GENERATION AND CHARACTERIZATION OF THE FIRST CONSTRUCT-VALID MODEL OF ADHD, THE DAT VAL559 KNOCK-IN MOUSE

By

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DEDICATION

To my grandfather, Victor Gausman,
for his love, support, and encouragement.
He wanted so much to see me achieve this goal.
I hope I have made him proud.

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CHAPTER I

INTRODUCTION

Introduction to Dopamine and Dopaminergic Circuitry

The neurotransmitter dopamine (3,4-dihydroxyphenethylamine; DA) plays an important role in brain function through its modulatory role on several functions including movement, motivation and reward, and attention (Giros and Caron, 1993; Bannon, 2005; Pamiter, 2008). DA is synthesized in a two-step process in which the amino acid tyrosine is first hydroxylated by tyrosine hydroxylase (TH) to form L-DOPA (L-dihydroxyphenylalanine) (Nagatsu et al., 1964). L-DOPA is then decarboxylated by aromatic acid decarboxylase (AADC) to produce DA (Blaschko, 1942) (Fig. 1A). TH is the rate-limiting enzyme in DA synthesis (Spector et al., 1967).

Once synthesized, DA is packaged into synaptic vesicles by vesicular monoamine transporter 2 (VMAT-2). VMAT-2 utilizes the proton gradient present across the vesicular membrane to pump DA into vesicles (Njus et al., 1986; Kanner and Schuldiner, 1987; Johnson, 1988). The other VMAT isoform, VMAT-1, is typically associated with large secretory granule vesicles utilized by endocrine/paracrine cells and in the sympathetic nervous system, whereas VMAT-2 is predominantly found on small synaptic vesicles in the central nervous system (Henry et al., 1994; Weihe et al., 1994). In most brain regions, including the striatum, free intracellular DA is degraded by monoamine oxidase into 3,4-dihydroxyphenylacetic acid (DOPAC) (Rutledge and Jonason, 1968). However, at some sites, such as the

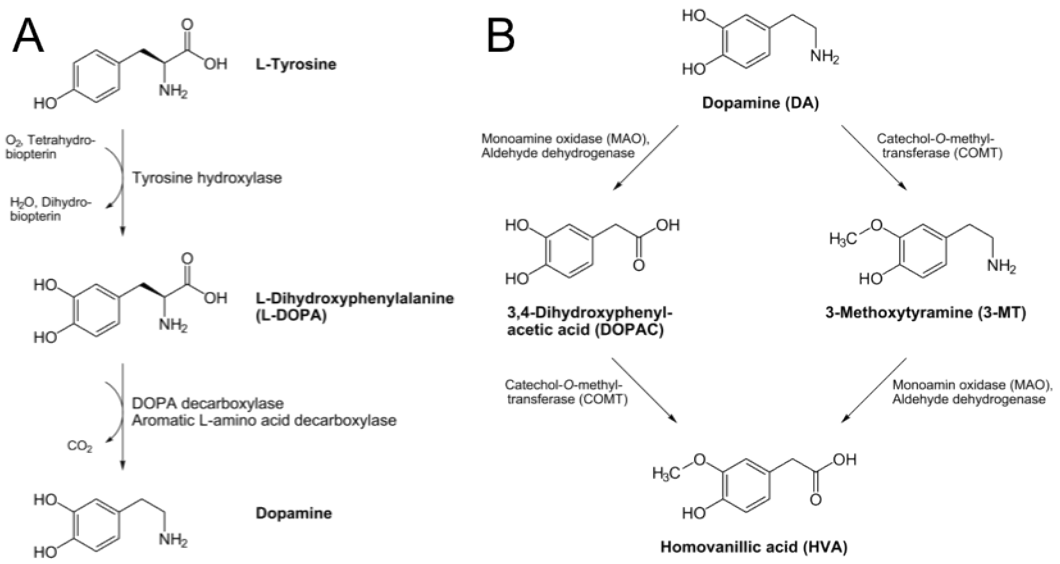

Figure 1. Synthesis and degradation of dopamine. (A) Tyrosine is converted to L-DOPA by TH, and L-DOPA to DA by AADC. (B) DA is metabolized by monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT).

prefrontal cortex, DA is degraded by catechol-*O*-methyl transferase (COMT) into 3-methoxytyramine (3-MT) (Yavich et al., 2007) (Fig. 1B). Furthermore, in some sites, especially the peripheral nervous system, DA is used as a precursor for the synthesis of norepinephrine (NE) and epinephrine.

DA is utilized as a neurotransmitter in four major brain circuits: the tuberoinfundibular, nigrostriatal, mesocortical, and mesolimbic pathways (Fig. 2). The tuberoinfundibular pathway originates in the arcuate and periventricular nuclei of the hypothalamus and projects to the median eminence (Gudelsky, 1981). There, DA provides tonic inhibition of prolactin secretion from the pituitary gland (Birge et al., 1970; Shaar and Clemens, 1974; Ben-Jonathan and Hnasko, 2001). The nigrostriatal pathway originates in the substantia nigra (SN), a brain region named for the distinct pigmentation of its neurons (Foley and Baxter, 1958), and projects to the striatum (in primates, the striatum is typically subdivided into the caudate nucleus and putamen) (Bédard et al., 1969; reviewed in Björklund and Dunnett, 2007). The mesocortical and mesolimbic pathways both originate in the ventral tegmental area (VTA), a midbrain nucleus, and project to the prefrontal cortex and nucleus accumbens (NAc), respectively (reviewed in Le Moal and Simon, 1991). The mesocortical pathway is associated with emotion, executive function, and modulation of emotions (reviewed in Floresco and Magyar, 2006; and Cools, 2008), while the mesolimbic pathway is typically implicated in pleasure and reward (reviewed in Salamone and Correa, 2012). Despite separate functions, the mesocortical and mesolimbic circuits are often collectively termed the mesocorticolimbic pathway, as they share a common origin. DA also has a role in

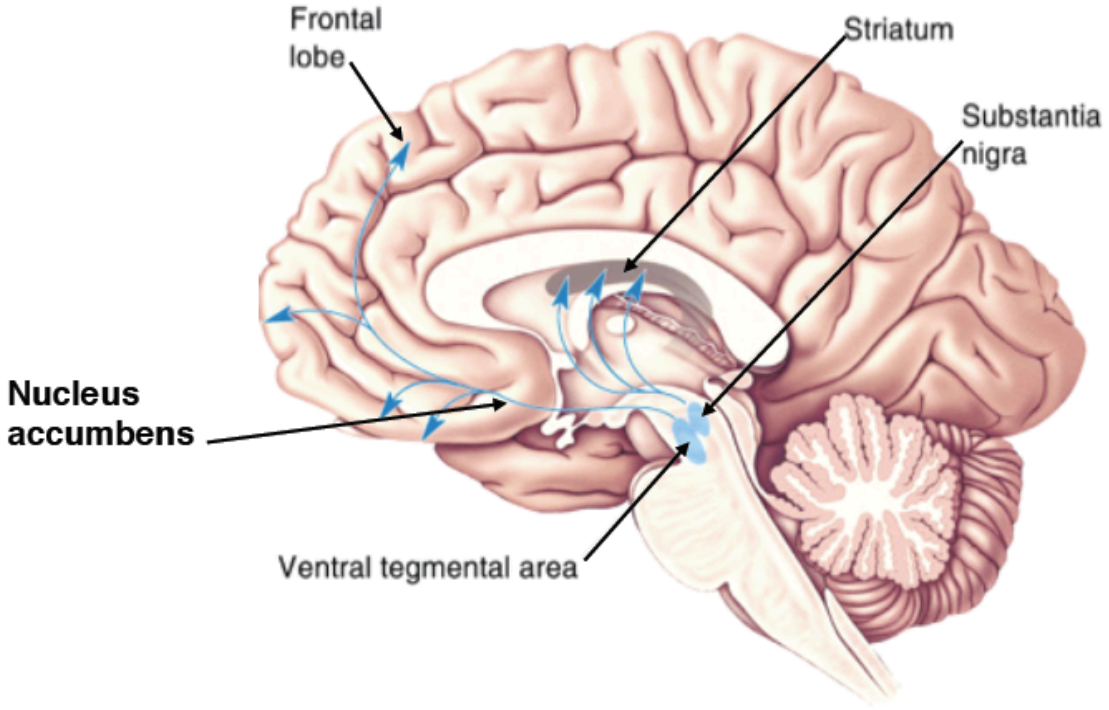

Figure 2. Schematic diagram of dopaminergic pathways in the human brain. DA neurons in SN project to the striatum via the nigrostriatal pathway. DA neurons in the VTA project to the nucleus accumbens and frontal cortex via the mesolimbic and mesocortical pathways, respectively. The tuberoinfundibular pathway is not pictured. Adapted from *Neuroanatomy, An Atlas of Structures, Sections, and Systems* (Haines, 2011).

several peripheral processes including olfactory (Dacks et al., 2012; Liu et al., 2012) retinal (Nir et al. 2000; Ruan et al., 2008), cardiovascular and renal (Jose et al., 1992; Zeng et al., 2007; Hussain and Likhandwala, 2008; Asico et al., 2011; Harris and Zhang, 2012), and immune system (Basu and Dasgupta, 2000; Kavelaars et al., 2005) function, as well as regulation of some hormone signaling (Ben-Jonathan and Hnasko, 2001) and the sympathetic nervous system (Hadjiconstantinou and Neff, 1987; Hollon et al., 2002).

DA Receptor Signaling

Dopamine typically acts in a relatively slow, modulatory fashion to fine-tune fast synaptic transmission mediated by the excitatory neurotransmitter glutamate and inhibitory neurotransmitter gamma-aminobutyric acid (GABA). In order to exert its modulatory effects, DA activates members of a family of G protein-coupled receptors. Initially, two populations of DA receptors were identified based on their ability to modulate adenylate cyclase activity (Kebabian and Calne, 1979). Later characterization identified multiple receptor subtypes (Bunzow et al., 1988; Dearry et al., 1990; Monsma et al., 1990; Sokoloff et al., 1990; Zhou et al., 1990; Sunahara et al., 1991; Tiberi et al., 1991; Van Tol et al., 1991) that are subdivided into D1-like (D1 and D5) and D2-like (D2, D3, and D4) receptors based on their pharmacological and biochemical properties (Andersen et al., 1990; Tiberi et al., 1991; Niznik and Van Tol, 1992; Sibley and Monsma, 1992; Sokoloff et al., 1992; Van Tol et al., 1992; Civelli et al., 1993; Vallone et al., 2000). It is generally accepted that D1-like receptors activate $G\alpha_{s/olf}$ G proteins and stimulate cAMP production, while D2-like receptors are coupled to $G\alpha_{i/o}$ G proteins and act to inhibit adenylate cyclase.

Furthermore, D1 and D5 receptors are exclusively expressed postsynaptically, while D2 and D3 receptors are expressed both postsynaptically on target cells as well as presynaptically on dopaminergic neurons (Sokoloff et al., 2006; Rondou et al., 2010).

DA receptors have broad expression profiles in the brain and the periphery. In the brain, D1 receptors are highly expressed in nigrostriatal and mesocorticolimbic projection areas such as the striatum (caudate and putamen), NAc, SN, and frontal cortex, and appear at lower levels in hippocampus, cerebellum, thalamus, and hypothalamus (Missale et al., 1998; Gerfen, 2000). D2 receptors are also highly expressed in the SN, VTA, hypothalamus, cortex, and hippocampus (Missale et al., 1998; Gerfen, 2000; Vallone et al., 2000; Seeman, 2006). D3 receptors have a more limited distribution, with the highest expression in the NAc and olfactory tubercle and lower expression in the striatum, SN, VTA, hippocampus, and cortical areas (Missale et al., 1998; Sokoloff et al., 2006). The D4 receptor has the lowest expression in the brain, with expression in the frontal cortex, hippocampus, SN, and thalamus (Missale et al., 1998; Rondou et al., 2010). D5 receptors are also expressed at low levels in several brain regions including prefrontal cortex pyramidal neurons, cingulate cortex, substantia nigra, and hippocampus (Missale et al., 1998; Gerfen, 2000).

The functional roles of DA receptors vary widely, as DA is involved in numerous physiological processes. Several lines of evidence demonstrate that D1, D2, and D3 receptors control locomotor activity (Missale et al., 1998; Sibley, 1999). D1 receptor activation has a stimulatory effect on locomotion, while D2 and D3

receptors have more complex functions as they are expressed both pre- and postsynaptically. Presynaptic D2 autoreceptors form a negative feedback loop that attenuates neuron firing rate (Lacey et al., 1987; Beckstead et al., 2007; Jang et al., 2011) as well as DA synthesis (Lindgren et al., 2001; Anzalone et al., 2012) and release (Anzalone et al., 2012; Zhang and Sulzer, 2012) in response to extracellular DA levels. Activation of presynaptic D2 receptors leads to less DA release and a decrease in locomotor behavior, whereas activation of postsynaptic D2 receptors stimulates locomotion.

Interestingly, the D2 receptor is the only DA receptor with multiple functional splice isoforms (Giros et al., 1989). The long isoform, D2L, has a 29 amino acid insertion in the third intracellular loop that the short isoform, D2S, lacks. D2L is predominantly postsynaptic while D2S is predominantly presynaptic (Usiello et al., 2000; De Mei et al., 2009). In addition, D2L and D2S differ in their sensitivity to D2 receptor agonists, including DA; D2S is activated by lower agonist concentrations than needed to activate D2L (Drukarch and Stoof, 1992). This can result in biphasic locomotor responses, as low agonist concentration favors D2S and therefore inhibits locomotion, while higher agonist concentration favors D2L and thus stimulates locomotor activity.

Along with locomotor activity, D1 and D2 receptors also play key roles in response to rewards and addictive drugs (Hyman et al., 2006; Di Chiara and Bassareo, 2007; De Mei et al., 2009) and working memory mediated by the prefrontal cortex (Goldman-Rakic et al., 2004; Xu et al., 2009). Similarly, D3, D4, and D5 receptors contribute a minor modulatory influence on cognitive functions

mediated by the hippocampus (Sibley, 1999; Sokoloff et al., 2006; Rondou et al., 2010). Other specific roles for D3, D4, and D5 receptors are unclear; D3 receptors have been shown to have a minor influence on several functions typically attributed to D2 receptors (Sibley, 1999; Joseph et al., 2002; Sokoloff et al., 2006; Beaulieu et al., 2007; De Mei et al., 2009). DA receptors also have modulatory roles in regulating attention, impulse control, motor learning, sleep, reproductive behavior, and regulation of food intake (Missale et al., 1998; Di Chiara and Bassareo, 2007; Koob and Volkow, 2010; Rondou et al., 2010).

The signaling pathways downstream of DA receptors vary greatly and are highly complex. For example, D1 and D2 receptors both modulate protein kinase A (PKA) activity, and therefore impact a vast array of PKA target molecules (DA receptor regulation and downstream signaling reviewed in Beaulieu and Gainetdinov, 2011). A pair of interesting and important pathways through which dopamine signals include dopamine and cAMP-regulated phospho-protein (DARPP-32) and glycogen synthase kinase 3 (GSK3). DARPP-32 plays a key role in integrating dopaminergic and glutamatergic signaling, as it is regulated by both PKA (downstream of D1 and D2 receptor activation) and cyclin dependent kinase 5 (CDK5; downstream of NMDA receptor activation). DARPP-32 also regulates protein phosphatase 1 (PP1) activity and can therefore affect other proteins by regulating phosphorylation state (Hemmings et al., 1984; Desdouits et al., 1995; Bibb et al., 1999; reviewed in Greengard et al., 1998; Greengard, 2001; Svenningsson et al., 2004; Le Novère et al., 2008).

Since DA receptors are all G protein-coupled receptors (GPCRs), they are inactivated by desensitization, internalization, and signal termination. Upon GPCR activation, receptors are quickly phosphorylated by GPCR kinases (GRKs), which leads to recruitment of scaffolding proteins known as arrestins (Lohse et al., 1990; Benovic et al., 1991; Arriza et al., 1992; Shenoy and Lefkowitz, 2003; Gainetdnov et al., 2004;) and subsequent uncoupling of the receptor from the G proteins. Association with an arrestin protein leads to clathrin-mediated internalization of a GPCR (Lohse et al., 1990; Ferguson et al., 1996; Laporte et al., 2002; Shenoy and Lefkowitz, 2003). However, β-arrestin 2 can associate with D2 receptors and regulate Akt and GSK3 independent of PKA activation (Beaulieu et al., 2004, 2005, 2006). D2 receptor activation leads to formation of a protein complex containing β-arrestin, Akt, and protein phosphatase A (PP2A) (Beaulieu et al., 2005). PP2A deactivates Akt, thus disinhibiting GSK3 (Beaulieu et al., 2004, 2005). GSK3β, once activated, facilitates DA-associated locomotor behaviors (Beaulieu et al., 2004).

The Dopamine Transporter

Discovery and Cloning

Although MAO and COMT metabolize some DA as addressed above, most DA (and other catecholamine transmitters) is cleared from synapses by transporter proteins. Early studies actually focused on NE reuptake; Hertting and Axelrod demonstrated that [^{3}H]-NE could be selectively taken up and sequestered in sympathetic nerve terminals (Hertting and Axelrod, 1961), and went on to demonstrate that antidepressants (i.e. imipramine) and psychostimulants (i.e. AMPH) block NE accumulation (Axelrod et al., 1961; Hertting et al., 1961). Several

studies later demonstrated that both NE and DA accumulate in brain slices and that psychostimulants blocked this process (Glowinski and Axelrod, 1966; Ross and Renyi, 1967). Ross and Renyi also differentiated DA uptake from NE uptake, showing that cocaine and AMPH inhibit both DA and NE uptake in the striatum, but desipramine (a norepinephrine transporter (NET) inhibitor) only weakly inhibits DA uptake in striatum while potently blocking cortical NE uptake (Ross and Renyi, 1967). Coyle and Snyder went on to show that AMPH isomers were differential inhibitors of DA (equally inhibited by L- and D-AMPH) and NE (preferentially inhibited by D-AMPH) (Coyle and Snyder, 1969), further supporting the idea that DA and NE transport are separate processes. Nearly two decades later, Ritz and colleagues showed that cocaine's potency to block DA clearance was significantly higher than it's ability to block NE clearance (Ritz et al., 1987).

Since DA and NE uptake were pharmacologically distinct, efforts turned to identification of the dopamine transporter. The first approach was to identify cocaine binding sites in the brain. Reith and colleagues used plasma membranes from mouse brain to identify and define the kinetic parameters of the cocaine binding site using [^{3}H]-cocaine (Reith et al., 1980, 1981). Subsequent work drew correlations between occupancy of the cocaine binding site and the effects (locomotor activity, self-administration, local anesthetic properties) of cocaine (Sershen et al., 1983). Further autoradiographic studies using a tritiated cocaine analog, [^{3}H] 2 beta-carbomethoxy-3 beta-(4-fluorophenyl)tropane ([^{3}H]-CFT), localized cocaine binding to the striatum of squirrel monkeys (Canfield et al., 1990). Today we understand that the cocaine binding sites reflect the presence of DAT.

In an effort to identify and characterize neurotransmitter transporter genes, Blakely and colleagues expressed mRNAs from several brain regions in *Xenopus laevis* oocytes and demonstrated the transport of radiolabeled neurotransmitters (glutamate, GABA, glycine, DA, serotonin (5-HT), and choline) (Blakely et al., 1988). The expression patterns of the mRNAs that expressed proteins competent for neurotransmitter transport were consistent with the neuroanatomical distribution of the cell bodies for particular neurotransmitter systems and paved the way for expression cloning of the neurotransmitter transporters (Blakely et al., 1991b).

Shortly thereafter, genes for the human norepinephrine transporter (NET) (Pacholczyk et al., 1991), rat serotonin transporter (Blakely et al., 1991a) and rat and bovine dopamine transporter (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991; Usdin et al., 1991) were successfully cloned. Furthermore, DAT expression in dopamine neuron cell bodies in the SN and VTA was confirmed by *in situ* hybridization (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991). Further evidence that the cloned gene was, in fact, DAT was that *in vivo* application of DAT inhibitors blocked DA transport with similar potencies as previously reported in synaptosomes (Ritz et al., 1987).

