012). In the case of the DAT Val559 striatum, release through DAT by AMPH treatment in was significantly impaired; however, we surprisingly observed an increase in [^{3}H]DA release through DAT following raclopride application
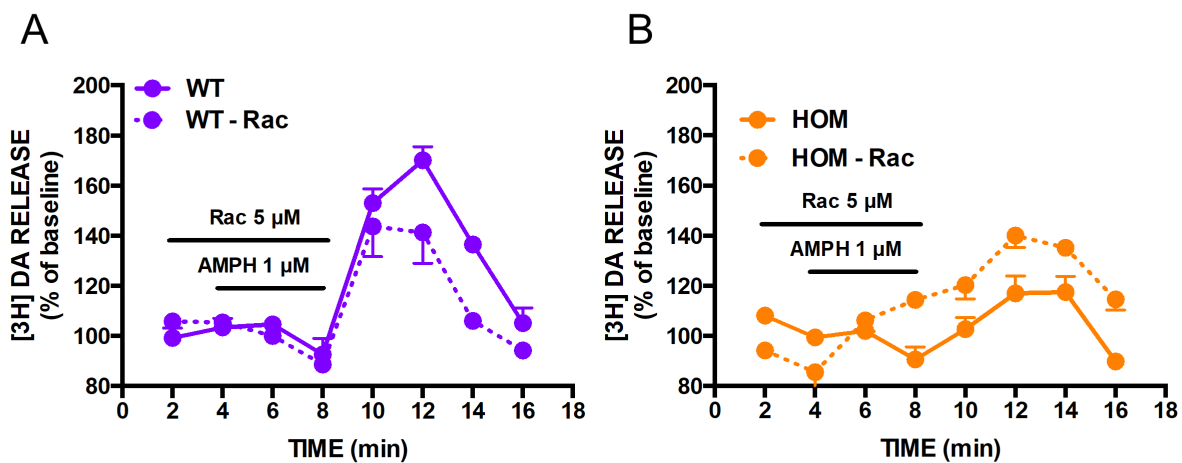
Figure 17. DAT Val559-induced reduction in AMPH-stimulated DA efflux can be rescued by D2AR antagonism. (A) In WT, raclopride pretreatment reduces AMPH-stimulated [^{3}H]DA release (P(time)<0.05, P(raclopride)<0.05 and P(interaction)<0.05; N=3). **(B)** In DAT Val559, raclopride shows a trend towards rescuing the ability of AMPH to induce [^{3}H]DA release (P(time)<0.05, P(raclopride)=0.06 and P(interaction)<0.05; N=3).

(Figure 17B), suggesting that preventing ADE-driven, tonic D2AR activity in the DAT Val559 leads to a loss in the amplification of ADE, and a subsequent reversal in the response to AMPH. Release did not return to levels seen in WT striatal slices treated with AMPH alone, but this could be attributed to dose and treatment time of raclopride.

We also attempted to reverse the reductions we observed in 4-AP-stimulated vesicular release in the DAT Val559 striatum. We provided support for D2AR activity-driven reductions in DA release, and hypothesized that DAT Val559-driven ADE resulted in tonic D2AR activity. Hence, we posited that we would be able to also reverse the inhibition in evoked-vesicular DA release by blocking ADE at the source, via pretreatment of striatal slices using a DAT-specific blocker, GBR 12909. In WT striatal slices, GBR 12909 pretreatment did not affect vesicular DA release (Figure 18A), suggesting that our experimental conditions, with the slices under constant perfusion, precludes the ability for us to detect any DAT blocker-induced changes in DA levels. GBR 12909 pretreatment in the DAT Val559 striatum marginally elevated 4-AP-evoked [^{3}H]DA release from DA terminals, but did not rescue the release to levels induced in the WT (Figure 18B). One possibility for this could be the existence of enduring alterations owing to ADE that are unable to be overcome by just acute blockade of ADE and DAT, although raclopride treatments in the same preparations rescued release to WT levels. Another explanation could deal with the duration of GBR 12909 pretreatment utilized in this experiment, with the possibility of longer treatments yielding stronger reversal. Moreover, our results from [^{3}H]DA uptake in DS slices also demonstrated a reduced efficacy for GBR 12909 in blocking uptake, suggesting another potential
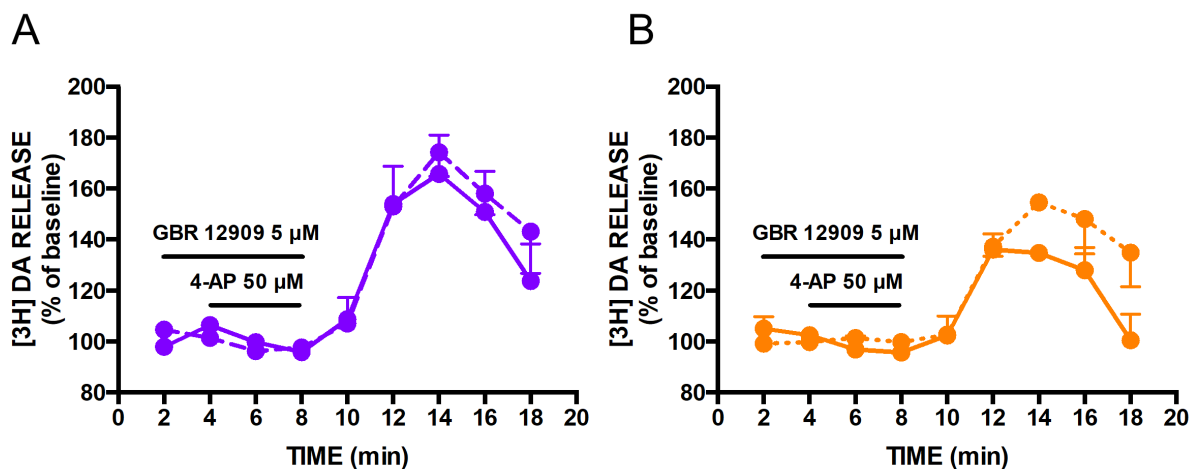
Figure 18. DAT Val559-induced reduction in 4-AP-evoked DA release may be reversed by DAT blockade. (A) In WT, GBR12909 pretreatment does not affect 4-AP-evoked [^{3}H]DA release (P(time)<0.05, P(GBR 12909)>0.05 and P(interaction)>0.05; N=3). **(B)** In DAT Val559, GBR 12909 treatment shows a trend towards reversing the deficit in 4-AP-induced [^{3}H]DA release (P(time)<0.05, P(GBR 12909)=0.051 and P(interaction)>0.05; N=3).

contributing factor to the results we observed on DA release. Additionally, we have as yet not determined if sensitivity of D2ARs to DA in the DAT Val559 striatum is elevated, something that would be difficult to directly pursue. Hence, our results could also be borne out of a heightened sensitivity of D2ARs, that we are able to prevent only with D2AR antagonism, thereby rescuing evoked-vesicular DA release.

Altogether, the above results serve to underscore the complex adaptations induced to the control of DA release at DAT Val559 DA terminals. Behaviorally, the loss in the ability of AMPH to promote non-vesicular DA release via transporter reversal is translated to a reduction in locomotor hyperactivity post i.p injection of AMPH. With the expert help of Dr. Gwynne Davis, we attempted to test the effects of raclopride co-injections, along with AMPH and found a trend in the reversal of blunted AMPH-induced locomotor activity in the DAT Val559 mice, compared to WT, where raclopride actually reduced locomotor activity (Blakely and Davis, unpublished observations). The interpretations for this result are complex, considering that the raclopride injection serves to antagonize both D2 auto and heteroreceptors, targets D2ARs somatodendritically and presynaptically, and finally also antagonizes D2 receptors in all regions they are present in. Nonetheless, this result corroborates our slice release observations suggesting that the loss of AMPH effects, and its putative rescue by D2AR antagonism, may very well translate to behavior as well.