Based on the rat DAT sequence, oligonucleotide probes were designed and used to screen a cDNA library derived from substantia nigra, resulting in the cloning of human DAT (hDAT) (Giros et al., 1992; Vandenbergh et al., 1992). Human DAT is a 620 amino acid protein that is 92% homologous with the previously identified rat DAT as well as 66% homologous to hNET and 50% homologous to hSERT. Hydropathicity analysis of DAT predicts 12 transmembrane domains (TMD) and

both N- and C-termini oriented intracellularly (Fig. 3). A novel splice variant of DAT exists in human blood cells (Sogawa et al., 2010). This splice isoform lacks exon 6, which results in deletion of TMD5 and re-orientation of the C-terminus to the extracellular side of the cell membrane. When expressed in a heterologous system, the splice isoform lacks DA transport activity and, when co-expressed with WT DAT, acts in a dominant negative fashion. In addition, Talkowski and colleagues reported a cassette exon located within intron 3 in a schizophrenic population (Talkowski et al., 2010). This alternate exon introduces multiple stop codons, resulting in a truncated protein product that is likely subject to nonsense-mediated decay. Other factors that favor expression of this alternate cassette exon could reduce the amount of functional DAT expressed and may contribute to risk for schizophrenia.

DAT Structure/Function Relationships

DA transport is an ion-coupled process with a transport stoichiometry of 2 Na^+, 1 Cl^-, and 1 DA molecule (McElvain and Schenk, 1992; Kilty, 1993; Gu et al., 1994). With this ion stoichiometry, DA transport is a voltage-dependent, electrogenic process where hyperpolarization increases DA transport (Sonders et al., 1997). Electrophysiological studies reveal that DA transport through DAT actually produces a larger current flux than the charge transfer that the ion-dependence of DA transport would predict (Sonders et al., 1997; Ingram et al., 2002). Subsequent research identified a channel-like state for DAT that permits excess ion permeation during the DA transport cycle (Carvelli et al., 2004; Kahlig et al., 2005).

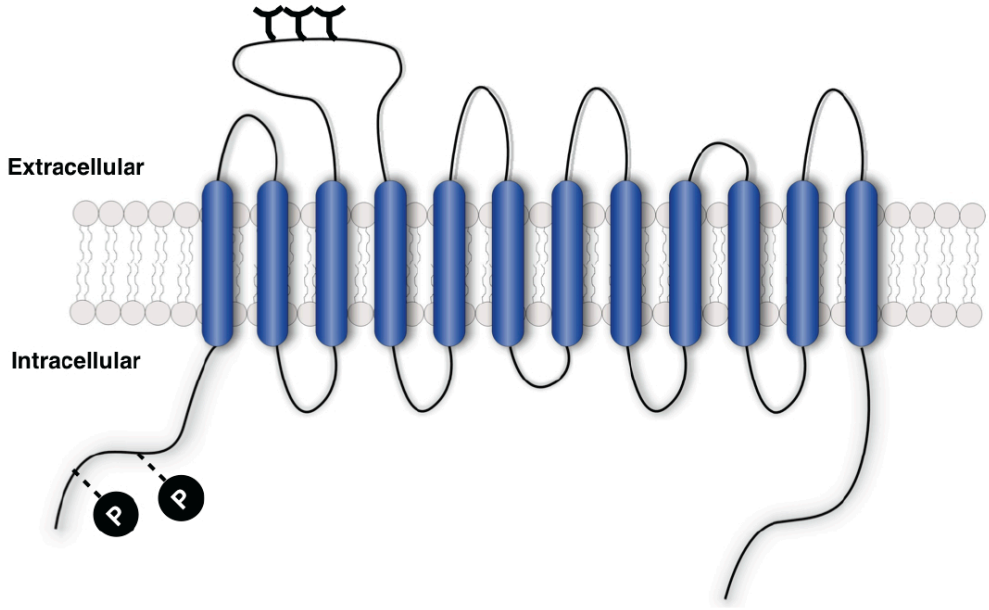

Figure 3. Schematic illustration of DAT topology. The DAT protein has 12 TMDs and both N- and C-termini oriented to the intracellular side of the plasma membrane. EL2 contains 3 *N*-linked glycosylation sites. Several phosphorylation sites, shown as black circles labeled P, are found throughout the protein, including both N- and C-termini and intracellular loops.

Several studies have pursued structure/function relationships for DAT. Initially, DAT and NET chimeric proteins were studied *in vitro* and provided the first evidence for distinct functional domains of the transporter linked to substrate recognition, translocation, and affinity (Buck and Amara, 1994; Giros et al., 1994; Syringas et al., 2000). Specifically, inhibitor selectivity was attributed to TMDs 5-8 and substrate affinity determined by TMDs 1-3 and 10-11 (Buck and Amara, 1994; Giros et al., 1994). Subsequent work found that TMDs 1-3 and 9-12 are important for the Na^+ and Cl^- dependence of substrate transport by DAT and the other SLC6 monoamine transporters (Syringas et al., 2000).

Another functional domain of DAT is the large extracellular loop between TMD3 and TMD4 (extracellular loop 2; EL2). This region of the protein contains three N-glycosylation sites that are essential for maturation and appropriate plasma membrane trafficking (Lis and Sharon, 1993; Ramamoorthy et al., 1998). Mutation of the canonical glycosylation sites, as well as enzymatic degradation or blockade of glycosylation resulted in reduced surface expression, DA transport, and inhibitor sensitivity (Li et al., 2004). Recent studies have gone on to show that maintenance of DAT glycosylation is involved in DA neuron vulnerability in Parkinson's disease (Afonso-Oramas et al., 2009), suggesting that DAT glycosylation has implications beyond basic DAT function. In addition, EL2 has a role in inhibitor binding, as DAT inhibitors but not substrates protect DAT from trypsin digestion (Vaughan and Kuhar, 1996; Gaffaney and Vaughan, 2004). EL2 may have a role in substrate translocation, as well. Norregaard identified a zinc-binding site in DAT (Norregaard et al., 1998); when a Zn^{2+} ion is coordinated between histidine residues in EL2 and

EL4, DA transport is blocked. The authors speculate that constraining EL2 via Zn^{2+} binding may lead to structural changes in TMD3 and/or TMD4.

In order to further study how DAT interacts with both substrates and antagonists, mutagenesis studies have sought to identify the amino acid residues necessary for ligand coordination. In a series of studies, Lin and coworkers performed alanine substitutions for all phenylalanine (Lin et al., 1999), proline (Lin et al., 2000a), or tryptophan (Lin et al., 2000b) residues in or near DAT TMDs. Several of the engineered mutants show changes in substrate or antagonist binding affinity, suggesting that the mutated amino acids are important for ligand binding. Other experiments using similar mutagenesis strategies defined roles for Asp79 (Kitayama et al., 1992) and Phe105 (Wu and Gu, 2003) in cocaine binding, as well.

To date, the crystal structure for neither DAT nor any other SLC6 family protein has been solved. However, Yamashita and colleagues crystallized the leucine transporter ($LeuT_{Aa}$), a bacterial homolog of the SLC6 transport proteins, from *Aquifex aeolicus* (Yamashita et al., 2005). Although $LeuT_{Aa}$ possesses the 12 TMD topology of DAT and other monoamine transporters, the N- and C-termini are both small and unstructured in the crystal structure. The observed arrangement of $LeuT_{Aa}$ is displayed in Figure 4. Computer models based on $LeuT_{Aa}$ have predicted the cocaine binding site on DAT to be located between TMD 1, 3, 6, and 8, as well as conformational changes that occur when cocaine binds then moves to occlude DA transport (Beuming et al., 2008; Ravna et al., 2009; Huang et al., 2009). Several $LeuT_{Aa}$-based DAT models have identified a second substrate binding pocket (Schmitt et al., 2010; Shan et al., 2011), binding sites for AMPH-like drugs

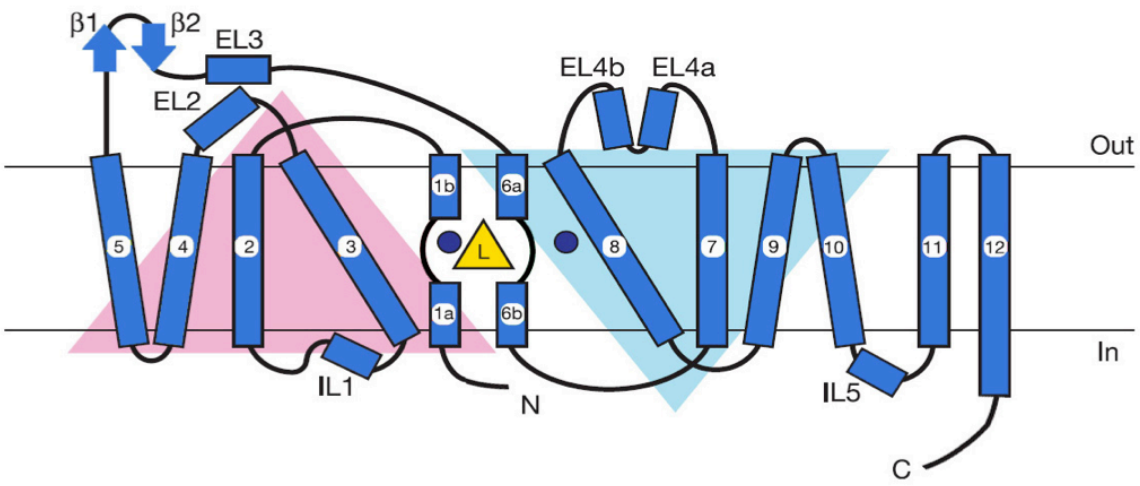

Figure 4. Predicted topology of DAT based on the crystal structure of the bacterial leucine transporter $LeuT_{Aa}$. TMDs 1-5 and 6-10 form a pseudo two-fold axis in the plane of the plasma membrane (inverted pink and blue triangles) and fold over to form the substrate translocation pathway. The yellow triangle and blue circles depict leucine and Na^+ ions, respectively. Adapted from Yamashita et al., 2005.

(Severinsen et al., 2012), benztropine-based inhibitors (Bisgaard et al., 2011), and a non-competitive inhibitor (Bulling et al., 2012), and predicted conformational changes required for DA binding and transport across the plasma membrane (Gedeon et al., 2010; Merchant and Madura, 2012). Recently, $LeuT_{Aa}$ was re-crystallized in outward-open and inward-open states (i.e. no substrate bound) (Krishnamurthy and Gouaux, 2012). By comparing the two transporter conformations, the authors predict similar conformational changes likely occur during DA transport as were suggested by previous computer simulations (Gedeon et al., 2012; Merchant and Madura, 2012).

DAT Regulation

Given DAT's role for clearing DA upon synaptic release, efforts turned to defining how DAT itself is regulated. Kuhar's group reported that, after irreversible inhibition of DAT, the time course for newly synthesized DAT to replace the inactivated transporters is very long, with only ~50% of transporters replaced after 2-3 days (Fleckenstein et al., 1996; Kimmel et al., 2000). The time course of synthesis of new DATs is far too slow to support the rapid changes in DAT function or expression in response to various challenges (reviewed in Zahniser and Doolen, 2001). Now, evidence exists that DAT is subject to rapid regulation by presynaptic receptors, interacting proteins, and various intracellular signaling networks to fine-tune DA clearance capacity (Zahniser and Doolen, 2001; Torres, 2006; Chen et al., 2010).

As reviewed above, presynaptic D2 autoreceptors exert inhibitory influence on DA signaling by modulating DA neuron firing rate (Lacey et al., 1987; Beckstead

et al., 2007; Jang et al., 2011), and DA synthesis and release (Lindgren et al., 2001; Anzalone et al., 2012; Zhang and Sulzer, 2012). D2 autoreceptors also directly regulate DAT activity (Meiergerd et al., 1993; Batchelor and Schenk, 1998; Dickinson et al., 1999; Gulley and Zahinser, 2003), an effect mediated via activation of extracellular signal-regulated kinases (ERK1/2) (Bolan et al., 2007). Other studies have demonstrated that presynaptic metabotropic glutamate receptors (mGluRI) (Falkenberger et al., 2001) and nicotinic acetylcholine receptors (nAChR) (Hart and Ksir, 1996; Middleton et al., 2004) regulate DAT function, as well. Furthermore, Lee and colleagues have reported a direct interaction between the DAT N-terminus and the presynaptic D2S receptor (Lee et al., 2007) that can modulate DAT function. This interaction may be important for coordinating DA release and clearance at the synaptic membrane.

Protein-Protein Interactions

Several DAT-interacting proteins have been identified via yeast two-hybrid (Y2H) screens that used portions of DAT as bait, and then confirmed observed interactions by co-immunoprecipitation in cultured cells or brain tissue preparations (reviewed in Torres, 2006). These interacting proteins are thought to regulate somatic export, synaptic localization, plasma membrane trafficking, and functional characteristics of DAT. None of the identified interactions, however, have been demonstrated to influence DAT function *in vivo*.

In a Y2H study using the DAT C-terminus as bait, Torres and colleagues first identified an interaction with Protein Interacting with C Kinase-1 (PICK1) (Torres et al., 2001). Since PICK1 also interacts with protein kinase C (PKC) (Staudinger et al.,

1995), this interaction may be important for mediating PKC regulation and trafficking of DAT. The same Y2H screen also identified Hic-5 as a DAT interactor (Carneiro et al., 2002). Hic-5 is an adhesion-associated adaptor protein that has been shown to interact with several signaling molecules (reviewed in Shibanuma et al., 2012), so it may be an important integrator that connects DAT to intracellular signaling pathways.

Another DAT C-terminal interacting protein is α-synuclein (Lee et al., 2001). This protein has been implicated in genetic forms of Parkinson's disease (Chua and Tang, 2006; Covy and Giasson, 2011). DAT/α-synuclein interactions have been shown to cluster DAT at the plasma membrane, increase transport activity, and enhance DA-induced apoptosis (Lee et al., 2001), an effect attributed to increased oxidative stress resulting from accumulation of too much DA.

In another Y2H screen, Fog and colleagues identified an interaction between calcium/calmodulin dependent kinase II (CaMKII) and the DAT C-terminus (Fog et al., 2006). Further study revealed that CaMKII binds to the C-terminus and phosphorylates DAT, likely at sites on the N-terminal tail. Furthermore, CaMKII binding and DAT phosphorylation were shown to be necessary for AMPH-induced DA efflux through DAT, providing evidence that DAT is stabilized in an "efflux-willing" state.

Most recently, the Ras-like GTPase Rin was shown to interact with the DAT C-terminus (Navaroli et al., 2011). In characterizing this interaction, it was suggested that the DAT/Rin interaction is necessary for PKC-induced DAT internalization, though the underlying mechanism remains to be determined. Rin is also known to

bind to and activate calmodulin (Lee et al., 1996). In light of the aforementioned DAT/CaMKII interaction, it is reasonable to speculate that Rin might have a role in activating DAT-bound CaMKII.

Researchers have used the Y2H approach with the DAT N-terminal as bait, as well. Lee and colleagues identified Receptor for Activated C Kinase (RACK1) and syntaxin 1A as DAT interactors (Lee et al., 2004). RACK1 binds activated PKC and recruits it to the plasma membrane, thus putting PKC in close proximity to its substrates (Ron et al., 1994; Rodriguez et al., 1999). The precise role of the DAT/RACK1 interaction is unclear, but may facilitate PKC regulation of DAT. Syntaxin 1A is a component of the SNARE complex, the machinery needed for synaptic vesicle fusion and neurotransmitter release (Bennett et al., 1992; Wu et al., 1999). Cervinski and colleagues demonstrated the presence of DAT/syntaxin 1A complexes in rat striatal synaptosomes and that syntaxin 1A cleavage enhances DAT activity, supporting a role for these complexes in DA neurons (Cervinski et al., 2010). Interestingly, syntaxin 1A also interacts with GABA transporters (GAT1) (Beckmann et al., 1998), glycine transporters (GLYT1) (Geerlings et al., 2000), NET (Sung et al., 2003), and SERT (Quick, 2002), suggesting that interactions between syntaxin and transporters may be a conserved regulatory mechanism.

Membrane Microdomain Sequestration

Plasma membranes are not a homogenous environment. Within the lipid bilayer, sphingolipids and cholesterol can cluster together to form microdomains known as lipid rafts. These rafts impose order onto the plasma membrane and are proposed to function as anchors to localize and/or stabilize proteins at the

membrane (Simons and Ikonen, 1997; Ikonen, 2001; Lingwood et al., 2009). By sequestering proteins to discrete regions of the membrane, lipid rafts are able to influence intracellular signaling (Brown and London, 1998; Helms and Zurzolo, 2004). Adkins and colleagues demonstrated DAT association with membrane rafts and that this membrane association limits DAT's lateral mobility within the membrane and supports DA transport (Adkins et al., 2007). Other groups have confirmed this finding and shown that lipid raft association can affect DAT's trafficking in response to PKC activation (Foster et al., 2008) and conformation (Hong and Amara, 2010). In addition, DAT has been shown to interact with the raft-associated protein flotillin-1 (Cremona et al., 2011), and that this interaction is required for localization to rafts and AMPH-induced DA efflux (Cremona et al., 2011; Pizzo et al., 2013). Furthermore, flotillin-1 phosphorylation was found to be required for PKC-mediated DAT internalization. This finding may explain the finding that PKC activation results in DAT internalization independent of DAT phosphorylation (Gränäs et al., 2003).

DAT regulation by association with lipid rafts is particularly interesting due to the loss of raft association and functional dysregulation of a DAT variant, Arg615Cys (Sakrikar et al., 2012). This DAT mutant was identified in a human subject diagnosed with ADHD and will be addressed later in the context of rare, ADHD-associated DAT variants.

Intracellular Signaling

In addition to regulation by interacting proteins and membrane microdomain association, DAT is regulated by covalent modifications including

phosphorylation, ubiquitination, and lipid modification. The impact of these modifications on DAT trafficking and function are based largely on *in vitro* studies in cultured cells or *ex vivo* in brain synaptosomes (Vaughan et al. 1997). Numerous model systems continue to be utilized to study DAT function, including the genetically tractable nematode, *C. elegans* (Nass et al., 2001, 2005; Carvelli et al., 2004, 2008; Hardaway et al., 2012), *Drosophila melanogaster* (Pörzgen et al., 2001; Chen et al., 2007; Makos et al., 2010; Vickrey et al., 2013), and DAT mutant mice (Chen et al., 2005; Zhou et al., 2009; Rao et al., 2012; Mergy et al., 2013).

DAT contains several canonical serine/threonine phosphorylation sites on the N- and C-termini, as well as many potential non-canonical phosphorylation sites. Several studies have shown that DAT activity is regulated by phosphorylation by a number of kinases including PKC (Huff et al., 1997; Zhang et al., 1997), PKA (Page et al., 2004), mitogen activated protein kinases (MAPKs) such as ERK (Bolan et al., 2007) and MEK (Lin et al., 2003; Moró), CaMKII (Page et al., 2004; Fog et al., 2006), and phosphoinositide 3 kinase (PI3K) (Carvelli et al., 2002). To date, limited evidence suggests that tyrosine kinase signaling can regulate DAT activity (Simon et al., 1997; Doolen and Zahniser, 2001; Hoover et al., 2007), however, there is no evidence that DAT is directly phosphorylated by tyrosine kinases.