Appendix B – DAT Val559-Induced Electrophysiological Adaptions In Dorsal And Ventral Striatal Medium Spiny Neurons

As opposed to how dysfunctions in DA homeostasis in the case of the DAT Val559, driven by ADE, affect presynaptic DA signaling, we will now consider potential changes induced in postsynaptic targets, specifically in the electrophysiological properties of medium spiny neurons (MSNs) in the DS and VS. A systematic understanding of the specific role of DA in the electrophysiological properties of medium spiny neurons (MSNs) has remained elusive with studies reporting that DA may both enhance or dampen MSN activity, primarily because the MSNs in both the DS and VS are not homogenous. MSNs can be largely be differentiated by the expression of DA D1Rs or D2Rs, with studies suggesting the presence of a small population expressing both (Surmeier et al., 2010). The first theory advanced to how DA affects MSN excitability is that DA enhances activity in the D1R MSNs and inhibits it in the D2R MSNs (Albin et al., 1989), although evidence for this hypothesis has still largely been indirect. D1Rs couple to Gs proteins, resulting in the accumulation of cyclic AMP (cAMP) and the subsequent activation of protein kinase A (PKA) (Missale et al., 1998). Intrinsic excitability in MSNs is controlled by a variety of Na^+ and Ca^{2+} channels (Surmeier et al., 2007), with studies showing that D1R activation in enhances intrinsic MSN activity (Hernandez-Lopez et al., 1997). Excitation/action potential evoked depolarization is mediated primarily by glutamate release onto ionotropic receptors such as AMPA and NMDA receptors. Whereas studies have shown that NMDAR phosphorylation by PKA enhances MSN activity (Blank et al., 1997), and that D1R activation has the potential to phosphorylate NMDARs via PKA (Hallett et al., 2006),

definitive studies demonstrating the fast modulation of AMPAR or NMDAR activity by DA and D1R activation still remain unclear. Perhaps the only definitive studies showing that D1R activity enhances MSN responsiveness in slices has been when MSNs are held in their "up-state" (Wickens and Wilson, 1998), a potential they are normally found at when active. In the case of D2R MSNs, D2R couples to Gi/o proteins (Stoof and Kebabian, 1984), resulting in the inhibition of cAMP production. Additionally, D2Rs, via $G\beta\gamma$ activation also activate phospholipase C, enhancing diacylglycerol (DAG) production and protein kinase C activity, and the increase of intracellular Ca^{2+} stores (Hernandez-Lopez et al., 2000). Studies have shown in striatal slices that D2R activity reduces AMPAR-mediated currents (Cepeda et al., 1993), via the dephosphorylation of AMPARs, normally thought to downregulate AMPAR surface trafficking (Hakansson et al., 2006). Furthermore, D2R activity also negatively modulates calcium channels (Hernandez-Lopez et al., 2000), and enhances the opening of K^+ channels (Greif et al., 1995). Both D1R and D2Rs have also been implicated in altering short-term and long-term plasticity states of MSNs too, however some of these effects have been attributed to the actions of D2Rs on cholinergic and GABAergic interneurons (Surmeier et al., 2007).