PKC activation, either by application of phorbol esters such as phorbol 12-myristate 13-acetate (β-PMA) (Huff et al., 1997; Vaughan et al., 1997; Zhang et al., 1997; Foster et al., 2008) or indirect stimulation via activation of substance P receptors (Grånäs et al., 2003), has been shown to produce downregulation of DAT transport activity and cell surface expression. It was initially thought that this

downregulation was a direct result of PKC-dependent DAT phosphorylation and subsequent intracellular sequestration of DAT (Zhang et al., 1997; Zhu et al., 1997; Melikian and Buckley, 1999; Gränäs et al., 2003; Loder and Melikian, 2003). Although PKC activation in striatal synaptosomes does increase DAT phosphorylation (Huff et al., 1997; Vaughan et al., 1997; Foster et al., 2002), later research demonstrated that deletion of N-terminal phosphorylation sites does not abolish PKC-mediated DAT downregulation (Gränäs et al., 2003). It is possible that N-terminal phosphorylation is part of a redundant system in place to insure appropriate transporter trafficking. Thus, studies of regulation of DAT trafficking should be interpreted cautiously, as observed effects may be the result of alternate processes.

After demonstrating that DAT phosphorylation was not required for PKC-mediated DAT internalization, efforts turned to finding other mechanisms that could be in play. Ubiquitination is typically thought of as a signal leading to protein degradation (Hershko and Ciechanover, 1992), however, attachment of single or short chain ubiquitin moieties rather than long ubiquitin chains can control other cellular events such as endocytosis and membrane trafficking (Hicke, 2001; Hicke et al., 2005; Staub and Rotin, 2006). Miranda and colleagues demonstrated that PKC activation leads to increases in DAT N-terminal ubiquitination, and that the ubiquitin signal is required for DAT endocytosis (Miranda et al., 2005, 2007).

Although PKC does not directly control DAT trafficking, PKC-induced phosphorylation does have a role in other processes, such as DAT-mediated DA efflux (Kantor and Gnegy, 1998; Cowell et al., 2000; Johnson et al., 2005). These data

suggest that DAT is subject to trafficking-dependent and -independent regulation by PKC. This conclusion parallels observations that NET (Jayanthi et al., 2004) and SERT (Zhu et al., 2005; Steiner et al., 2008) undergo both trafficking-dependent and trafficking-independent regulation, as well.

Interestingly, DAT is sensitive to insulin signaling. Carvelli and colleagues demonstrated that insulin treatment stimulates DAT activity in a PI3K-dependent manner in serum-starved, DAT-transfected cells (Carvelli et al., 2002). Later studies showed that a dominant negative form of Akt (a kinase activated by PI3K) blocks insulin's effect on DAT (Garcia et al., 2005). These findings coupled with data that hypoinsulinemic rats exhibit decreased DA uptake (Patterson et al., 1998) support the idea that insulin- and PI3K-linked signaling pathways regulate DAT function.

The Galli lab has continued to study the role of PI3K/Akt signaling in transporter regulation, and implicated a downstream target of Akt, mTOR, in NE transport and NET trafficking (Robertson et al., 2010; Siuta et al., 2010). Beyond altered NET trafficking, they demonstrated that conditional mTOR KO mice had behavioral changes (disrupted pre-pulse inhibition) that could be corrected by the NET antagonist desipramine (Siuta et al., 2010). In light of PI3K's ability to regulate DAT, further exploration of the PI3K-Akt-mTOR-DAT signaling pathway is warranted.

Taken together, the studies described above present a complicated and incomplete understanding of regulation of DAT activities. Multiple signaling pathways and interacting proteins intersect at DAT. Further efforts to characterize

DAT regulation will continue to inform our understanding of neuropsychiatric disorders such as addiction, schizophrenia, and ADHD.

Psychostimulants

Given the key role of DAT in controlling DA signaling, it is not a surprise that drugs targeting DAT have profound effects. These effects can be beneficial, as is the case with methylphenidate (e.g. Ritalin ®) and amphetamine formulations (e.g. Adderall ®) used to treat ADHD. However, other psychostimulants can be highly addictive and have profound negative consequences for the drug abuser, their family, and the community at large.

Cocaine is the archetypical competitive DAT antagonist, simply blocking DA reuptake through DAT, whereas the mechanism of AMPH action is more complicated. In addition to blocking DA transport and/or inducing DA efflux, these drugs can both influence subcellular distribution of DATs (Saunders et al., 2000; Daws et al., 2002). Cocaine's effects on DAT cell surface distribution are inconsistent; Daws and colleagues reported that cocaine application increases DAT surface expression and DA transport (Daws et al., 2002). However, Vaughan's group observed no changes in DAT expression or activity after cocaine application (Gorentla and Vaughan, 2005). Results from studies of the brains of cocaine abusers are inconsistent, as well, showing both reduced (Wilson et al., 1996) and increased (Mash et al., 2002) DAT levels. In the intact brain of a cocaine abuser, one might expect chronically elevated DA levels due to chronic DAT blockade by cocaine to result in downregulation of DAT. The data, however, indicate that this is not the case (Little et al., 1999, 2002). Further investigation is certainly warranted, as

understanding compensatory alterations in DAT expression and/or function could inform our understanding of drug addiction.

AMPH has a far more complicated mechanism of action than cocaine. AMPH acts as a competitive inhibitor of DA uptake, but also acts as a DAT substrate, getting transported into neurons through DAT (Azzaro et al., 1974), where it acts as a weak base and disrupts vesicular proton gradients (Sulzer et al., 1995), resulting in release of vesicular DA into the cytoplasm. This excess cytosolic DA is then transported out of the neuron through DAT, a process known as DA efflux. AMPH-induced DA efflux can also be observed in cultured cells transfected with DAT and loaded with DA (Khoshbouei et al., 2003).

Prolonged exposure to AMPH leads to a reduction in DAT surface expression and DA transport (Saunders et al., 2000; Gulley et al., 2002). This process is dependent on AMPH transport and PKC activity, as cocaine or PKC inhibition can block AMPH effects (Kantor and Gnegy, 1998; Saunders et al., 2000). AMPH-induced DA efflux is regulated by both PKC (Kantor and Gnegy, 1998) and CaMKII (Fog et al., 2006). Activation of either kinase results in DAT phosphorylation on the N-terminal tail that is required for AMPH-induced efflux; deletion of the entire N-terminus (Foster et al., 2002; Gränäs et al., 2003) or alanine substitution for all of the N-terminal serine residues (Khoshbouei et al., 2004) abolishes AMPH-induced efflux. It appears that AMPH acts to inhibit DA clearance, but eventually gets transported into neurons where it induces DA release and activation of PKC and CaMKII. The activated kinases then phosphorylate DAT and stabilize an “efflux-willing” state, At

the same time, AMPH is emptying DA from vesicles and the efflux-willing DAT transports DA into the synapse.

DAT and the DA System in Disease

Parkinson's Disease

Dopaminergic dysfunction is associated with several neuropsychiatric disease states. Perhaps the best studied is Parkinson's disease (PD) (Temlett, 1996). PD involves the death of SN DA neurons and the resulting loss of dopaminergic tone leads to involuntary movements and motor tremors (Simões et al., 2012). Other motor symptoms include bradykinesia, rigidity, shuffling gait, and balance problems (reviewed in Fritsch et al., 2012). In addition, patients with PD often suffer from depression (Chen and Marsh, 2013; Lord et al., 2013), affective disturbances (Aminian and Strafella, 2013), and cognitive impairment (Hanganu et al., 2013; Kudlicka et al., 2013; Kulisevsky et al., 2013). Supplementation with the DA precursor L-DOPA is the most effective treatment for the motor symptoms associated with PD (Poskanzer, 1969; Worth, 2013).

Drug Abuse and Addiction

The DA system is also implicated in drug abuse and addiction (Kalivas and Volkow, 2005). A large body of research implicates dopaminergic (Comings et al., 1994; Noble et al., 2000; Gorwood et al., 2012) and other (Wang et al., 2012) genes with addiction. Furthermore, psychostimulant drugs such as cocaine (Giros et al., 1993), amphetamine (Sulzer et al., 1993), and methamphetamine (Fleckenstein et al., 1997) act directly on the DAT. Although it appears straightforward, drugs of abuse also elicit circuit-level changes in the brain (Hanlon and Canterberry, 2012;

Jentsch and Pennington, 2013; Keramati and Gutkin, 2013) and form an exquisitely complex disorder.

Schizophrenia and Bipolar Disorder

Dopaminergic signaling is also considered a primary factor underlying affective disorders such as schizophrenia and bipolar disorder (Diehl and Gershon, 1992; Manji and Lenox, 2000; Jones and Craddock, 2001; Ross et al., 2006; Eyles et al., 2012; Kuepper et al., 2012). Numerous genetic linkage and association studies have connected dopamine-related genes to these disorders (Lewis et al., 2003; Seguardo et al., 2003; Owen et al., 2004). In addition, a variant of COMT, Val158Met, has been associated with schizophrenia (Kunugu et al., 1997; Nolan et al., 2004; Ohnishi et al., 2006), though further studies did not observe any association (Turnbridge et al., 2006; Hosák, 2007; Singh et al., 2012). Interestingly, the DAT Ala559Val variant that is the focus of the research to be described herein was first reported in a patient with bipolar disorder (Horschitz et al., 2005). Lastly, dopamine's role in bipolar disorder and schizophrenia is supported by the fact that antipsychotic drugs used to treat the disorders primarily target D2 receptors (Boyd and Mailman, 2012; Ginovart and Kapur, 2012). Early studies observed a correlation between antipsychotic efficacy and drug affinity for D2 receptors (Seeman and Lee, 1975; Creese et al., 1976), but not other DA receptor subtypes (Seeman, 1987). Further research revealed that antipsychotics were most effective when greater than 65% of striatal D2 receptors are blocked, suggesting that antipsychotic effects are driven by D2 antagonism (Farde et al., 1992; Kapur et al., 2000). Clearly, these

few examples support the role of dopaminergic dysfunction in bipolar disorder and schizophrenia, but are a mere peek into a very active research field.

Dopamine Transporter Deficiency Syndrome (DTDS)

Loss of DAT function leads to a severe clinical phenotype, as well. Though rare, humans with two loss-of-function DAT alleles, DTDS, develop a complex movement disorder characterized by infantile hyperkinesia that evolved into generalized dystonia and parkinsonian symptomatology (Kurian et al., 2009, 2011). Several missense mutations were identified from these patients and deficits in DA transport were attributed to deficits in DAT expression, DA affinity for DAT, or glycosylation and subsequent trafficking to the plasma membrane. These DAT variants may prove useful for future studies of DAT structure-function relationships or design of new neuromodulatory therapeutic strategies.

ADHD

Alterations in DA signaling and DAT function have also been associated with attention deficit hyperactivity disorder (ADHD). ADHD is the most commonly diagnosed neuropsychiatric disorder, affecting an estimated 4-12% of school-age children (Biederman and Faraone, 2005; Polanczyk et al., 2007; Willcutt, 2012). Adult ADHD is also fairly common, estimated at 4-5% of adults (Kessler et al., 2006; Fayyad et al., 2007; de Graaf et al., 2008), though more recent studies suggest rates of adult ADHD may actually be greater than 10% (Garnier-Dykstra et al., 2010; Cahill et al., 2012). The disorder is characterized by motor hyperactivity, impulsivity, and/or inattention (American Psychiatric Association, 1994). There are no biomarkers for ADHD, so diagnosis is based on clinical observation as well as

parent and teacher reports (Wolraich et al., 2003; Visser et al., 2013; Wolraich et al., 2013). Also of note, ADHD diagnoses exhibit an approximately 3:1 male:female bias (Gaub and Carlson, 1997; Getahun et al., 2013a). It is unclear if this sex bias arises from reinforcement of behavioral patterns or sex-linked biological factors underlying the disorder. Interestingly, rates of ADHD diagnosis span different cultural groups, as studies of populations in Africa (Bakare, 2012), Asia (Chien et al., 2012), and Europe (Bianchini et al., 2013; Ezpeleta et al., 2013) report similar prevalence.

Many lines of evidence implicate DAT and DA receptors in ADHD. Previous studies have demonstrated an association between ADHD and genes supporting DA signaling, including D1 (Bobb et al., 2005; Ribases et al., 2012), D2 (Nyman et al., 2007), D4 (Roman et al., 2001; Bidwell et al., 2011) and D5 (Manor et al., 2004) receptors, though alterations of receptor function in ADHD remain unclear. Several studies have also observed association between ADHD and the DAT gene (Cook et al., 1995; Gill et al., 1997; Waldman et al., 1998; Barr et al., 2001). Furthermore, positron emission tomography (PET) methods have afforded a direct inspection of DAT levels in the brain of human ADHD subjects (Varrone and Halldin, 2010; Zimmer, 2009). However, the findings with this approach have been mixed, possibly due to prior drug exposure in some studies (Fusar-Poli et al, 2012). Thus, studies have observed increased DAT binding in the basal ganglia of both children (Cheon et al., 2005) and adults (Dougherty et al., 1999; Dresel et al., 2000; Krause et al., 2000), although others have seen no change (van Dyck et al., 2002) or decreased DAT density in ADHD (Volkow et al., 2007).

The clear link between DAT and ADHD led researchers to use DAT as a candidate gene for further study. Five studies have examined the coding sequence and splice junctions of the human dopamine transporter (hDAT) for polymorphisms (Cargill et al., 1999; Grünhage et al., 2000; Vandenbergh et al., 2000; Mazei-Robison et al., 2005; Mergy MA and Blakely RD, unpublished data). Six non-synonymous variants were identified between the four studies: Val24Met (V24M), Val55Ala (V55A), Arg237Gln (R237Q), Val382Ala (V382A), Ala559Val (A559V), and Glu602Gly (E602G). In addition, in a study of inattentive characteristics in children diagnosed with ADHD (Bellgrove et al., 2009), a seventh coding variant, Arg615Cys (R615C), was identified (Sakrikar et al., 2012). Screening of proband DNAs for new DAT coding variants is still ongoing in various diseases including bipolar disorder and autism with comorbid ADHD (Rommelse et al., 2011; Davis and Kollins, 2012; Mahajan et al., 2012).

Disease-associated DAT coding variants are listed in Table 1 and their locations on the DAT protein are shown in Figure 5. Furthermore, recent advances in whole-exome and whole-genome sequencing (1000 Genomes Project Consortium, 2010) have resulted in a plethora of new DAT coding variants being deposited into databases. Since most exome screening projects are merely searching for genetic variation, the sample population is not associated with any particular disorder. DAT coding variants without known disease association are as follows (D = found in dbSNP database, G = found by 1000 Genomes, N = found in National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project): K3N (D, G, N), M11I (D, G, N), M11V (D, G), S12P (D, G, N), V14M (D, G), A16T (G), P17L (G,N), E20(stop) (D),

Variant	Disease Association	Reference
Val24Met	ADHD	M. Mergy, unpublished findings
Thr48Ser	Autism	Liu et al., 2013; Neale et al., 2013
Arg51Trp	Autism	Liu et al., 2013; Neale et al., 2013
Val55Ala	?	Vandenbergh et al., 2000
Val158Phe	DTDS	Kurian et al., 2011
Leu167Phe	ADHD	M. Mazei-Robison, unpublished findings
Leu224Pro	DTDS	Kurian et al., 2011
Arg237Gln	?	Cargill et al., 1999
Gly327Arg	DTDS	Kurian et al., 2011
Ala346Val	Autism	Liu et al., 2013; Neale et al., 2013
Thr356Met	Autism	Liu et al., 2013; Neale et al., 2013
Leu368Gln	DTDS	Kurian et al., 2009, 2011
Val382Ala	?	Vandenbergh et al., 2000
Pro395Leu	DTDS	Kurian et al., 2009, 2011
Val464Ile	Autism	Liu et al., 2013; Neale et al., 2013
Arg521Trp	DTDS	Kurian et al., 2011
Pro529Leu	DTDS	Kurian et al., 2011
Pro554Leu	DTDS	Kurian et al., 2011
Ala559Val	Bipolar, ADHD	Grunhage et al., 2000; Mazei-Robison et al., 2005
Glu602Gly	Bipolar	Grunhage et al., 2000
Arg615Cys	ADHD	Sakrikar et al., 2012

Table 1. Disease-associated DAT coding variants. ADHD = attention deficit hyperactivity disorder; DTDS = dopamine transporter deficiency syndrome.

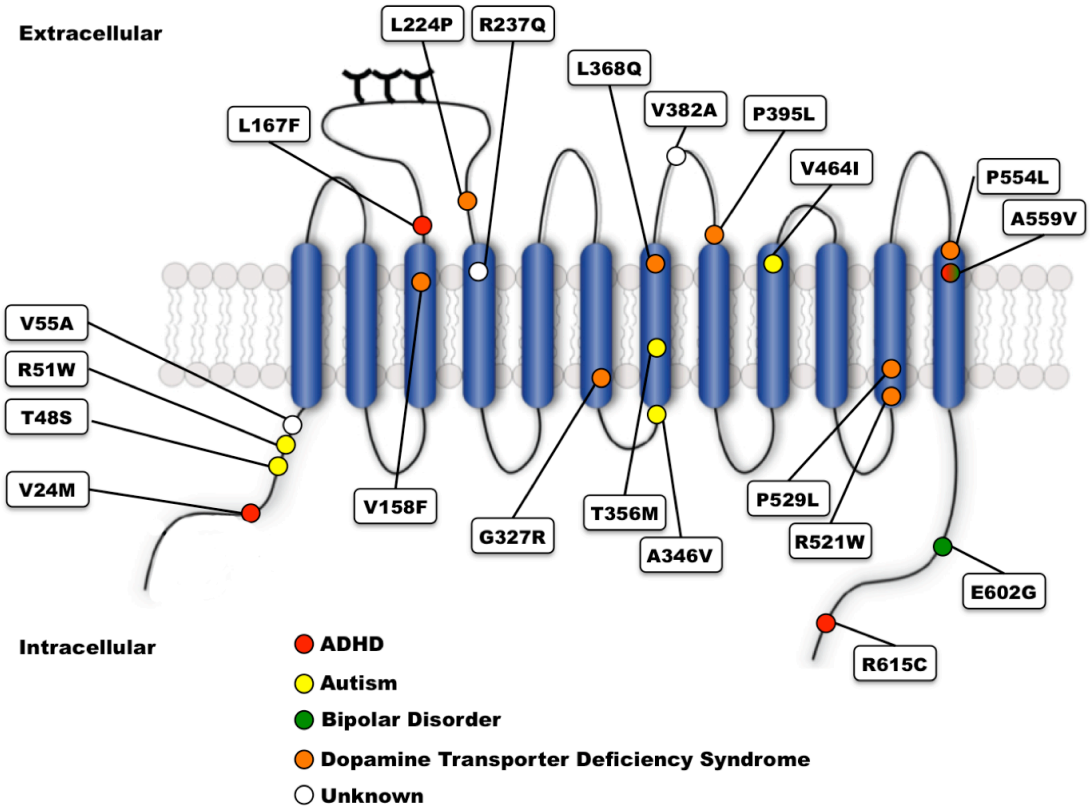

Figure 5. Location of disease-associated hDAT variants. ADHD-associated variants are marked with red circles, ASD-associated variants with yellow circles, bipolar disorder-associated variants with green circles, DAT deficiency syndrome-associated variants with orange circles, and variants without disease association with white circles.

E20V (G, N), I32M (G, N), V24A (G), G39R (G), L42F (D, G), P50L (D, G, N), S53R (D, G, N), V73I (D, G, N), L104I (D, G, N), G121S (G), V131I (D, G), L138P (G), L138R (G), A161T (G), A163V (G), A192T (D, G), S198T (G), S202L (D, G), S202W (D, G, N), G209R (G, N), V221M (D, G, N), R237W (G, N), V245A (D, G), I268V (G, N), T271N (G), V275L (D, G, N), L281P (G), G289R (G), G293S (G), V300I (D, G, N), E307K (D, G), A308V (G), A314V (G), D345G (G, N), A346T (G, N), A346V (D, G, N), F362L (G), L368Q (G), Q373R (D, G, N), G380R (G, N), P395L (G) G433R (G), D436N (G), R445G (G), A455V (G, N), V464I (D, G, N), V471I (D, G), I490V (D, G, N), V501A (G, N), Q509H (D, G), R515W (G), S517T (G), G538A (D), V538I (G, N), R544S (G), P545T (G, N), H547Q (D, N), A559T (D, G), A576E (G), K579R (G, N), R588Q (G, N), G607W (G), R610H (D, G), T613M (G, N), K619N (D, G, N).