The pathological effects of elevated DA levels on MSN physiology can be gleaned from either alterations in their properties in response to drugs of abuse, or in transgenic models such as the DAT KO or KD. Bath applications of AMPH and cocaine have been shown to reduce the frequency of MSN firing (White and Kalivas, 1998; Wu et al., 2007), but most studies pertaining to drugs of abuse have been focused on NAcc MSNs following chronic, contingent or non-contingent drug administration suggesting

long lasting alterations in MSN physiology such as spontaneous EPSC amplitude, frequency, probability of release and AMPA/NMDA ratios (Luscher and Malenka, 2011). However, information regarding extracellular DA levels following chronic drug administration, and how it may result in these adaptations to MSN neurons is unclear. Very few studies have explored the impact of elevated DA in DAT KO or KD mice on MSN physiology. Wu and colleagues, in one of the few studies using DAT KD mice, reported that elevated extracellular DA induced an enhancement in amplitude, but a reduction in the frequency of spontaneous EPSCs (sEPSC), and a lack of response to AMPH and cocaine (Wu et al., 2007). Additionally, studies exploring the structure and morphology of MSNs have suggested reduced striatal volume and a loss in spine density (Berlanga et al., 2011). Interestingly, we observed a trend towards a lower volume in the DMS of DAT Val559 mice compared to WT, a result of collaboration with Dr. Jacob Ellegood and Jason Lerch at the University of Toronto (Figure 19). Hence, we sought to determine if there were any gross differences in the electrophysiological properties of MSNs in the DS and the VS of DAT Val559 mice, compared to WT. Upon determination of sEPSC amplitude and frequency via whole-cell patch clamp electrophysiology of MSNs, we found no changes in the DAT Val559 DS, compared to WT. However, we found an increase in sEPSC