Of the coding variants that have been characterized in any way, V24M, V55A, R237Q, and E602G do not demonstrate any changes in transporter expression or dopamine transport capacity. V382A hDAT, however, shows reduced protein expression, reduced capacity for both DA and NE transport, and a loss of phorbol ester (PMA)-induced trafficking (Mazei-Robison and Blakely, 2005). The authors suggest that, upon PMA treatment, V382A hDAT stabilizes an inactive conformation in the plasma membrane but is not appropriately internalized.

The A559V hDAT variant also displays a unique phenotype. *In vitro* characterization of DAT Val559 (note: the genetic variant is referred to as A559V hDAT, however the mutant DAT protein is named based on the amino acid at position 559; Ala559 refers to WT DAT and Val559 refers to the mutant DAT encoded by the mutant A559V DAT gene) revealed normal levels of both total and

cell-surface DAT expression, as well as normal DA uptake in a heterologous expression system (Mazei-Robison et al., 2008). However, amperometric studies revealed that DAT Val559 in transfected cells exhibited a spontaneous, DAT-mediated outward "leak" of cytoplasmic DA under basal conditions, a phenomenon termed anomalous DA efflux (ADE), as well as elevated voltage-dependent DA efflux. Furthermore, the ability of AMPH to induce DAT-mediated efflux (Sulzer et al., 2005) was lost in DAT Val559-transfected cells. Instead, AMPH acts like the competitive DAT antagonists methylphenidate and cocaine to block ADE. Further research has demonstrated that DAT Val559 displays increased channel activity and that ADE is dependent on D2 receptor signaling and increased phosphorylation of the DAT N-terminus (Bowton et al., 2010).

The most recently identified hDAT coding variant, R615C, also displays functional alterations. Although R615C hDAT expresses at WT levels, surface R615C DAT is reduced by ~50% and the dopamine transport V_{max} is significantly reduced (Sakirkar et al., 2012). Despite reduced surface expression, upon treatment with AMPH, R615C supports similar amounts of DA efflux as the WT transporter, a finding attributed to a loss of AMPH-induced transporter internalization observed with WT DAT (Saunders et al., 2000; Chen et al., 2010). Sakrikar and colleagues went on to demonstrate that internalization and recycling rates for DAT Cys615 are significantly elevated. Furthermore, DAT Cys615 interaction with calcium/calmodulin-dependent protein kinase II (CaMKII) is increased, leading to increased CaMKII-mediated basal phosphorylation of DAT's N-terminal tail. Since DAT association with membrane microdomains is required for PKC-mediated

internalization and CaMKII-mediated DA efflux (Cremona et al., 2011), the authors examined DAT Cys615 localization to membrane rafts and found a reduced association with raft markers. These findings suggest that DAT localization and function within membrane microdomains may provide another mechanism of DAT dysfunction in ADHD.

Although fascinating from a mechanistic perspective, *in vitro* studies of ADHD-associated DAT variants in transfected cells have obvious limitations with respect to perturbations of DA signaling in the intact CNS. Animal models of rare DAT variants are the next step for understanding DAT-mediated changes in DA signaling *in vivo*.

Animal Models of ADHD

Animal models are a valuable tool for studying disease mechanisms. Rodent models of ADHD are typically focused on recapitulating the symptoms seen in human subjects with ADHD – locomotor hyperactivity, impulsivity, and inattention – and the response to psychostimulant therapy (amphetamine or methylphenidate). Here, I will review animal models of ADHD based on manipulations of candidate genes to generate transgenic animals, lesion-based approaches that target brain regions or circuits relevant to ADHD, developmental insult models, and models bred to select for ADHD-like phenotypes. The models and findings addressed below are summarized in Table 2.

Lesion/Insult Models			
	Hyperactivity	**Inattention**	**AMPH/MPH Response**
6-OHDA (rat)	+	ND	+
Prenatal nicotine exposure (mouse)	+	ND	+
Prenatal BrdU exposure (rat)	+	ND	-
Prenatal ethanol exposure (rat)	ND	+	ND
Neonatal hypoxia (rat)	+	-	ND
Neonatal PCB exposure (rat)	+	ND	ND
Neonatal BPA exposure (rat)	+	ND	-
Neonatal X-ray exposure (rat)	ND	+	+
Neonatal chronic GBR 12909 (rat)	+	+	-
Genetic KOs			
	Hyperactivity	**Inattention**	**AMPH/MPH Response**
DAT (mouse)	+	+	-
Steroid sulfatase (mouse)	+	-	ND
GIT1 (mouse)	+	+	+
NK1 receptor (mouse)	+	+	+
SNAP-25/coloboma (mouse)	+	ND	+
p35 (mouse)	+	ND	+
Guanylyl cyclase C (mouse)	+	ND	+
Other Genetic Manipulations			
	Hyperactivity	**Inattention**	**AMPH/MPH Response**
Grin1 (missense mutant mouse)	+	ND	+
SynCAM1 dominant negative	+	ND	+
Cocaine-insensitive DAT	+	ND	+
DAT knock-down	+	-	+
CK1-delta overexpression	+	ND	+
Thyroid hormone receptor beta-PV	+	+	+
Selective Breeding Models			
	Hyperactivity	**Inattention**	**AMPH/MPH Response**
SHR	+	+	+
Genetically Hypertensive (GH) rat	+	ND	ND
NHE rat	+	+	ND
WKHA rat	+	ND	-
Wig rat	+	ND	-
I/LnJ acallosal mouse	+	+	ND
Developmental Models			
	Hyperactivity	**Inattention**	**AMPH/MPH Response**
Social Isolation	+	+	+
Dirupted habenula development	+	+	+
Maternal separation	+	+	ND

Table 2. Animal models of ADHD. + = phenotype observed in model, - = phenotype absent from model, ND = phenotype not determined for model.

Transgenic Mouse Models

DAT Knockout

Since the DAT gene has been associated with ADHD (Gill et al., 1997; Barr et al., 2001), imaging studies in human subjects with ADHD have suggested alterations in DAT density (Swanson et al., 2007), and the DAT is the primary target for psychostimulant therapies for ADHD, it was a prime candidate for generation of a knockout (KO) mouse model. Perhaps one of the most widely studied models of ADHD, the DAT KO mouse (Giros et al., 1996) displays a fivefold elevation in extracellular DA concentrations due to the inability to recapture released DA from the synapse. Subsequent characterization demonstrated that DAT KO mice have a greater than 95% reduction in total tissue levels of DA, greater than 90% reduction in tyrosine hydroxylase (TH) expression (Jones et al., 1998), and significant reductions in D2R mRNA levels (~50%) (Giros et al., 1996) and protein expression (~55%) (Jones et al., 1999). Interestingly, these changes are accompanied by a reduction in striatal volume and the total number of neurons (Cyr et al., 2005) as well as a loss of dendritic spines in proximal portions of dendrites (Berlanga et al., 2011). Taken together, these finding demonstrate that deletion of DAT results in significant structural and biochemical changes that alter DA signaling.

The biochemical changes in the DAT KO mouse manifest in several behavioral alterations, most notably extreme (5-6 fold increase) locomotor hyperactivity in a novel environment (Giros et al., 1996). Interestingly, although AMPH's ability to cause DA efflux is lost in striatal slices from DAT KO mice (Giros et al., 1996), these mice still show a reduction in locomotor activity following AMPH

treatment (Gainetdinov et al., 1999), an effect attributed to serotonergic signaling. Furthermore, DAT KO mice display increased impulsivity as measured by a greater number of head dips over an unprotected edge of the elevated plus maze (Carpenter et al., 2012) and a reduced cliff avoidance reaction (Yamashita et al., 2013). DAT KOs also have impaired cognitive function as assessed in the eight-arm radial maze (Gainetdinov et al., 1999) and the Morris water maze (Morice et al., 2007), impaired social interactions with other mice (Rodriguiz et al., 2004), increases in stereotyped and perseverative behaviors (Gainetdinov et al., 1999; Rodriguiz et al., 2004), and deficits in sensorimotor gating as determined by a reduction of prepulse inhibition of the acoustic startle response (Yamashita et al., 2006; Arime et al., 2012).

Due to the extreme behavioral phenotypes of a complete deletion of DAT, it has been suggested that the DAT KO is not a model for ADHD, but perhaps a better model for schizophrenia (Spielewoy et al., 2000; Wong et al., 2012). Due to poor performance in the forced swim test, the DAT KO mouse has even been suggested as a model for depression (Perona et al., 2008). A major caveat of the DAT KO mouse, however, is that homozygous loss-of-function of DAT (DTDS) in humans results in a complex motor phenotype characterized by infantile dystonia and Parkinsonian symptoms (Kurian et al., 2011). Despite displaying ADHD-like symptoms and responding to psychostimulant medications, the DAT KO mouse most closely models the symptoms seen in humans without a functional DAT.

DAT Knockdown

Since the DAT KO mouse is such an extreme perturbation to the DA system, efforts were made to create a more moderate disruption in DAT function. This led

to the creation of the DAT knockdown (KD) mouse that has an approximately 90% reduction in DAT expression (Zhuang et al., 2001). The restoration of just a 10% of DAT expression greatly attenuated many of the features observed in DAT KO mice. For instance, extracellular DA is elevated two-fold, spontaneous locomotor activity is between DAT KO and WT levels, and psychostimulant treatment improves locomotor hyperactivity. A significant DAT reduction, however, is not a key feature of ADHD, as evidenced by several brain imaging studies showing increased (Spencer et al., 2007) or unchanged DAT density (van Dyck et al, 2002) in ADHD subjects. The DAT KD has been of limited utility for studying the disorder, although some groups have utilized the DAT KD as a model for bipolar mania (Ralph-Williams et al., 2003; Young et al., 2011).

GIT1 Knockout

Another putative model for ADHD is the G protein-coupled receptor (GPCR) kinase-interacting protein 1 (GIT1) KO mouse. An intronic single nucleotide polymorphism (SNP) that causes reduced GIT1 gene expression was associated with ADHD in a Korean population (Won et al., 2011). GIT1 KO mice display a two-fold increase in spontaneous locomotor activity as well as deficits in recognition memory (novel object recognition paradigm) and spatial learning and memory (Morris water maze). GIT1 KO mice display similar alterations to electroencephalogram (EEG) rhythms in the theta range (4-8 Hz) as human subjects with ADHD (Barry et al., 2003). The aforementioned characteristics were all reversed by AMPH treatment (Won et al., 2011), providing predictive as well as face validity to this model.

Due to GIT1's role in regulating G protein-coupled receptors (reviewed in Hoefen and Berk, 2006) and the association of DA receptors (all GPCRs) to ADHD (Gill et al., 1997; Barr et al., 2001; Manor et al., 2004; Bobb et al., 2005; Nyman et al., 2007; Bidwell et al., 2011), Won and colleagues investigated several signaling pathways to see if neurotransmission was altered in GIT1 KO mice. They found that excitatory transmission was normal, but that presynaptic mechanisms regulating inhibitory transmission were altered in GIT1 KO mice. These findings suggest a possible imbalance between excitation and inhibition may be contributing to the ADHD-like symptoms seen in GIT1 KO mice. Such changes in excitatory/inhibitory balance are an interesting departure from the dopamine theory of ADHD. Further studies are needed to understand the precise mechanisms underlying the ADHD-like symptoms and how deficits in inhibitory neurotransmission on dopaminergic neurons might modulate the behaviors affected in ADHD.

Neurokinin Receptor 1 Knockout

The substance P-preferring neurokinin receptor (NK1R) KO mouse was first reported to exhibit locomotor hyperactivity (Herpfer et al., 2005), though was not touted as a model for ADHD. More recent research identified four single nucleotide polymorphisms (SNPs) in the TACR1 (human NK1R) gene that are associated with ADHD (Yan et al., 2010), thus generating interest in the NK1R KO mouse as an ADHD model. In addition to hyperactivity, the performance of NK1R KO mice on the 5-choice serial reaction time task (5-CSRTT) reveals inattentive, impulsive, and perseverative behaviors, as well (Yan et al., 2011). Upon treatment with AMPH, hyperlocomotion and perseveration were improved (Yan et al., 2010, 2011), but

impulsive responding was exacerbated (Yan et al., 2011). Furthermore, NK1R KO mice have deficits in AMPH-induced DA efflux in the striatum, but not prefrontal cortex (Yan et al., 2010) and it was speculated that the loss of NK1R on cholinergic interneurons may contribute to altered striatal DA signaling. Although NK1R KO mice exhibit the core behavioral features of ADHD, the lack of improvement in inattentive and impulsive symptoms after AMPH treatment raises questions about the validity of this model.

Steroid Sulfatase Knockout

Steroid sulfatase (STS) is an X-linked gene that is responsible for converting the sulfated steroid hormone dehydroepiandosterone (DHEAS) to its non-sulfated form, DHEA, in the brain (Compagnone et al., 1997), and thus modulating GABAergic signaling (Park-Chung et al., 1999). Furthermore, deletion of the STS gene (Tobias et al., 2001; Doherty et al., 2003) and DHEA/DHEAS levels (Strous et al., 2001) have previously been associated with attention problems, and was thus suggested as a candidate gene for ADHD (Davies et al., 2007). Subsequent work using either pharmacological manipulation or genetic deletion of STS in mice revealed performance deficits on the 5-CSRTT (Davies et al., 2009) and locomotor hyperactivity (Trent et al., 2012). To date, however, the effects of amphetamine or methylphenidate on 5-CSRTT performance or locomotor activity have not been tested. Recently, two SNPs in STS were associated with ADHD risk and inattentive symptoms (Stergiakouli et al., 2011), but the precise mechanisms underlying the connection between STS and ADHD remain to be determined.

p35 Knockout

Cyclin dependent kinase 5 (Cdk5), along with its cofactor p35 (Lew et al., 1994; Tsai et al., 1994), regulates dopaminergic signaling through phosphorylation of DARPP-32 (Bibb et al., 1999). While Cdk5 KO mice are not viable (Ohshima et al., 1996; Gilmore et al., 1998), p35 KO mice do survive, but display severe morphological defects including inversion of cortical lamination and reduced corpus callosum volume (Chae et al., 1997; Kwon and Tsai, 1998). Despite such severe abnormalities, two independent groups found that p35 KO mice display spontaneous locomotor hyperactivity (Drerup et al., 2010; Krapacher et al., 2010). Both amphetamine and methylphenidate elicit a paradoxical calming effect, as observed in ADHD. While p35 KO mice do display a key clinical phenotype and pharmacological responses relevant to ADHD, the major caveat of structural abnormalities still remains, and testing for other features of ADHD such as inattention and impulsivity are needed.

Guanylyl Cyclase C Knockout

Although originally identified as a membrane receptor for the peptides guanyline and uroguanyline in the gut (Currie et al., 1992; Hamra et al., 1993), guanylyl cyclase C (GC-C) was later found to be selectively expressed in DA neurons in the midbrain (Lein et al., 2007). GC-C KO mice display a greater than two-fold increase in locomotor activity as well as attention deficits, both of which are corrected by treatment with a low dose of AMPH (Gong et al., 2011). Furthermore, protein kinase G (PKG) activators can alleviate the locomotor hyperactivity of GC-C KO mice, suggesting another possible therapeutic target for modulating DA signaling

in ADHD. Further research is needed to understand how PKG signaling might impact ADHD-like behavior in other model systems.

Coloboma/SNAP-25 Knockout

The coloboma (Cm) mutation consists of a 2 cM deletion of mouse chromosome 2 that includes the synaptosomal-associated protein 25 kDa (SNAP-25) locus (Hess et al., 1992). Further fine mapping identified the genes for SNAP-25 and brain-specific phospholipase C-β1 within the Cm deletion (Hess et al., 1994). SNAP-25 is widely expressed in neurons (Oyler et al., 1989) and is suggested to have a key role in synaptogenesis and synaptic remodeling (Geddes et al., 1990; Catsicas et al., 1991; Oyler et al., 1991). Cm mice exhibit profound locomotor hyperactivity (Hess et al., 1992), thus making them a possible model for ADHD.

Total levels of DA in Cm mice are not different from WT controls (Jones et al., 2001), but *in vivo* microdialysis studies showed a nearly two-fold increase in basal *extracellular* DA in Cm mice (Fan and Hess, 2007). SNAP-25 plays a key role in vesicular fusion and neurotransmitter release (Bark et al., 1995; Schiavo et al., 1997); Cm mice display a decreased capacity for K^+-induced DA release (Raber et al., 1997). In addition to locomotor hyperactivity, Cm mice display deficits in latent inhibition and are highly impulsive (Bruno et al., 2007), thus manifesting the core clinical features of ADHD. While Cm mice display ADHD-like behaviors, their response to psychostimulant treatment varies: amphetamine decreases locomotion in Cm mice, but methylphenidate enhances locomotor activity (Hess et al., 1996). Subsequent studies suggested that the behavioral characteristics and AMPH response of Cm mice arise from dysregulation of DA D2 receptors (Fan et al., 2010).

Interestingly, transgenic replacement of SNAP-25 in Cm mice will rescue the behavioral deficits observed in these animals (Hess et al., 1996). However, although the Cm mutation is semi-dominant (that is, Cm/Cm homozygotes are not viable so all Cm phenotypes are measured in Cm/+ heterozygotes) (Hess et al., 1994), SNAP-25 heterozygotes do not display any of the behavioral abnormalities seen in Cm mice (Oliver and Davies, 2009). This finding suggests that SNAP-25 is not solely responsible for the behavioral deficits observed in Cm mice. Further studies are needed to define the roles of genes other than SNAP-25 that are disrupted by the Cm mutation, as they may have important roles in mediating the phenotypes of Cm mice.

Thyroid Receptor βPV

Thyroid receptor (TR) βPV mutant mice were originally developed to understand the effects of a mutant thyroid hormone receptor from patients with severe resistance to thyroid hormone (RTH) on growth and development (Zhu et al., 1999; Kaneshige et al., 2000). TRβPV mice display locomotor hyperactivity, albeit mild and significantly greater than WT controls only after multiple sessions (Siesser et al., 2005), as well as attention deficits and increased impulsivity (Siesser et al., 2006). In addition, TRβPV mice have elevated striatal DA turnover. The locomotor hyperactivity of TRβPV mice is ameliorated by methylphenidate treatment, as well, but only at high (> 30 mg/kg) doses (Siesser et al., 2005, 2006). Interestingly, human subjects with RTH frequently have comorbid ADHD (Hauser et al., 1993), but it remains unclear how dysfunctional thyroid hormone signaling manifests as ADHD-like symptoms.

Other Genetic Models

In addition to the models described above, several new mouse models for ADHD have been suggested, but have not been well studied. Those models are described below:

Grin1 Knock-in

Furuse and colleagues reported locomotor hyperactivity that was ameliorated by methylphenidate treatment in mice harboring a mutation in Grin 1 (NMDA receptor subunit 1) (Furuse et al., 2010). Further characterization revealed deficits in sensorimotor gating, working memory problems, reduced anxiety-like behaviors, and deficient contextual and cued fear learning (Umemori et al., 2013). The behavioral changes observed are not exclusive to ADHD, thus the Grin1 mutant mouse may prove a useful model for other neuropsychiatric disorders such as schizophrenia and bipolar disorder, as well.

SynCAM1 Dominant Negative

SynCAM1 is a synaptic adhesive protein that plays a significant role in astrocyte-to-astrocyte and astrocyte-to-neuron adhesive communication (Sandau et al., 2011b) and is important for female sexual development (Sandau et al., 2011a). In order to understand the role of SynCAM1 function in astrocytes, Sandau and colleagues generated transgenic mice that express an astrocyte-specific dominant negative SynCAM1 (DNSynCAM1) (Sandau et al., 2012). During the course of studies relating to female reproductive function, the DNSynCAM1 mice displayed high levels of locomotor activity and impulsive behaviors, reminiscent of ADHD. Formal characterization revealed elevated locomotor activity and disruption of

diurnal patterns of locomotor activity and reduced anxiety. Furthermore, AMPH treatment normalized locomotor hyperactivity. Since DNSynCAM1 was specific to astrocytes, these findings suggest that astrocytic signaling may contribute to ADHD phenotypes.