frequency in the DAT Val559 VS, specifically from MSNs in the NAcc core. A reduction in sEPSC frequency could indicate altered presynaptic properties of glutamatergic afferents onto the MSNs; but we failed to detect any changes in paired-pulse ratios (PPR) in the NAcc core. Surprisingly, we observed an increase in PPR from MSNs in the DAT Val559 DS, suggesting a reduction in the probability of release from afferents to the DS. We then ascertained potential

changes in currents induced through AMPA and NMDA receptors, but found no changes in AMPA/NMDA ratios by region or genotype. Overall, my preliminary results raise as many questions as they answered. Whereas we observed some modest changes in sEPSC frequency and PPR, albeit in different regions, that could be attributed to DAT Val559 expression, systematic analyses of electrophysiological properties remain needed, particularly comparing D1R vs. D2R MSNs in DS vs VS. Furthermore, input-specific differences may well arise upon more specific estimations of afferent-dependent modulation of MSN properties in the DAT Val559, via activation of specific glutamatergic terminals using optogenetics.
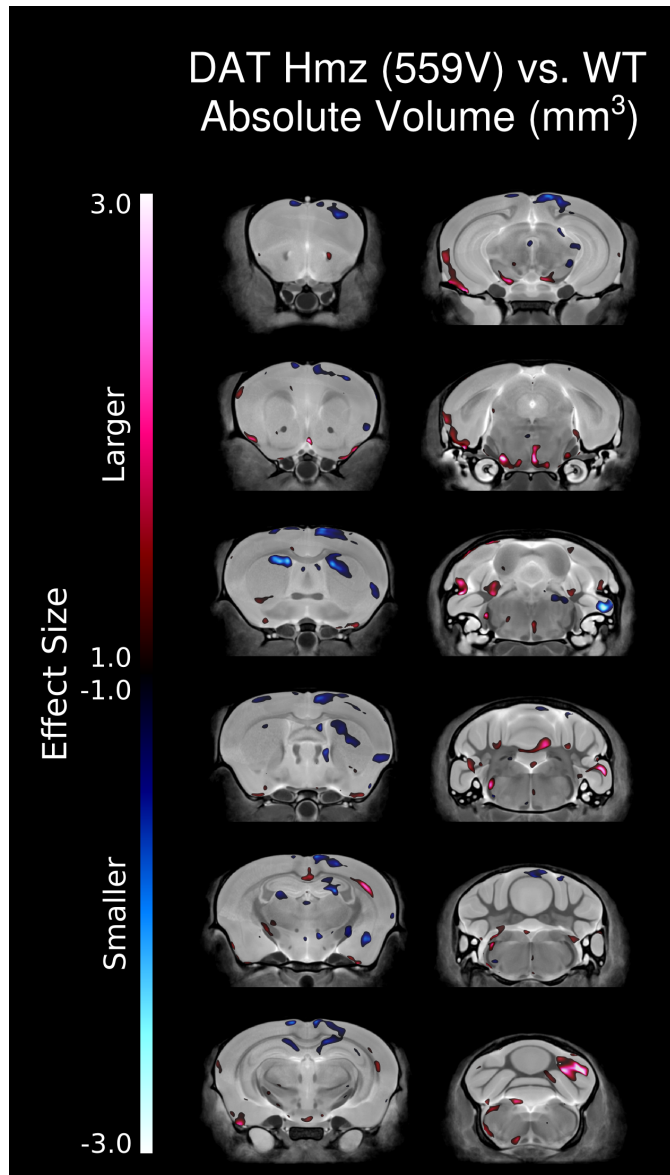
Figure 19. DAT Val559 expression results in lesser volume of the Dorsal Medial Striatum. Data above was obtained from Drs. Jacob Ellegood and Jason Lerch that shows a reduction in the volume of the DMS (in addition to other cortical regions), as indicated by the cooler colors, in brains obtained from DAT Val559 animals compared to WT

Figure 20. No changes in sEPSC amplitude in MSNs from both DS and VS of DAT Val559 mice. sEPSCs were recorded from MSNs in slices containing either the DS or NAcc core, part of the VS prepared from WT and DAT Val559 animals. No changes in sEPSC amplitude were observed in the DS (*P*>0.05, unpaired *t* Test) or the VS (*P*>0.05, unpaired *t* Test)