Casein Kinase 1 Overexpression

Casein kinase 1 δ (CK1δ) is a brain-enriched protein kinase that has been associated with bipolar disorder (Matsunaga et al., 2012) and implicated in psychostimulant-induced behaviors (Li et al., 2011) via phosphorylation of DARPP-32, a key integrator of dopaminergic and glutamatergic signals (Desdouits et al., 1995; Greengard et al., 1998; Le Novère et al., 2008). In order to understand the importance of CK1δ signaling *in vivo*, Zhou and colleagues generated a mouse with inducible overexpression of CKI1δ in the striatum, cortex, and hippocampus (Zhou et al., 2010). These mice display locomotor hyperactivity that is reduced by either AMPH or MPH treatment, as well as reduced anxiety. Interestingly, CK1δ overexpression did not alter glutamatergic signaling, but did result in significant reductions in D1R and D2R expression. These findings suggest that CK1δ may play a role in regulating dopaminergic signaling that could contribute to ADHD.

Selectively Bred Models

Spontaneously Hypertensive Rat

One of the most frequently studied ADHD models is the spontaneously hypertensive rat (SHR). Originally derived from selective breeding of Wistar rats (Okamoto and Aoki, 1963) and used to study essential hypertension (Yamori, 1977), researchers soon recognized the spontaneous hyperactivity of SHRs (McCarty and

Kopin, 1979). It was then demonstrated that SHRs display the paradoxical locomotor response to AMPH (Myers et al., 1982) and MPH (Wultz et al., 1990) and the SHR was suggested as a model for ADHD. Further studies have demonstrated increased impulsivity (Sagvolden et al., 1992b; Fox et al., 2008; Sagvolden 2011), impaired attention (Sagvolden and Xu, 2008; Sagvolden 2011), and cognitive deficits (Clements and Wainwright, 2006; Kantak et al., 2008; Brackney et al., 2012). Accordingly, the SHR has been lauded as the best validated model of ADHD (Sagvolden, 2011).

While the SHR displays a number of ADHD-like characteristics, these traits are not all improved by psychostimulant treatment. For instance, psychostimulants improve SHR performance on attention tasks in some studies (Kantak et al., 2008; Sagvolden et al., 2011) but not in others (van den Bergh et al., 2006; Ferguson et al., 2007). Similarly, AMPH typically reduces locomotor hyperactivity (Sagvolden and Xu, 2008), but has been shown to potentiate locomotor behavior in some studies (Calzavara et al., 2011). Finally, SHRs display deficits in sensorimotor gating, as measured by prepulse inhibition (Li et al., 2007; Levin et al., 2011), but humans with ADHD typically do not (Feifel et al., 2009; Hanlon et al., 2009; Holstein et al., 2011), unless the task requires sustained attention (Hawk et al., 2003). The PPI deficits observed in SHRs are perhaps a better model for schizophrenia than ADHD (Levin et al., 2011).

A major caveat to using the SHR to model ADHD is the lack of understanding as to the mechanisms underlying the observed behavior changes. Li and colleagues demonstrated a reduction of D4 receptor expression in the prefrontal cortex (Li et

al., 2007), an interesting finding in light of the association of the D4 receptor gene and ADHD (Bidwell et al., 2011). In addition, SHRs have elevated DAT expression (Roessner et al., 2010), while human imaging studies suggest a reduction in DAT density in ADHD subjects (Volkow et al., 2009). The SHR is certainly a valuable tool for understanding how perturbations in DA signaling manifest behaviorally, but perhaps is not an ideal model for ADHD.

Genetically Hypertensive Rat

In an effort to demonstrate that the hyperactivity observed in SHRs is not an artifact of background strain (Wistar-Kyoto, WKY), Wickens and colleagues report the independently generated New Zealand genetically hypertensive (GH) rat (Wickens et al., 2004). GH rats do not display basal locomotor hyperactivity, but have similar levels of lever pressing when tested on a fixed-interval schedule to SHRs. This finding suggests that GH rats, like SHRs, are abnormally sensitive to reward. Further research demonstrated that both GH rats and SHRs have a strong preference for immediate reinforcement (Sutherland et al., 2009), a finding consistent with studies of humans with ADHD (Tripp and Alsop, 2001). Thus, GH rats may be a valuable complementary model to SHRs for studying delayed reinforcement deficits in ADHD.

Naples High Excitability Rat

In order to understand hippocampal function, selective breeding for high and low arousal in response to novelty was undertaken and resulted in the creation of the Naples High Excitability (NHE) and Naples Low Excitability (NLE) rat strains (Cerbone et al., 1993). NHE rats display increased locomotor activity, deficits in

unreinforced learning, and impaired attention (Cerbone et al., 1993; Viggiano et al., 2002a, b). Furthermore, NHE rats have larger dopaminergic neurons and higher TH expression in the VTA without any changes in the substantia nigra (Viggiano et al., 2002a), suggesting an imbalance between nigrostriatal and mesolimbic DA circuits. While the NHE rat displays some characteristics of ADHD as well as underlying changes in the brain, it has not proven to be a very productive model for ADHD.

Wistar-Kyoto Hyperactive Rat

In order to separate hypertensive and hyperactive traits, SHRs were bred with their progenitor Wistar-Kyoto strain, the selectively bred for hypertensive or hyperactive phenotypes, resulting in Wistar-Kyoto Hyperactive (WKHA) and Wistar-Kyoto Hypertensive (WKHT) rats (Hendley and Ohlsson, 1991). WKHA rats display elevated hyperactivity, reduced anxiety behavior, and impaired attentional processing (Hendley and Ohlsson, 1991; Sagvolden et al., 1992a; Courvoisier et al., 1996; Chess et al., 2005). Subsequent analysis identified a quantitative trait locus on rat chromosome 8, linking locomotor hyperactivity to a chromosomal region containing genes for the α3 nicotinic acetylcholine receptor and serotonin 5-HT1B receptor genes (Moisan et al., 1996). The genes are not typically considered candidates for influencing hyperactivity, however, it was shown that an antagonist of the 5-HT1B receptor can block locomotor hyperactivity induced by a 5-HT1B agonist, but not locomotor hyperactivity induced by AMPH treatment (Chaouloff et al., 1999). These findings suggest that serotonergic signaling can contribute to hyperactivity in WKHA rats, but it is unclear if serotonin receptors are involved in

the tonic hyperactivity of these animals. It was later demonstrated the hyperactivity of WKHA rats is not improved upon methylphenidate treatment (Drolet et al., 2002).

Wig Rat

"Wiggling" rats were initially identified as a spontaneously hyperactive sub-strain of the Long-Evans Cinnamon rat, a rat strain that spontaneously develops acute hepatitis and hepatocellular carcinoma (Kamimura et al., 2001). Initial characterization revealed locomotor hyperactivity, impaired working memory, and impulsivity. Furthermore, the hyperactive trait was transmitted in an autosomal recessive pattern that is not linked to the causative gene for hepatitis. Subsequent research confirmed the hyperactive phenotype observed in Wig rats (Masuo et al., 2007), and found an increase in DAT gene expression in the dorsal midbrain, suggesting that abnormal development of dopamine neurons may underlie the locomotor hyperactivity. Furthermore, methamphetamine exacerbated locomotor activity, indicating that Wig rats do not display a paradoxical response to psychostimulants. Most recently, Hirano and colleagues reported several differentially expressed genes and proteins in the brain of the Wig rat (Hirano et al., 2008). Tyrosine hydroxylase was the only dopamine-related gene differentially expressed in Wig rats, but the other genes and proteins identified could be useful targets for future studies into the pathogenesis of ADHD.

Lesion and Insult Models

6-Hydroxydopamine

The compound 6-hydroxydopamine (6-OHDA) is a neurotoxin that targets catecholaminergic neurons, leading to degeneration of nerve terminals and eventual

neuron death (Tranzer and Thoenen, 1968; Uretsky and Iversen, 1969, 1970). Behavioral characterization of 6-OHDA-treated rats revealed locomotor hyperactivity in juvenile, but not adult, animals (Evetts et al., 1970; Lipton et al., 1980; Pappas et al., 1980; Shaywitz et al., 1981), as well as a paradoxical response to amphetamine, methylphenidate, and atomoxetine (Shaywitz et al., 1976a; Wool et al., 1987; Luthman et al., 1989; Davids et al., 2002b; Moran-Gates et al., 2005). Furthermore, even though 6-OHDA affects both noradrenergic and dopaminergic neurons, it was shown that loss of DA signaling after 6-OHDA lesion was responsible for the behavioral defects observed in these animals (Shaywitz et al., 1984). As such, 6-OHDA-treated juvenile rats were put forth as a model for ADHD (Shaywitz et al., 1976b; Kostrzewa et al., 1994) and lauded for both face and predictive validity (Sagvolden et al., 2005; van der Kooij and Glennon, 2007).

Subsequent work in both mice and rats has focused on the brain response to 6-OHDA lesion. Masuo and colleagues reported on gene expression changes in the striatum and midbrain, finding increases in striatal glutamate transporter (GluT) and midbrain DAT and D4 dopamine receptor expression (Masuo et al., 2002). Induction of DAT expression was in agreement with other studies showing increased DAT expression in ADHD subjects (Dougherty et al., 1999; Madras et al., 2002). Further studies demonstrated that the D4 receptor is essential for mediating locomotor hyperactivity in 6-OHDA-lesioned mice (Avale et al., 2004a).

Several studies have also investigated alterations in serotonin signaling after 6-OHDA lesions. Davids and colleagues reported that selective serotonin transporter (SERT) inhibitors (SSRIs) reduce the hyperlocomotion of 6-OHDA-

lesioned rats (Davids et al., 2002a), a finding reminiscent of the effect of SSRIs on DAT KO mice (Gainetdinov et al., 1999). In addition, 6-OHDA lesion leads to increased SERT binding in the striatum (Zhang et al., 2002) and induces sprouting of serotonergic axons and increases 5-HT levels in the striatum (Avale et al., 2004b). In light of a growing body of evidence implicating alterations in serotonin signaling to ADHD (Comings et al., 2000; Arnsten, 2006; Sonuga-Barke et al., 2011), this model may continue to be a useful tool.

In spite of the obvious face and predictive validity of 6-OHDA-lesioned rodents, the nature of the insult has limited relevance to ADHD. 6-OHDA lesions lead to degeneration of dopaminergic neurons, a clinical profile similar to Parkinson's disease (reviewed in Poewe and Mahlknecht, 2009). The ADHD-like characteristics of this model are transient (Pappas et al., 1980), and adult animals are perhaps best used as a Parkinson's disease model (Tieu, 2011; Blandini and Armentero, 2012; Blesa et al., 2012).

Prenatal Nicotine

Current studies are not clear if cigarette smoking during pregnancy causes cognitive impairments such as ADHD. Many studies find a significant effect of cigarette smoking (Milberger et al., 1996, 1998; Ernst et al., 2001; Fried and Watkinson, 2001), but some see no such effect (Thapar et al., 2009; Obel et al., 2011). While cigarette smoke contains many chemical compounds, evidence suggests that the effect of nicotine on the developing brain causes future behavioral problems (Slotkin et al., 1987; Navarro et al., 1989). Recent research in both mice (Zhu et al., 2012) and rats (Schneider et al., 2012) finds that prenatal exposure to

nicotine does, in fact, result in ADHD-like locomotor hyperactivity, and MPH ameliorates that hyperactivity in mice (Zhu et al., 2012). Nicotine-exposed rats also showed impulsive responses on the 5-choice serial reaction time task, indicating that nicotine also affects inhibitory control.

Polychlorinated Biphenyls

A substantial body of evidence suggests that exposure to polychlorinated biphenyls (PCBs) is associated with development of ADHD in humans (Sagiv et al., 2010, 2012; reviewed in Eubig et al., 2010). Studies in rats have demonstrated that early postnatal exposure to sub-toxic levels of PCBs leads to hyperactive and impulsive behavior similar to that observed in SHRs (Holene et al., 1998). Further studies examined changes in gene expression following PCB exposure and identified a number of potential target genes underlying the ADHD-like behavioral induced by PCBs (DasBanerjee et al., 2008; Sazonova et al., 2011). Such findings reiterate the concept that environment has a direct impact on gene expression and function and that the combination of genes and environment contribute to disease phenotypes.

Bisphenol A

Early postnatal exposure to the endocrine disruptor and environmental txin bisphenol A (BPA) has been suggested as a model for ADHD since BPA-treated rats dose-dependently develop locomotor hyperactivity, particularly during the dark phase of the light/dark cycle (Masuo et al., 2004). Subsequent studies confirmed that BPA exposure does indeed cause locomotor hyperactivity (Kiguchi et al., 2007, 2008), but that the hyperactivity is not improved by treatment with methylphenidate. In fact, BPA-treated mice displayed dose-dependent increases in

MPH-induced locomotor activity. More recently, Zhou and colleagues report that early postnatal BPA treatment leads to functional alterations in the basolateral amygdala (BLA) (Zhou et al., 2011). It appeared that BPA treatment causes disinhibition of GABA signaling and enhancement of dopaminergic signaling, but that application of D1 receptor or NMDA receptor antagonist or GABA-A receptor agonist in the BLA improve hyperactive and inattentive symptoms. Although such signaling changes are not typically considered for ADHD, they may provide interesting avenues for future study.

BrdU

5-bromo-2'-deoxyuridine (BrdU) is a nucleotide analog that is incorporated into DNA during cell division and is known to affect neurogenesis and cellular differentiation (Biggers et al., 1987; Kolb et al., 1999; Kuwagata et al., 2001). Rats exposed prenatally to BrdU are similar to 6-OHDA lesioned animals (Avale et al., 2004b) in that they display locomotor hyperactivity, decreased DA levels, and increased 5-HT levels (Kuwagata et al., 2004). Such changes in the DA and 5-HT systems led to the suggestion of BrdU-treated rats as an ADHD model. Further research observed no changes in DAT density in BrdU-treated rats and that their locomotor hyperactivity was increased by methylphenidate treatment (Muneoka et al., 2006; Orito et al., 2009). Similar to DAT KO mice (Gainetdinov et al. 1999), however, the selective serotonin reuptake inhibitor paroxetine did ameliorate the hyperactivity of BrdU-treated rats (Orito et al., 2009). These findings suggest changes in serotonergic signaling may be an adaptive response to reduced dopaminergic signaling.

Neonatal Hypoxia

Previous studies have suggested that children born prematurely commonly go on to develop ADHD (Szatmari et al., 1990; Botting et al., 1997; Bhutta et al., 2002), and recent evidence found an association between ischemic-hypoxic conditions at birth and ADHD, particularly in preterm births (Getahun et al., 2013b). Efforts to understand the impact of premature birth and hypoxic conditions on brain development in rats have demonstrated that acute neonatal hypoxia leads to locomotor hyperactivity (Speiser et al., 1983; Oorschot et al., 2007) but the impact on attention remains unclear (Decker et al., 2003; Oorschot et al., 2007). Interestingly, rats with neonatal hypoxic injuries display an upregulation of D1 receptor and vesicular monoamine transporter (VMAT), suggesting downregulated DA signaling, but normal levels of DAT and TH (Decker et al., 2003). These findings suggest that neonatal hypoxia induces neurochemical and behavioral changes consistent with ADHD, but further studies are needed to identify the precise lesion that yields such alterations.

Prenatal Ethanol

Several studies have recognized the similarity in behavior of children diagnosed with fetal alcohol syndrome and ADHD (Nanson and Hiscock, 1990; Coles et al., 1997; O'Malley and Nanson, 2002). Prenatal exposure to ethanol also produces ADHD-like characteristics in rats – locomotor hyperactivity, impulsivity, and attention are all negatively affected by ethanol in a dose-dependent fashion (Hausknecht et al., 2005; Kim et al., 2013). Furthermore, prenatal ethanol exposure alters the development of electrical activity in DA neurons of the VTA (Choong and

Shen, 2004). It remains unclear, though, if prenatal ethanol exposure contributes to ADHD, or if ADHD symptoms are merely a component of a larger ethanol-related clinical syndrome.

X-Ray

A single study has examined the effects of early postnatal x-ray exposure on ADHD-like phenotypes in rats (Highfield et al., 1998). Hippocampal irradiation resulted in significant cell death in the hippocampus and produced deficits in patterned single alternation, a form of memory-based learning. Interestingly, treatment with amphetamine improved performance on the learning task. Regardless, it is difficult to separate a learning deficit due to hippocampal damage from poor task performance due to inattention. As such, the x-irradiated rat is only of minor importance for furthering the understanding of ADHD.

Chronic DAT Inhibition

Sub-chronic treatment with the potent DAT inhibitor GBR 12909 and subsequent withdrawal has been suggested as a model for ADHD (Hewitt et al., 2005, 2009). This model is based on the rebound hypothesis – DAT inhibition will initially reduce DAT activity, but upon withdrawal DAT expression will rebound and be over-expressed. After short-term GBR 12909 treatment, rats maintain a small but significant elevation in locomotor activity and poor performance on an object discrimination task, a result attributed to inattention. While this model is based on alteration of DAT function, the behavioral phenotypes observed are transient and may limit the utility of this model system.

Social Isolation

Social isolation has been associated with subsequent development of locomotor hyperactivity (Wilkinson et al., 1994; Võikar et al., 2005), anxiety (Hellemans et al., 2004; Wei et al., 2007), aggression (Miczek and O'Donnell, 1978; Wongwitdecha and Marsden, 1996; Ibi et al., 2008), learning and memory deficits (Lu et al., 2003; Ibi et al., 2008), and impairment of prepulse inhibition of the acoustic startle response (Wilkinson et al., 1994; Day-Wilson et al., 2006). Socially isolated rats treated with methylphenidate showed reduced anxiety-like behaviors, but no change in aggression (Koike et al., 2009). Further study revealed an apparent attention deficit, as socially isolated rats performed poorly on a water finding test (a test of latent learning) (Ouchi et al., 2013). Interestingly, some of the behavioral deficits were improved by treatment with the selective serotonin reuptake inhibitor fluoxetine or antipsychotic clozapine (Koike et al., 2009). The diverse repertoire of behavioral deficiencies and response to a variety of drugs suggests that social isolation may be a model for a very complex psychiatric syndrome and could prove useful for studies relevant to schizophrenia or depression in addition to ADHD.

Maternal Separation

Separation of infant rats from their mothers during the first two weeks of life has been used to study the effects of early life stress because newborn rats are entirely dependent on the mother at this age (Janus, 1987). Maternal separation (MS) has been suggested as a model of ADHD based on subsequent locomotor hyperactivity (von Hoersten et al., 1993; Arnold and Siviy, 2002; Braun et al., 2003;

Colorado et al., 2006; Kwak et al., 2009), however several studies have observed either no change or a decrease in locomotor behavior following MS (Janus, 1987; Stanton et al., 1992; Rhees et al., 2001; Shalev and Kafkafi, 2002; Matthews and Robbins, 2003). Such conflicting findings raise questions regarding the validity of MS rats as an ADHD model.

Recent studies have utilized maternal separation and early weaning (MSEW) in mice to model early life neglect and abuse (ENA) (George et al., 2010; Duque et al., 2012), a complex syndrome that can manifest later in life as ADHD (Ouyang et al., 2008) as well as anxiety, depression, and substance abuse (reviewed in Heim et al., 2010). Such mice do display locomotor hyperactivity, increased anxiety, depressive-like symptoms (George et al., 2010), and subtle deficits related to attention (Carlyle et al., 2012). While perhaps not an ideal model for ADHD, MSEW mice may be a useful tool for studying environmental/experiential influences on complex behaviors.

Interestingly, efforts have been made to study the effects of maternal separation on SHRs. Using this compound model, Womersley and colleagues demonstrated that MS did not alter SHR behavior (i.e. MS does not increase locomotor behavior or induce anxiety) (Womersley et al., 2011). However, MS did alter DAT function in SHRs; the time to clear DA was increased in maternally separated SHRs, an effect attributed to a decrease in either DAT surface expression or affinity for DA. Further study using the MS SHR model revealed neurochemical changes in GABA-A receptor-mediated modulation of norepinephrine release in the hippocampus (Sterley et al., 2013). However, the behavior of MS SHRs is unaffected

(Womersley et al., 2011), leading to the hypothesis that additional adaptive changes likely occur in response to MS (Sterley et al., 2013), thus further highlighting the fact that environmental inputs can interact with genetic factors in some manifestations of ADHD.

Habenula Disruption

The habenula consists of a pair of nuclei – lateral (LHb) and medial (MHb) habenula – that receive inputs primarily from limbic structures and serve to regulate the activity of monoaminergic neurons in several midbrain nuclei (Hikosaka, 2010). Pharmacological inactivation of the lateral habenula has been shown to increase DA release in the prefrontal cortex and nucleus accumbens (Lecourtier et al., 2008), suggesting that the LHb is inhibiting dopaminergic neurotransmission. Rats that received either chemical (Lee and Goto, 2011) or genetic (Kobayashi et al., 2013) lesions of the habenula resulted in locomotor hyperactivity, impulsivity, and inattention (Lee and Goto, 2011; Kobayashi et al., 2013). Furthermore, low dose AMPH treatment attenuated hyperactive and impulsive traits in juvenile rats, and dopamine D3 receptor and DAT expression were reduced in juvenile rats (Lee and Goto, 2011). Taken together, these findings suggest that lesion of the habenula disrupts dopaminergic signaling and results in ADHD-like behaviors. Habenular dysfunction has been associated with schizophrenia (Sandyk, 1992; Caputo et al., 1998; Shepard et al., 2006), but may prove to be an interesting target for future studies related to ADHD.

Specific Aims

The work presented herein seeks to generate and characterize a new construct-valid mouse model for ADHD, the knock-in mouse expressing the ADHD-associated DAT coding variant Val559. To achieve these goals, I pursued the following aims:

1) Generate the DAT Val559 knock-in mouse.
2) Characterize biochemical features of DAT Val559 and validate that *in vitro* findings regarding DAT Val559 (Mazei-Robison et al., 2008) are maintained in the mouse model.
3) Characterize baseline behavioral profile of DAT Val559 mice.
4) Test behavioral effects of amphetamine, a psychostimulant medication commonly used in the treatment of ADHD.
5) Examine DAT Val559 function via *in vivo* and *ex vivo* preparations.

CHAPTER II

GENERATION AND INITIAL CHARACTERIZATION

Introduction

Increasing evidence supports the concept that risk for neuropsychiatric disorders is derived from a complex interplay of genetic variation and environmental factors (Nigg et al., 2010; Wermter et al., 2010). Although difficult to identify, rare variants may be of larger effect than common variants, and, when localized to functionally annotated regions of the genome, such as protein coding sequences, afford the generation of animal models that may be used for mechanistic studies (Boerio et al., 2010; Hassouna et al., 2012; Veenstra-VanderWeele et al., 2012). In addition, as described in Chapter 1, ADHD is the most common neuropsychiatric disorder diagnosed in children, and a preponderance of evidence associates altered DA signaling, in general, and the dopamine transporter, specifically, to the disorder. Therefore, we hypothesized that the ADHD patient population may be enriched for rare, highly penetrant coding variants. To that end, we screened the DAT coding sequence and splice junctions of ADHD probands for rare, non-synonymous variation absent from control samples (Mazei-Robison et al., 2005) and identified DAT A559V in a pair of brothers diagnosed with ADHD (note: the genetic variant is referred to as A559V hDAT, however the mutant DAT protein is named based on the amino acid at position 559; Ala559 refers to WT DAT and Val559 refers to the mutant DAT encoded by the mutant A559V DAT gene). DAT

Val559 exhibits a unique alteration of function, basal DA efflux (Mazei-Robison et al., 2008). We speculate that DAT Val559 may contribute to risk for ADHD, as well as suggest a more general mechanism by which altered DA signaling and the resulting compensatory changes contribute to the disorder.

Since *in vitro* studies of DAT Val559 have obvious limitations regarding applicability to an intact nervous system, we engineered knock-in mice to express DAT Val559 in order to better study the biochemical and behavioral effects this variant *in vivo.* This chapter will describe the generation of the DAT Val559 knock-in mouse and initial characterization of its viability and basic sensorimotor functions (note: experiments were performed in both hetero- and homozygous DAT Val559 animals; hetero- or homozygosity will be noted for all results).

Methods

Production of DAT Val559 Knock-In Mice

Transgenic mice were produced using a linearized DNA construct derived from the genomic DNA sequence of the 129S6/SvEvTac mouse strain to match the strain background of the mouse stem cells (TL-1) that are used for gene targeting experiments. The targeting construct contains 5' and 3' homologous arms from the *Slc6a3* gene, derived from 129S6 mouse genomic DNA, with a mutation in the 5' arm to encode a valine at amino acid position 559 introduced by oligonucleotide-mediated site-directed mutagenesis (Stratagene Quik-Change Mutagenesis Kit, Agilent Technologies, Santa Clara, CA). The targeting construct also included self-excising Cre recombinase and neomycin resistance (Neo^R) cassettes in the 3' intron and a thymidine kinase cassette for negative selection. The DAT Val559 targeting

construct was electroporated into TL-1 embryonic stem cells in the ES/Transgenic Core Facility operated by the Vanderbilt University Center for Stem Cell Biology. Successful homologous recombination was confirmed by Southern blotting for both 5' (576 stem cell clones screened; 12 positive for 5' probe (2.08%)) and 3' probes (6 stem cell clones containing the 5' end of the construct screened; 2 positive for 3' probe (0.34% of all stem cell clones). The presence of the Val559 substitution was subsequently confirmed on both strands via Sanger sequencing (1 clone positive for 5' and 3' probes via Southern blotting and presence of Val559 substitution by sequencing (0.17% of all stem cell clones).

C57BL/6J blastocysts were injected with successfully targeted stem cells and then implanted into pseudo-pregnant females. The resulting chimeric offspring were mated with WT C57BL/6J mice to test for germline transmission of the DAT Val559 allele. Thereafter, genotypes of all mice were determined by PCR (forward primer: CAG CAT GGA AAA AAT CCA TGA A; reverse primer: AGC TAT ATT CAC CAT CAA AAG G; 490 base pair (BP) product = WT, 561 bp product = homo, 490 and 561 bp products = het). The difference in PCR product size between WT and Val559 DAT arises from 71 bp remaining in DAT intronic sequence after the excision of the Cre and Neo^R cassettes.

A single male derived from the testcross was then mated with a WT 129S6 female to generate the initial colony for breeding, resulting in a hybrid background strain consisting of 25% C57BL/6J and 75% 129S6 genomic character. All animals used for subsequent studies were derived from heterozygous breedings at this and subsequent generations. Mice were maintained under standard housing conditions

on a 12-hour light/dark cycle (lights off 1800-0600 for breeding colony; lights off 0200-1400 for behavior colony) with food (irradiated LabDiet 5L0D chow (PMI Nutrition International, St. Louis, MO)) and water available *ad libitum*. In order to assess growth and development, male mice of all three genotypes were weighed weekly from 3-12 weeks of age.

Irwin Screen

Basic sensorimotor function and reflexes of DAT Val559 hetero- and homozygous mice were examined using a modified version of the Irwin test battery (Irwin, 1968). Mice were recorded over 2 consecutive days during the light phase (1600-2000). General physical condition (presence of whiskers, quality of fur (general coat condition, bald patches, piloerection), limb tone, body tone) for individual mice was observed in a clean cage containing corncob bedding.

Motor abilities and reflexes were assayed as follows: *Trunk curl*: each mouse was lifted 12-18 inches by the base of the tail and the presence of nose-to-tail curling of the body assessed. *Forepaw reaching*: each mouse was lifted by the base of the tail and moved horizontally towards a metal wire food bin/cage insert. The degree to which the mouse extended its forepaws as it approached the wire food bin was scored (no reaching = 0, reaching upon nose contact = 1, reaching upon whisker contact = 2, reaching before whisker contact (18-20 mm) = 3, early, vigorous reaching (> 25 mm) = 4). *Inverted screen*: Two to four mice were placed on a metal grid screen (10 x 14 cm) into individual compartments. After placement, the mice were given time to establish a grip on the screen before it was inverted 60 cm above a clean plastic cage containing fresh corncob bedding. The latency to fall was

recorded for up to 1 minute, at which point the mice were removed from the apparatus and returned to the home cage. *Positional passivity*: Each mouse was subjected to sequential handling and the reaction to handling (e.g. struggle to escape) assessed. Mice were first restrained by the tail (score = 0), then gently restrained by the neck (score = 1), restrained by the nape of the neck (scruffed) and held supine (score = 2), and finally restrained by the hind legs (score = 3). Data are represented as the number of mice showing any positional passivity. *Rotarod*: Motor coordination and balance were assessed using an accelerating rotarod apparatus (Ugo Basile, Comerio VA, Italy). Mice were placed on the rotating cylinder (3 cm in diameter) and confined to a segment of the cylinder (approximately 6 cm wide) by gray plastic dividers. The rotational speed of the cylinder increased from 5 to 40 rpm over the 5-minute testing period. The latency at which mice fell off of the rotating cylinder was measured. Each mouse was given a trial on the rotarod before performance was assessed. *Grip strength*: Grip strength was measured using a force gauge attached to a small metal grate (8 x 8 cm). Each mouse was allowed to grip the metal grate with its forepaws, then gently pulled backwards by the base of the tail until it released the grate. Grip strength was recorded with a digital gauge that returned the maximum force during each trial. The average grip strength, given in Newtons, is the average of three trials. *Righting reflex*: Mice were inverted to a supine position in the research's hand. The mouse was then released and the ability of the mouse to right itself was assessed. *Air righting reflex*: Mice were inverted to a supine position in the researcher's hand while being held approximately 30 cm above a cage containing 8-10 cm of clean corncob bedding. The mouse was released

and allowed to drop into the cage and bedding below. The ability of the mouse to right itself while falling was assessed. *Ear twitch*: Mice were gently restrained and the auditory meatus gently touched with the tip of a size 6.1 Touch Test (Von Frey) Sensory Evaluator (Stoelting, Wood Dale, IL). The presence of a reaction (active retraction and/or flick of the ear) was assessed. *Petting escape*: Mice were stroked down the length of the body, starting with a light touch and gradually increased pressure as the researcher approached the mouse's tail. The escape reaction was assessed as follows: no response (score = 0), mild (escape from moderate pressure, score = 1), moderate (escape from light pressure, score = 2), or vigorous (escape from approach, score = 3). Data are presented as the number of mice showing an escape response. Data for all measures of sensorimotor function were analyzed using a one-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

Backcrossing to C57 Background

Since the primary DAT Val559 colony is maintained on a hybrid background, we opted to backcross to a pure C57BL/6J background. In order to accelerate the generation of congenic C57BL/6J-DAT Vall559 mice, we utilized the commercially available MAX-BAX® speed congenic service (Charles River Laboratories International, Inc., Wilmington, MA) (for a review of the basic speed congenic process, see Wong, 2002). Briefly, heterozygous hybrid DAT Val559 male mice were bred with WT C57BL/6J females. Offspring were genotyped and DNA samples from those carrying the DAT Val559 allele were subjected to screening on a microarray-based microsatellite panel. This microsatellite marker panel contains DNAs that are polymorphic between the 129S6 and C57BL/6J strains. Using a fluorescence-based

genotyping assay, the genome is analyzed to determine which of the mice screened possesses the most markers associated with the target (C57BL/6J) strain. The mouse with the highest percentage of C57BL/6J genome was selected for breeding with a WT C57BL/6J and the microsatellite analysis repeated. DAT Val559 mutant mice were backcrossed onto the C57BL6/J background for 4 generations.

Results

Successful Generation of DAT Val559 Mice

To express the DAT Val559 variant from the endogenous mouse *Slc6a3* locus, we performed homologous recombination with a construct bearing an altered codon encoding the Val substitution in exon 13, as well as self-excising loxP-flanked Cre recombinase and neomycin resistance (Neo^R) cassettes in the 3' intron (Fig. 6). Chimeric animals positive for integration of the targeting construct (Fig. 7) were crossed with a WT C57BL/6J mouse to test for germline transmission. A founder animal positive for germline transmission of the Val559 variant and Neo excision (Fig. 8) was selected and bred as described in Methods.

Evaluation of Growth and Sensorimotor Function of DAT Val559 Mice

Before proceeding with full characterization of DAT Val559 mice, we assessed growth and development and basic sensorimotor function. Whereas DAT KO mice show reduced weight gain and survival (Giros et al., 1996), neither hetero- nor homozygous DAT Val559 mice displayed growth abnormalities relative to WT littermates (Fig. 9). Furthermore, general sensorimotor assessment of male mice at 7 weeks of age revealed no genotype differences in general health, reflexes, or basic motor function (Table 3). Although we observe normal growth of DAT Val559 mice,

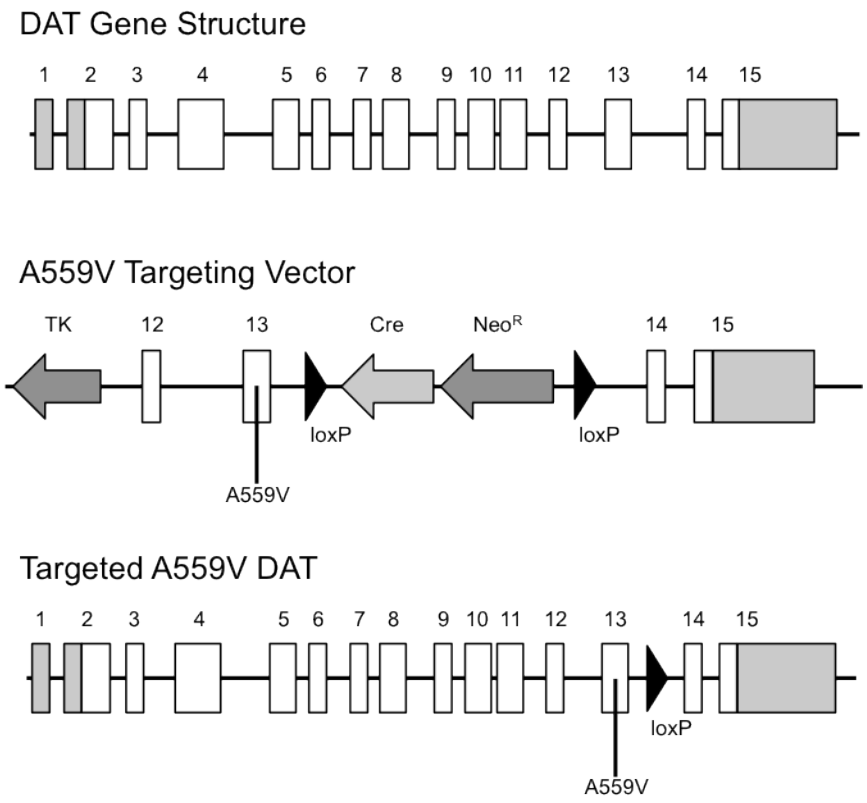

Figure 6. Targeting strategy to generate DAT Val559 knock-in mice. Structure of the DAT gene (top), DAT Val559 targeting construct (middle), and successfully recombined DAT gene (bottom). Coding exons are displayed as white boxes, non-coding exonic sequence appears as a gray box. Following successful recombination, the Cre recombinase and neomycin resistance cassette are excised from the DAT gene.

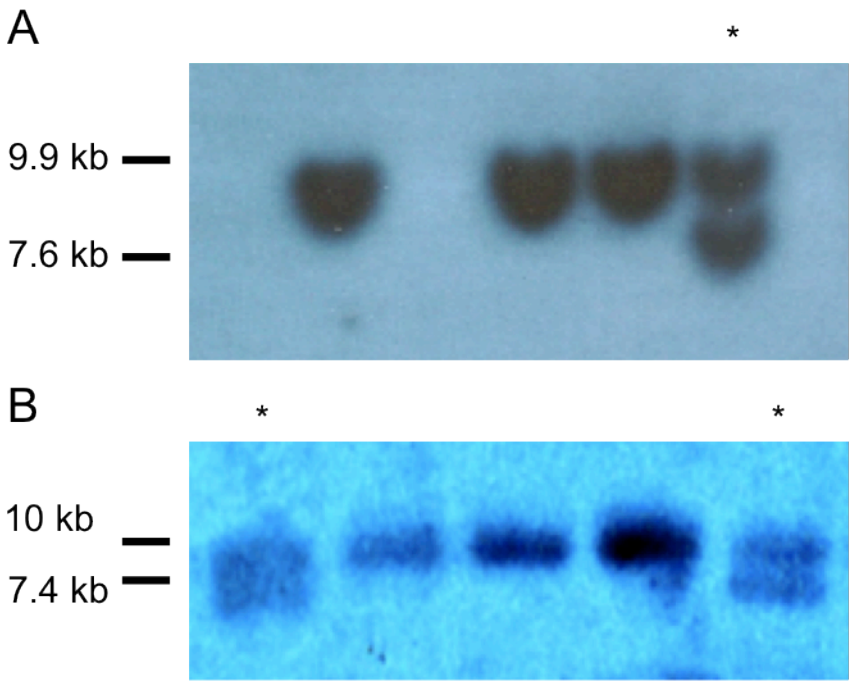

Figure 7. Southern blot analysis to screen for genomic integration of the targeting construct. Probes for the (A) 5' and (B) 3' ends of the targeting construct bind to a larger band for the WT allele and a smaller band for the DAT Val559 allele following the appropriate restriction enzyme digest (EcoRI for 5' probe; EcoRV for 3' probe).

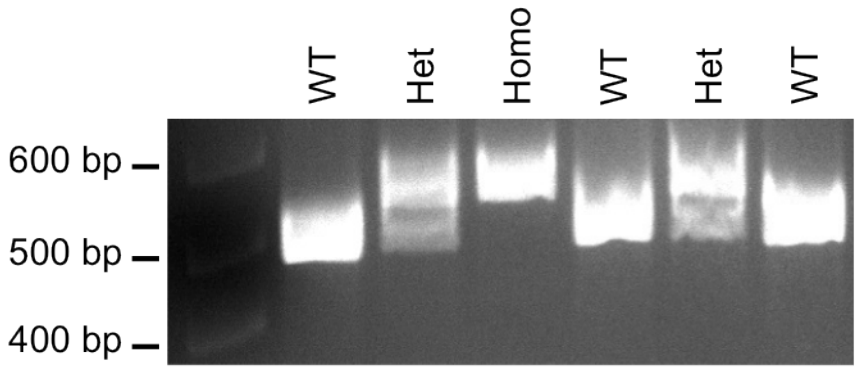

Figure 8. Genotype determination of DAT Val559 mice. Following successful recombination and excision of Cre recombinase and neomycin resistance cassettes, genotypes of all mice can be determined by PCR. The presence of a 490 bp band indicates a WT DAT allele, a 561 bp band indicates a DAT Val559 homozygote, and the presence of both 490 and 561 bp bands indicates a DAT Val559 heterozygote.