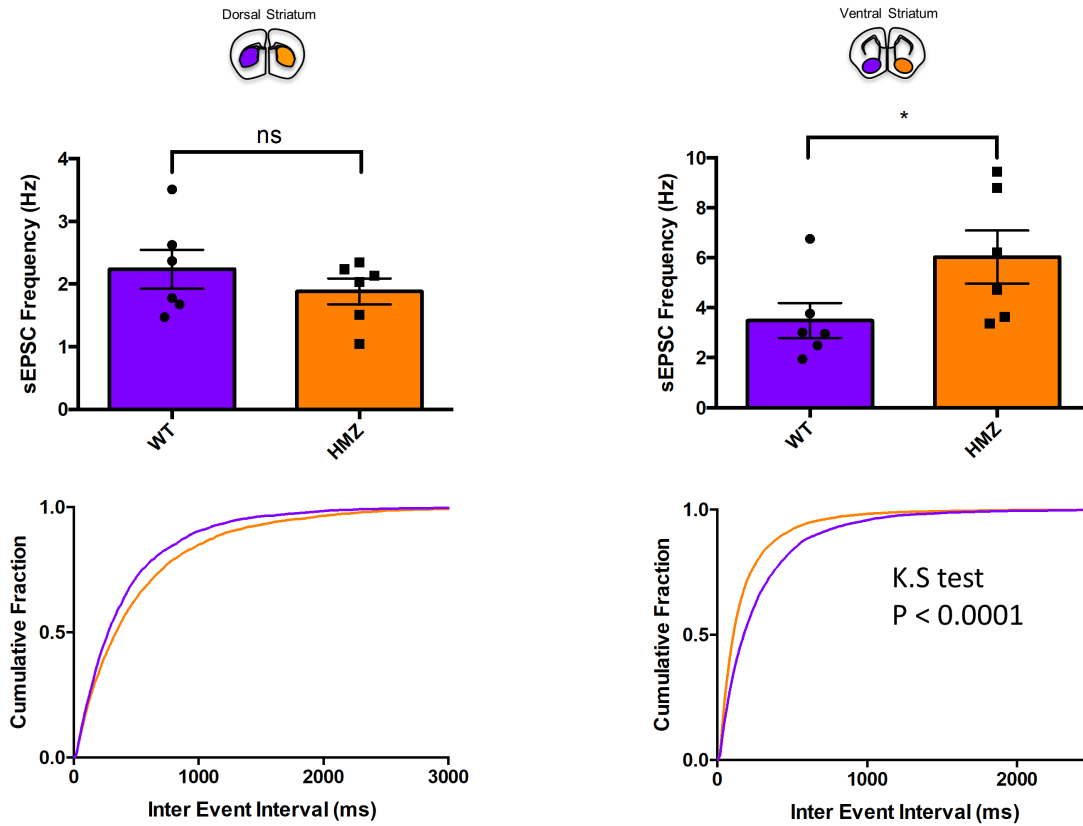
Figure 21. Increase in sEPSC frequency in MSNs from VS, but not DS of DAT Val559 mice. sEPSCs were recorded from MSNs in slices containing either the DS or NAcc core, part of the VS prepared from WT and DAT Val559 animals. No cha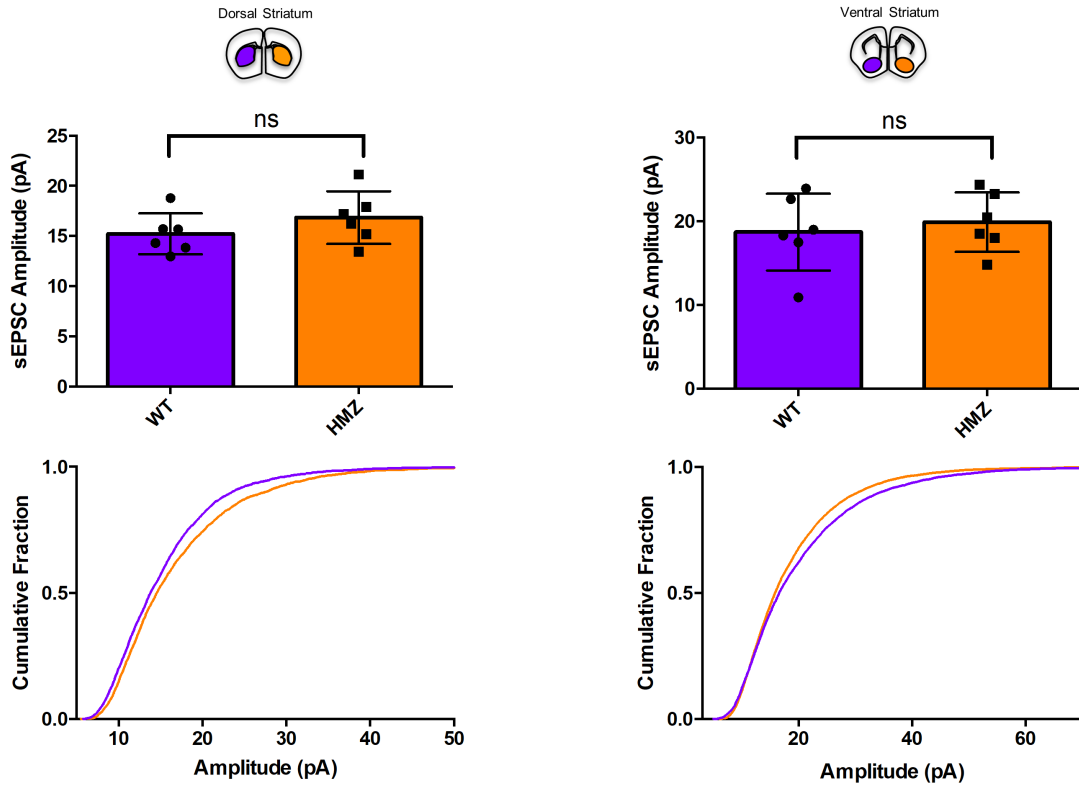nges in sEPSC frequency were observed in the DS ($P>0.05$, unpaired t Test), but a significant elevation was observed in the VS ($P<0.05$, unpaired t Test)
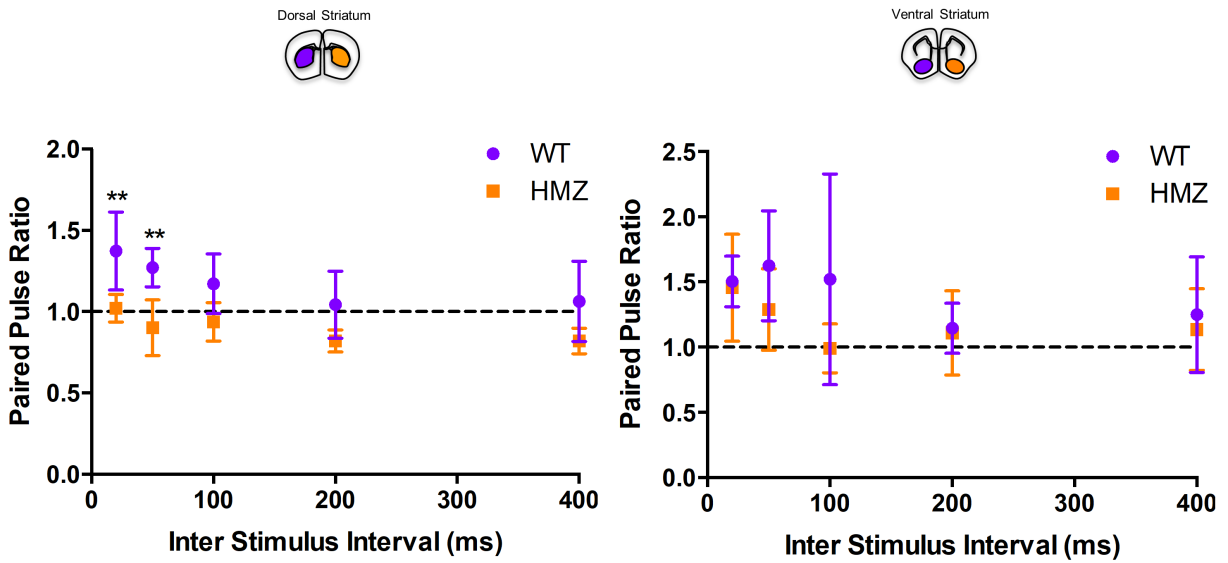
Figure 22. Elevated PPR and decreased probability of release in MSNs from DS, but not VS of DAT Val559 mice. Paired pulse ratios were recorded at different inter-stimulus intervals from MSNs in slices containing either the DS or NAcc core, part of the VS prepared from WT and DAT Val559 animals. DAT Val559 DS MSNs showed an elevated PPR at 20 and 50 ms, compared to WT (Two Way ANOVA with Sidak's multiple comparisons test revealed *P*<0.05, at 20 and 50 ms ISI), but no changes in PPR were found in the VS
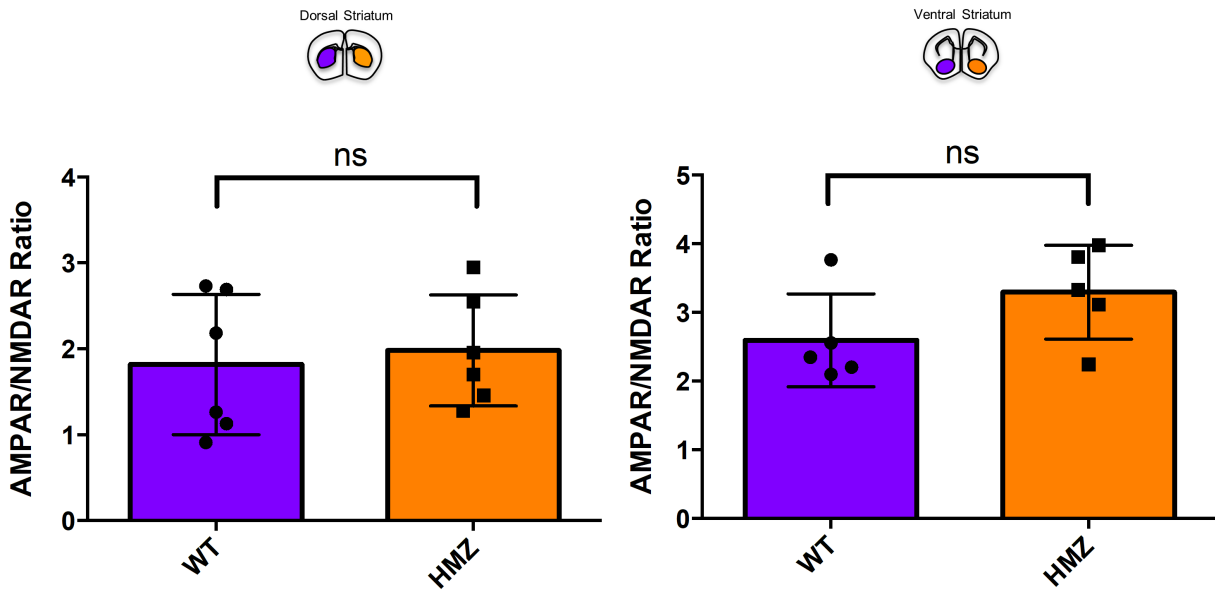
Figure 23. No changes in AMPAR/NMDAR ratios in MSNs from DS and VS of DAT Val559 mice. AMPAR currents were recorded at -70 mV and NMDAR+AMPAR currents at +40 mV, following which NMDAR currents were electrically isolated in slices containing either the DS or NAcc core, part of the VS prepared from WT and DAT Val559 animals. No changes were observed in AMPAR/NMDAR ratios in DS (*P*>0.05, unpaired *t* Test) or VS (*P*>0.05, unpaired *t* Test)

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