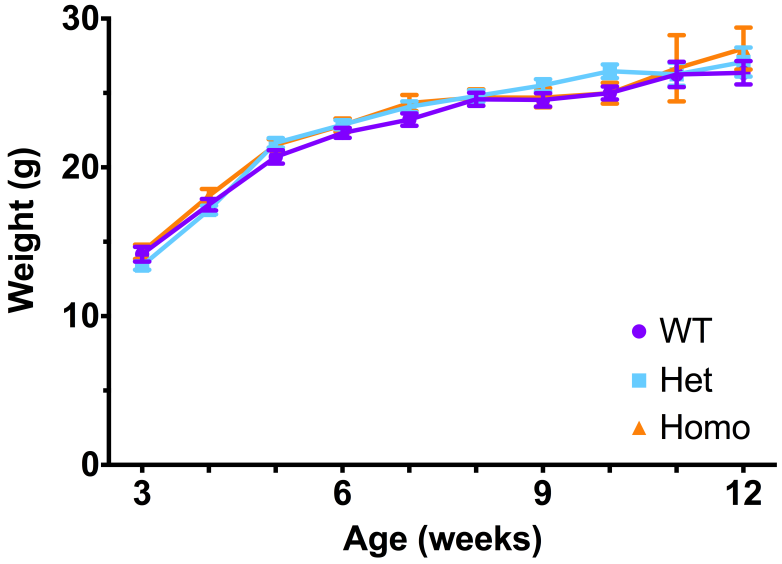

Figure 9. Growth of DAT Val559 mice after weaning (3-12 weeks) does not differ among genotypes.

General	***WT***	***Het***	***Homo***
Missing whiskers	1/8	0/8	0/8
Poor coat condition	0/8	0/8	0/8
Piloerection	0/8	0/8	0/8
Bald patches	0/8	0/8	1/8
Body tone (# with normal tone)	8/8	8/8	8/8
Limb tone (# with normal tone)	8/8	8/8	8/8
Respiration rate (# abnormal)	0/8	0/8	0/8
Heart rate (# abnormal)	0/8	0/8	0/8
Tremor	0/8	0/8	0/8
Gait (# abnormal)	0/8	0/8	0/8
Motor Ability	***WT***	***Het***	***Homo***
Trunk curl	1/8	0/8	3/8
Forepaw reaching	8/8	8/8	8/8
Horizontal wire hang	8/8	8/8	8/8
Vertical wire hang	8/8	8/8	8/8
Pole climb	8/8	8/8	8/8
Inverted screen (latency to fall, sec)	34.9 +/- 8.1	22.9 +/- 8.3	16.7 +/- 3.7
Positional passivity	0/8	0/8	0/8
Rotorod (latency to fall, sec)	171.9 +/- 25.8	204.0 +/- 10.1	166.9 +/- 23.5
Grip strength (N)	1.71 +/- 0.17	1.43 +/- 0.04	1.71 +/- 0.13
Reflexes	***WT***	***Het***	***Homo***
Righting reflex	8/8	8/8	8/8
Air righting reflex	8/8	8/8	8/8
Ear twitch	5/8	7/8	8/8
Petting escape	7/8	6/8	8/8

Table 3. Sensorimotor evaluation of DAT Val559 mice. DAT Val559 hetero- and homozygous mice display no deficiencies in measures of general health, motor capability, or reflexes (each test analyzed by one-way ANOVA, P = n.s. for all measures).

we detected a small but significant reduction in the number of DAT Val559 males present in the post-weaning population (Table 4) (chi-square test: male: $P = 0.006$; female: $P = 0.80$; overall: $P = 0.006$).

Generation of Congenic DAT Val559 Mice on C57BL/6J Background

As described in the methods, animals used for the experiments described herein were maintained on a hybrid background. We used a commercially available marker-assisted selection protocol ("speed congenic") to express DAT Val559 on a pure C57BL/6J background. After only four generations of backcrossing to the C57BL/6J target strain, we successfully generated DAT Val559 heterozygotes with 99% C57BL/6J genomic character (Table 5). These animals were then bred in order to establish the C57BL/6J DAT Val559 mouse colony.

Discussion

Rare genetic variation is increasingly recognized as source for insights into the etiology of complex disorders (Horschitz et al., 2005; Kauwe et al., 2008; Quast et al., 2012; Takata et al., 2013). Oftentimes, such variation is limited to a single, and sometimes small pedigree, compelling the demonstration of functional perturbations *in vitro* and *in vivo* to make reasonable conclusions regarding possible disease associations. Having analyzed the biochemical features of the DAT Val559 variant *in vitro* (Mazei-Robison et al., 2008), we pursued construction of a DAT Val559 knock-in mouse model to permit *in vivo* analyses of the functional impact of the DAT Val559 variant.

After successful generation of the DAT Val559 mice, we established heterozygous breeders and evaluated growth and development of offspring as a

Expected			
	Male	**Female**	**Total**
WT	332 (25%)	286.5 (25%)	618.5 (25%)
Het	664 (50%)	573 (50%)	1237 (50%)
Homo	332 (25%)	286.5 (25%)	618.5 (25%)
Observed			
	Male* **	**Female**	**Total** **
WT	386 (29.07%)	299 (26.09%)	685 (27.69%)
Het	650 (48.95%)	561 (48.95%)	1211 (48.95%)
Homo	292 (21.99%)	286 (24.96%)	578 (23.36%)

Table 4. Expected and observed allele frequencies of DAT Val559. The DAT Val559 allele is significantly under-represented in the total population of post-weaning mice (** = $P < 0.01$, chi-square test), driven primarily by a reduction in the male mice possessing the DAT Val559 allele (*** = $P = 0.001$, chi-square test). Males obtained from 446 litters, females obtained from 367 litters (for some litters, males were utilized for behavioral experiments, but females were not needed and, therefore, never genotyped).

Gen.	Expected % C57	Polymorphic Markers Only Observed % C57			All Markers Observed % C57		
		Average	Max	Min	Average	Max	Min
N0	25	27.69	34.54	21.989	63.1	66.667	60.26
N1	62.5	--	--	--	--	--	--
N2	81.25	86.95	91.1	83.07	93.2	95.39	91.21
N3	90.625	96.39	97.14	95.57	98.11	98.43	97.64
N4	95.3125	97.74	98.9637	96.6321	98.77	99.48	98.18

Table 5. Expected and observed C57BL/6J (C57) genomic content throughout backcross from hybrid 129S6/C57 background to congenic C57BL/6J background. At each generation, DAT Val559 heterozygous mice were crossed with WT C57 mice. C57 genomic character of each generation was determined via Charles River Laboratories MAX-BAX® speed congenic service. For each generation, the mouse with the highest percentage of C57 genomic character was bred to generate the subsequent generation.

function of genotype to ensure that perturbations in DA signaling resulting from the presence of DAT Val559 did not affect mouse viability. Whereas DAT KO mice display retarded growth and reduced survival (Giros et al., 1996), we found that DAT Val559 mice develop normally without any gross morphological abnormalities. We have also obtained brain sections from these mice from Neuroscience Associated (Knoxville, TN), and preliminary gross inspection shows no obvious alterations to brain structure. Furthermore, since DA signaling is a key modulator of motor function, we assayed basic motor function and reflexes in the DAT Val559 mice. No deficits in reflexes, sensory function, or motor function were observed.

Interestingly, we did observe a small but significant reduction in the number of male progeny expressing DAT Val559, suggesting a contribution of DAT function to neurodevelopmental processes. The reason for the male bias evident in this reduced recovery of DAT Val559 mice is unclear at present; it is reminiscent of the as-yet unexplained male predominance of ADHD, and could derive from perturbations of common pathways. For instance, we do know that DAT mRNA expression is evident in the mouse CNS by E13.5 (Allen Institute for Brain Science) and the rat CNS by E14-15 (Fujita et al., 1993; Perrone-Capano et al., 1994), and robustly expressed by E70 in the rhesus monkey (Fang and Rønnekliev, 1999). Other studies have shown the presence of DA and DA receptors in the periphery, including both rodent (Kim et al., 1997) and human (Elwan et al., 2003) placenta, possibly related to a role for D2-like receptors in regulating secretion of human placental lactogen (hPL), a growth factor that modulates the mother's metabolic state and helps to establish the energy supply for the developing fetus (Saxena et al.,

1969; Spellacy, 1969; Petit et al., 1990). DAT is also expressed by neural crest-derived neurons in the gut (Li et al., 2004). Further studies that restrict the spatial and temporal expression of the DAT Val559 variant are needed to infer when and where the precise role of the mutant protein in developmental processes. In addition, although the gross brain structure of DAT Val559 mice is normal, Stanwood and colleagues have shown that pharmacological (Stanwood et al., 2001) or genetic (Stanwood et al., 2005) manipulation of DA signaling during development leads to permanent alterations in cortical architecture and function. Such findings encourage a more refined analysis of cortical structure of DAT Val559 mice throughout development and into adulthood.

As addressed in Methods, the DAT Val559 mice were maintained and studied on a hybrid 129S6/C57BL/6J background. Over the course of my studies, however, I successfully backcrossed DAT Val559 to congenicity on a C57BL/6J background strain via speed congenic techniques. Interestingly, the background strain on which mutant mice are maintained can have sizable effects on both behavior and response to pharmacological manipulation (Loos et al., 2010; Kerr et al., 2013; O'Neill and Gu, 2013). Further studies are needed to identify other genetic factors that vary by background strain that may contribute to the phenotypes observed in DAT Val559 mice.

CHAPTER III

BIOCHEMICAL CHARACTERIZATION

Introduction

Prior to initiating our biochemical studies of the DAT Val559 model, we developed several hypotheses based on the *in vitro* behavior of the allele. As noted in Chapter 1, characterization of DAT Val559 in a heterologous expression system demonstrated that the mutant transporter expressed in HEK-293 cells at the same level as WT DAT and demonstrated normal DA transport kinetics (Mazei-Robison et al., 2008). However, DAT Val559 supported a spontaneous outward DA "leak", a process referred to as anomalous DA efflux (ADE). Accordingly, and assuming no unexpected impact of the addition of a loxP site in intron 13, we expected DAT Val559 to express at WT levels in our knock-in mouse model *in vivo* and to maintain normal DA transport kinetics.

Both DAT KO and knock-down (KD) mice have elevated extracellular DA due to their inability to recapture released DA (Jones et al., 1998; Zhuang et al., 2001), and likely rely more on newly synthesized DA to maintain DA signaling than WT animals. In DAT KO animals, the activity of TH is doubled relative to WT mice, though TH protein levels fall to ~10% of WT levels (Jones et al., 1998). Although we expected elevated extracellular DA in DAT Val559 mice due to ADE (addressed in Chapter 5), we did not anticipate changes in total tissue levels of DA (or DA

metabolites) or in TH expression because DA reuptake and vesicular packaging should be intact.

Further predictions regarding the impact of DAT Val559 expression on biochemical measures of the mouse brain are not based on *in vitro* observations, per se, but arise from predictions of compensations for the ADE exhibited by the mutant transporter. For example, potential compensatory changes could occur in DA receptor expression or function. DAT KO mice, for example, show reduced D1 and D2 receptor mRNA expression in both dopaminergic cell bodies in the midbrain and terminal fields of the striatum (Giros et al., 1996; Fauchey et al., 2000a, b), as well as reduced D2 receptor protein in the striatum (Jones et al., 1999). Such changes are believed to arise from the *excess* DA signaling that occurs as a result of the inability to clear DA out of the synapse or away from somatodendritic autoreceptors that act to constrain DA signaling (Giros et al., 1996). Since the DAT Val559 mice are expected to be at least mildly hyperdopaminergic as a result of ADE, we may also see downregulation of receptor expression. Such alteration of receptor expression could be interpreted as an adaptive change intended to dampen aberrant DA signaling and balance the increased dopaminergic tone derived from ADE.

Methods

Immunoblotting

Brains were harvested from mice following rapid decapitation. Brains were immediately placed on an ice-cold metal platform and the frontal cortex, midbrain, and striatum quickly dissected. Dissected tissue was placed into 3 mL of homogenization buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2

mM KH_2PO_4, 10 mM HEPES (pH 7.4), 10 mM glucose, 100 uM pargyline, ascorbic acid, 10 uM tropolone, 0.32 M sucrose) and homogenized using a Potter-Elvehjem homogenizer (Wheaton, Millville, NJ). Protein concentrations of all samples were determined by a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL). Equal amounts of protein were incubated with Laemmli sample buffer for 1 hr at room temperature and analyzed using SDS-PAGE and western blotting. DAT protein was visualized on samples blotted to Immobilon-P PVDF membrane (EMD Millipore Corporation, Billerica, MA) using a rat anti-hDAT antibody (MAB369, 1:2500 dilution; Millipore, Billerica, MA). Tyrosine hydroxylase (TH) was visualized using a rabbit anti-TH antibody (SAB4300675, 1:1000 dilution; Sigma-Aldrich, St. Louis, MO), and β-actin visualized using a mouse anti-β-actin antibody (A5316, 1:2000 dilution; Sigma-Aldrich, St. Louis, MO) as a loading control. Appropriate HRP-conjugated, secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Secondary antibody labeling was detected using Western Lightning Plus ECL (Perkin Elmer, Waltham, MA) and exposure to Hyblot CL Autoradiography Film (Denville Scientific, Inc., Metuchen, NJ). Multiple exposures were obtained to insure linearity of band detection. Western blots were quantified using NIH ImageJ software and data analyzed using one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons, with $P < 0.05$ considered significant.

Synaptosomal DA Transport Assays

Brains were harvested from mice following rapid decapitation. Brains were immediately placed on an ice-cold metal platform and the striatum quickly

dissected. Tissue samples were placed into 3 mL of homogenization buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES (pH 7.4), 10 mM glucose, 100 uM pargyline, ascorbic acid, 10 uM tropolone, 0.32 M sucrose) and homogenized using a Potter-Elvehjem homogenizer (Wheaton, Millville, NJ). Homogenates were centrifuged at 500 *x* g at 4°C for 10 min. Supernatants were removed and centrifuged at 12000 *x g* at 4°C for 10 min. The resulting synaptosome-enriched pellets were then resuspended in 2.5 mL of assay buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES (pH 7.4), 10 mM glucose, 100 uM pargyline, ascorbic acid, 10 uM tropolone) and protein concentrations determined using the Coomassie Plus (Bradford) Protein Assay (Thermo Fisher Scientific, Rockford, IL). This material (hereafter called synaptosomes) was then diluted to 30 ug of total protein per 100 uL. Synaptosomes were incubated with varying concentrations of [^{3}H]-DA (46.0 Ci/mmol; Perkin Elmer, Waltham, MA) ranging from 50 nM to 1 uM (50 nM was 100% labeled substrate, DA concentrations between 250 nM and 1 uM used 10% labeled and 90% unlabeled DA) for 5 min. at 37^{o}C. To determine non-specific transport, parallel samples were incubated with 10 uM cocaine for 10 min. before the addition of [^{3}H]-DA. Assays were terminated by rapid filtration over 0.3% polyethyleneimine-soaked GF/B glass microfiber filters (Whatman, Clifton, NJ) and washed 3X with ice-cold Krebs-Ringers-HEPES buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES (pH 7.4)). Filters placed into scintillation vials with 7 mL of Ecoscint H (National Diagnostics, Atlanta, GA) scintillation fluid, shaken overnight at room temperature, and then radioactivity

was quantified using a TriCarb 2900TR scintillation counter (Perkin Elmer, Waltham, MA). Specific [^{3}H]-DA transport was assayed in triplicate for all samples. Data were analyzed using one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons.

Receptor Binding Assays

Frozen tissue was dissected and frozen as noted above then placed in 2 mL of binding buffer (50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 120 mM NaCl) and homogenized using an Omni-Tip handheld homogenizer (Omni International, Kennesaw, GA) at 10000-12000 rpm for 10-15 secs. Homogenates were then centrifuged at 20000 *x g* at 4°C for 20 min. Membrane pellets were resuspended in 3 mL of binding buffer and homogenized again as described above. Homogenates were pre-incubated at 37°C for 15 min and then centrifuged at 20000 *x g* at 4°C for 20 min. Membrane pellets were again resuspended in 3 mL of binding buffer and homogenized as described above. Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL). Samples were stored at -80°C until used for binding assays. Membrane samples were then thawed and (100-150 ug for cortex, 150-200 ug for midbrain, and 100-150 ug for striatum) incubated in a final reaction volume of 1 mL at room temperature for 75 min in the presence of 3 nM [^{3}H]-SCH 23390 (D1 receptor assays; 84.3 Ci/mmol; Perkin Elmer, Waltham, MA) or 90 min in the presence of 3 nM [^{3}H]-raclopride (D2 receptor assays; 76 Ci/mmol; Perkin Elmer, Waltham, MA). Binding reactions were terminated by the addition of 8 mL of ice-cold wash buffer (50 mM Tris (pH 7.4)) followed by rapid filtration over water-moistened S&S (#5)

(Schleicher and Schuell Bioscience, Inc., Kenne, NH) or Whatman GF/B (Whatman, Clifton, NJ) glass fiber filter using a Millipore vacuum manifold. Each filter was then washed 2X with 8 mL of ice-cold wash buffer. Filters were placed in scintillation vials and 10 mL of Biosafe II scintillation fluid (Research Products International Corp., Mount Prospect, IL) was added to each vial. Vials were shaken overnight at room temperature, and then radioactivity was counted using a TriCarb 2900TR scintillation counter (Perkin Elmer, Waltham, MA). Non-specific binding was determined using parallel incubations as above with the addition of 2 uM butaclamol to samples with binding values from these samples subtracted from radioligand only samples to determine specific binding. All samples (total and non-specific binding) were assayed in triplicate. Data were analyzed using one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons.

Assessment of Tissue Biogenic Amines

Brain regions, harvested and dissected as noted above, were flash-frozen in liquid nitrogen and stored at -80°C. Frozen brain tissue was homogenized using a tissue dismembrator (Misonix XL-2000; Qsonica, LLC, Newtown, CT) in 100-750 uL of a solution containing 100 mM TCA, 10 mM $NaC_2H_3O_2$, 100 uM EDTA, 5 ng/mL isoproterenol (an internal standard), and 10.5% methanol (pH 3.8). Samples were spun in a microcentrifuge at 10000 *x g* for 20 min. and the supernatants were stored at -80°C until assayed (Cransac et al., 1996). Prior to assay, thawed supernatants were centrifuged at 10000 *x g* for 20 min. before being analyzed by HPLC. Twenty uL samples of each sample were injected using a Waters 2707 auto-sampler onto a Phenomenex Kintex (2.6 u, 100 A) C18 HPLC column (100 x 4.6 mm). Biogenic

amines were eluted with a mobile phase (89.5% 100 mM TCA, 10 mM $NaC_2H_3O_2$, 100 uM EDTA, and 10.5% methanol (pH 3.8)) delivered at 0.6 mL/minute using a Waters 515 HPLC pump. Analytes were detected utilizing an Antec Decade II (oxidation: 0.4) (3 mm GC WE, HYREF) electrochemical detector operated at 33°C. HPLC instrument control and data acquisition was managed by Empower software. Data were analyzed with one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons.

Results

DAT Val559 Expression and Transport Capacity

Previous *in vitro* studies demonstrated that DAT Ala559 (WT) and DAT Val559 express equivalently, both in total extracts and cell surface fractions (biotinylation), and did not differ in inward DA transport capacity (Mazei-Robison et al., 2008). Consistent with these findings, striatal extracts from DAT Val559 animals demonstrate normal transporter protein levels (Fig. 10). Similarly, striatal synaptosomes from both hetero- and homozygous DAT Val559 animals show equivalent DA transport kinetics as compared to WT samples (Fig. 11), with no differences in DA K_M or transport V_{MAX}.

DAT Val559 Effects on Neurochemistry and DA System Components

Whereas DAT KO mice show drastically reduced tissue levels of DA (less than 5% of WT levels) (Jones et al., 1998), DAT Val559 mice show no genotype-dependent changes in total levels of DA, DA metabolites (DOPAC, HVA, 3-MT), or DA turnover (DA:DOPAC ratio) in cortex, midbrain, or striatum (Fig. 12). Interestingly,

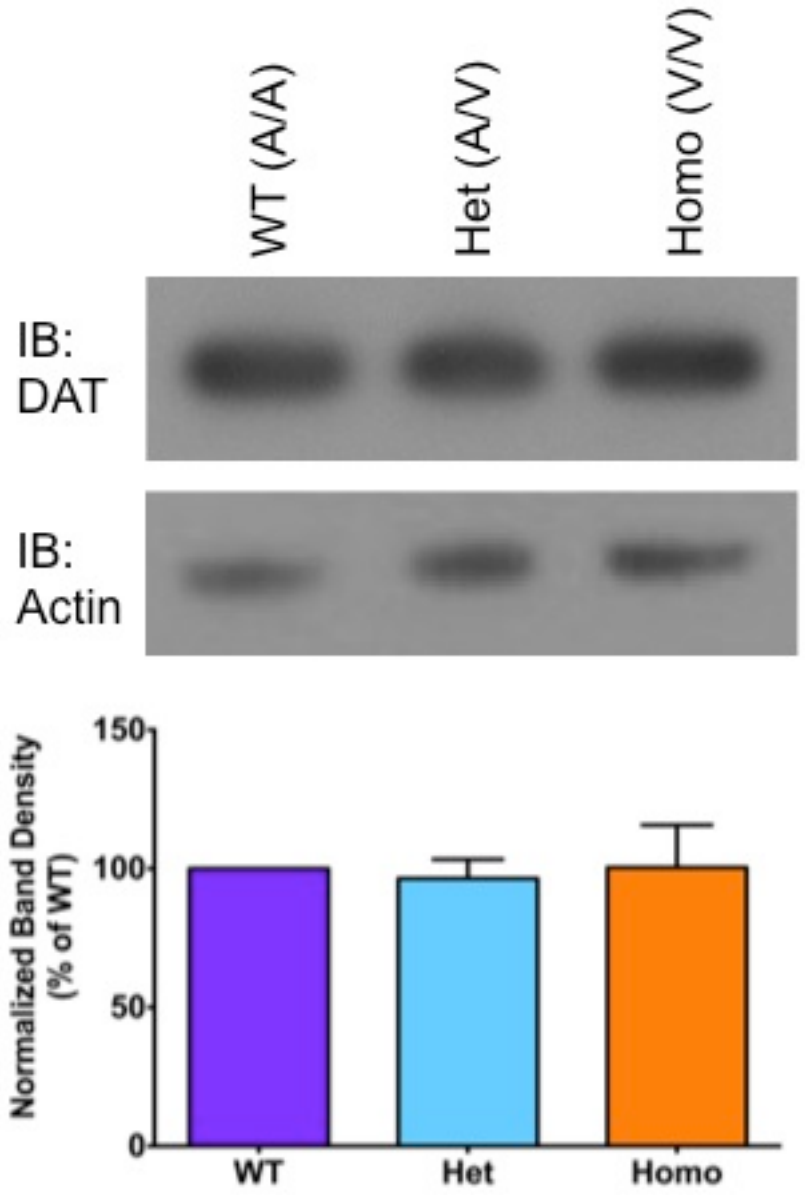

Figure 10. DAT Val559 mice have normal striatal DAT expression (n = 6 per genotype; $P > 0.05$, one-way ANOVA).

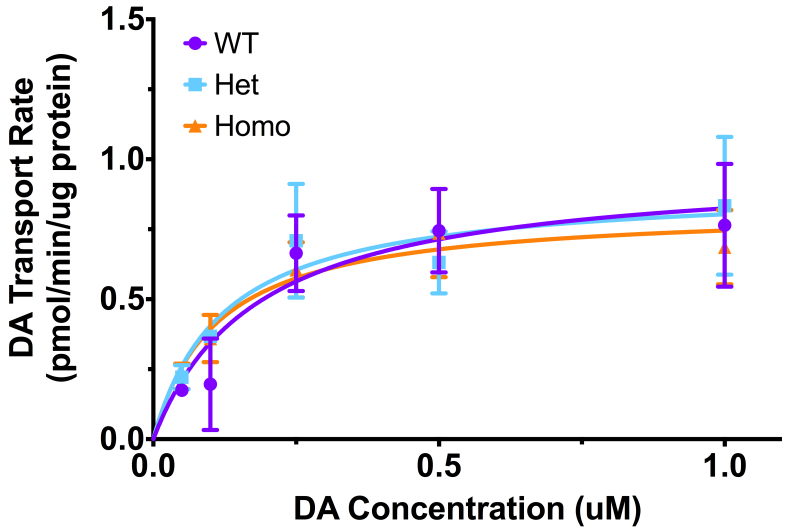

Figure 11. DAT Val559 in striatal synaptosomes supports normal DA transport kinetics (n = 5 WT, 4 het, 7 homo; V_{max}+/- SEM (pmol/min/ug protein): WT = 1.01 +/- 0.215, het = 0.8284 +/- 0.1822, homo = 0.8847 +/- 0.1770, $P > 0.05$, one-way ANOVA; K_m +/- SEM: WT = 0.1774 +/- 0.03839, het = 0.08762 +/- 0.0261, homo = 0.1407 +/- 0.04754, $P > 0.05$, one-way ANOVA).

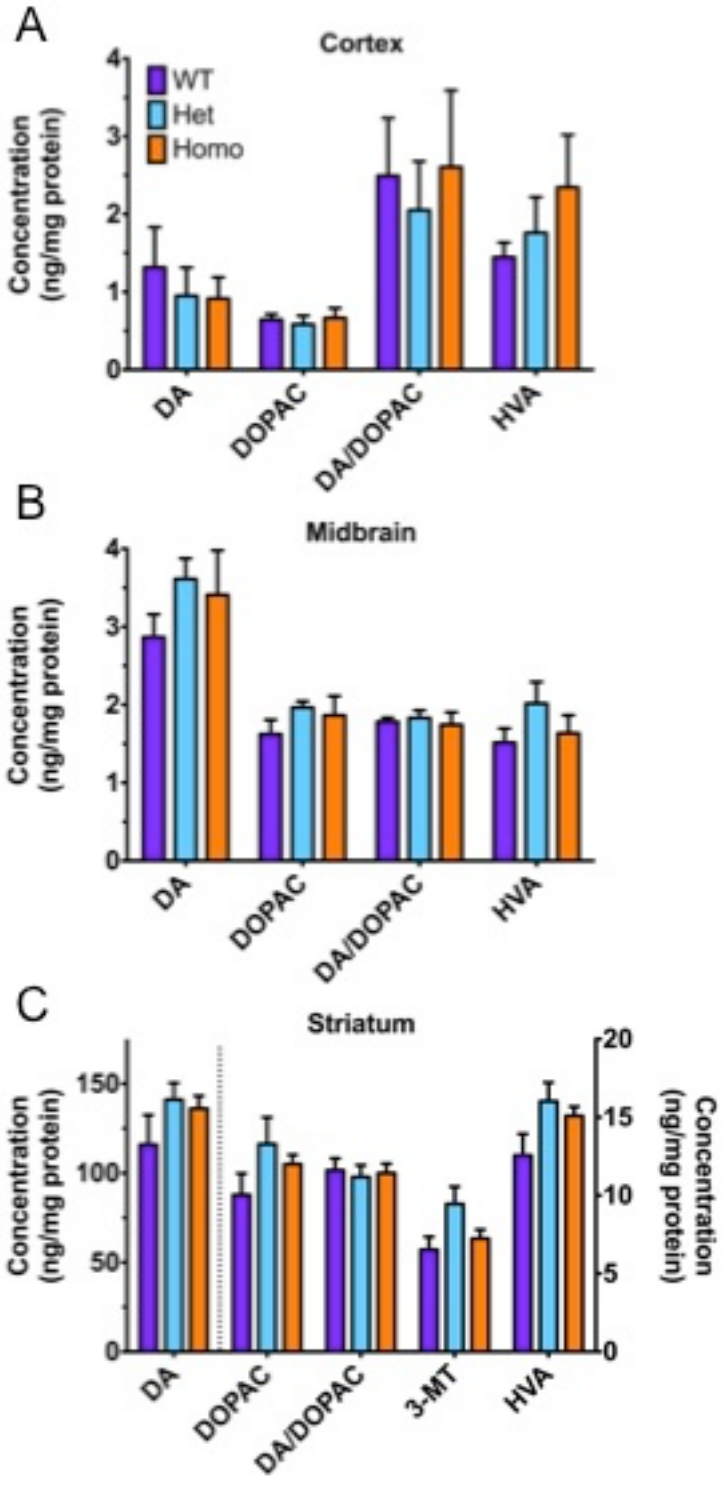

Figure 12. Dopaminergic neurochemistry is not significantly different in (A) cortex, (B) midbrain, or (C) striatum between WT and DAT Val559 mice (n = 8 WT, 8 het, 9 homo; $P > 0.05$, one-way ANOVA).

we detected small but significant changes in cortical and striatal 5-HT levels without concomitant changes in 5-HT metabolite 5-HIAA or 5-HT turnover (Fig. 13).

Since DAT KO mice are unable to recover released DA, they rely totally on the synthesis of DA to maintain dopaminergic signaling. Moreover, Jones and colleagues demonstrated that TH expression in DAT KO mice is reduced by 96%, though the rate of DA synthesis by available TH is doubled relative to the rate of synthesis in WT animals (Jones et al., 1998). In contrast, we found in DAT Val559 animals, that TH total protein levels are equivalent to WT levels (Fig. 14). Finally, DAT KO mice show reductions in D1 and D2 receptor mRNA expression in striatum (Giros et al., 1996; Fauchey et al., 2000a, b) and reduced D2 receptor levels in midbrain (Jones et al., 1999). In contrast, we observed no change in D1 or D2 receptors in the cortex, midbrain, or striatum of DAT Val559 hetero- or homozygotes (Fig. 15).

Discussion

All of the findings reported in this chapter derived from direct evaluation of DAT Val559 are consistent with previous *in vitro* studies of the mutant protein. DAT Val559 total expression and transport function in mouse striatum are equivalent to those of the WT protein. Whereas the loss of DA reuptake in DAT KO mice results in reduced tissue DA and TH protein levels (Giros et al., 1996; Jones et al., 1998), the normal inward DA reuptake of DAT Val559 mice, along with normal levels of TH, appears to sustain normal tissue DA levels. It is also possible that ADE occurs in the context of reduced DA neuron excitability, such that the loss of cytoplasmic DA is offset by reduced vesicular release, resulting in normal tissue DA levels. Thus,

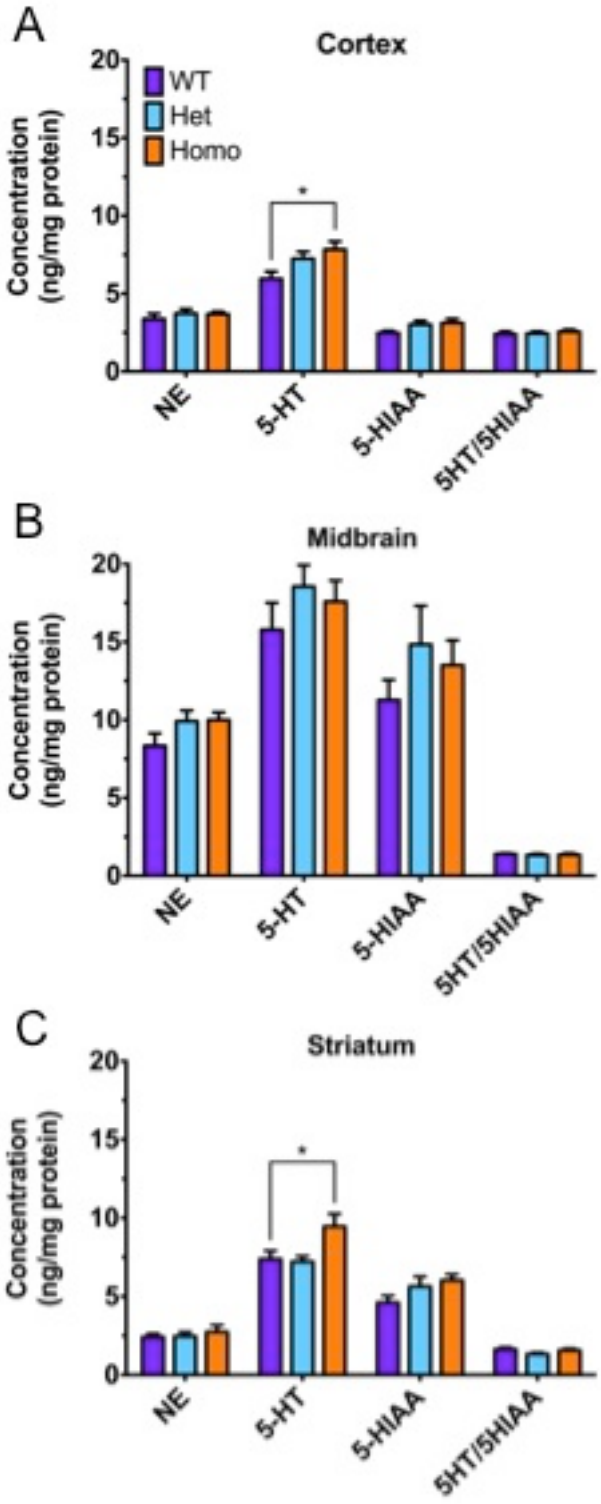

Figure 13. Noradrenergic and serotonergic neurochemistry of WT and DAT Val559 mice. (A) Cortical serotonin (5-HT) levels are significantly elevated in DAT Val559 homozygotes (n = 8 WT, 8 het, 9 homo; * = $P < 0.05$, one-way ANOVA), but 5-HT metabolite 5-HIAA levels and 5-HT turnover (5-HT/5-HIAA ratio) and noradrenaline (NE) levels are unchanged ($P > 0.05$, one-way ANOVA). (B) Midbrain noradrenergic and serotonergic neurochemistry is not changed in DAT Val559 mice ($P > 0.05$, one-way ANOVA). (C) Striatal 5-HT levels are also significantly elevated in DAT Val559 homozygotes (* = $P < 0.05$, one-way ANOVA) without changes in 5-HIAA or 5-HT turnover ($P > 0.05$, one-way ANOVA).

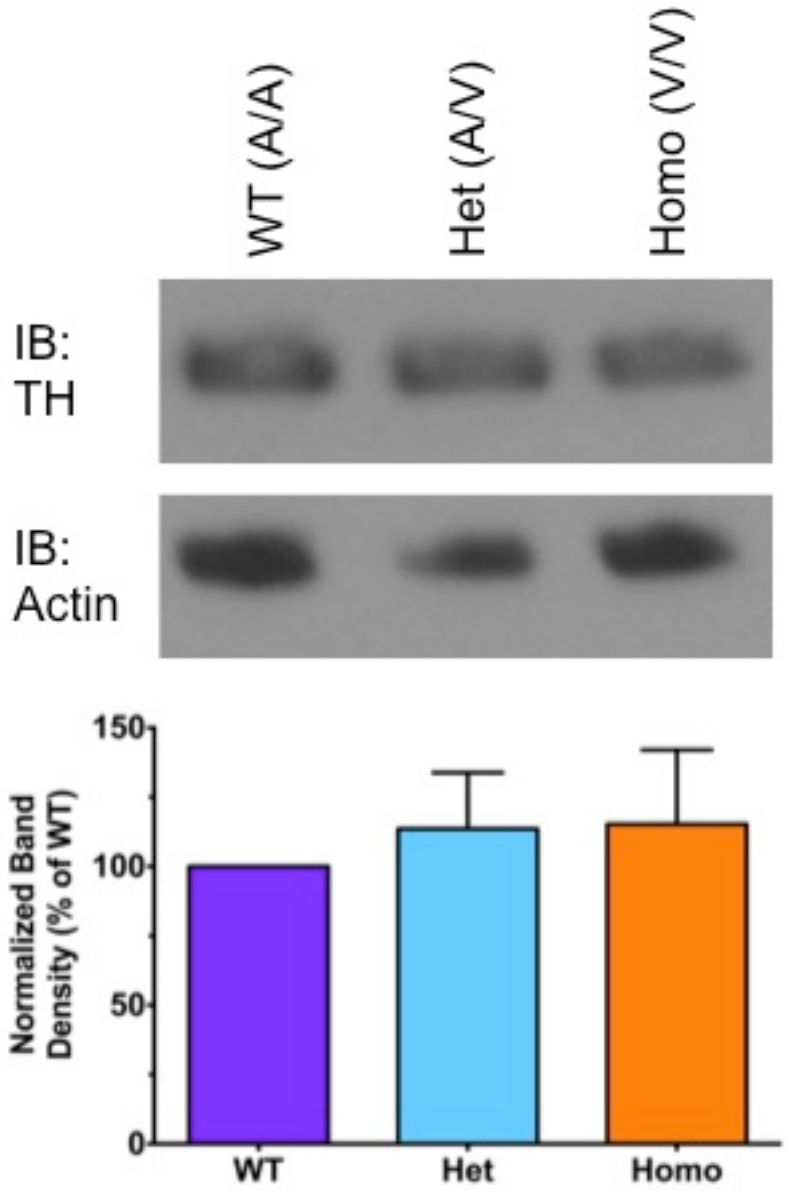

Figure 14. DAT Val559 mice have normal striatal TH expression (n = 3 per genotype; $P > 0.05$, one-way ANOVA).

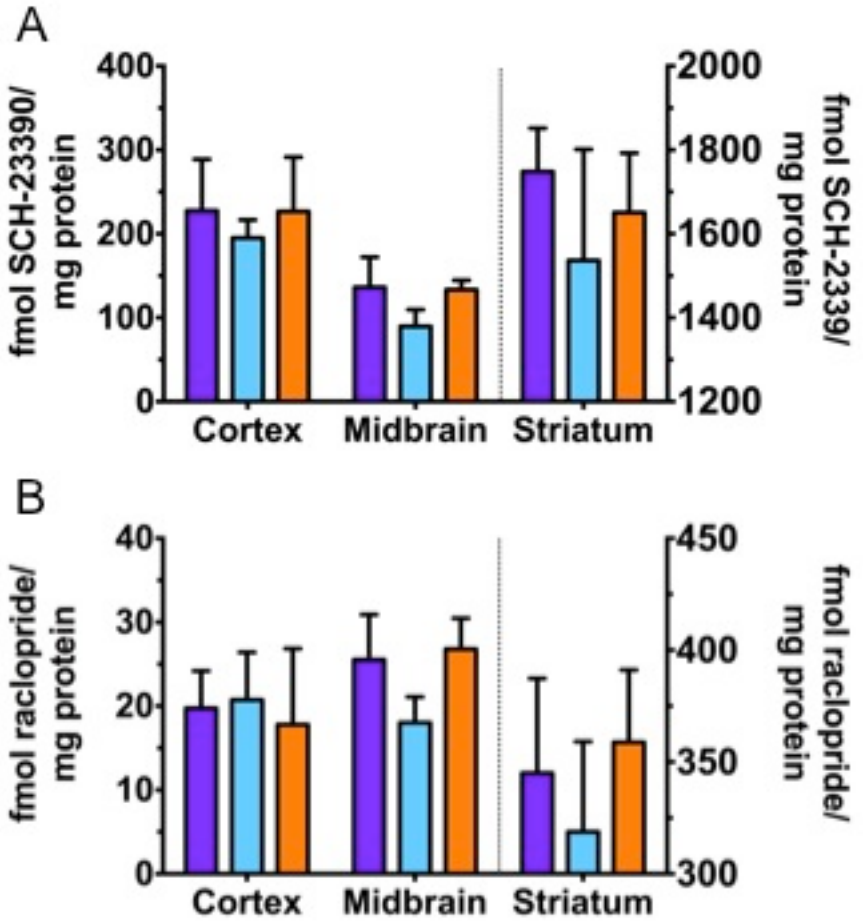

Figure 15. Radioligand binding assays reveal no differences in (A) D1 receptor (n = 3-5 per genotype per brain region; [^{3}H]-SCH-23390 binding, cortex and midbrain plotted on left y-axis, striatum plotted on right y-axis; $P > 0.05$ for each brain region, one-way ANOVA) or (B) D2 receptor (n = 3-5 per genotype per brain region; [^{3}H]raclopride binding, cortex and midbrain plotted on left y-axis, striatum plotted on right y-axis; $P > 0.05$ for each brain region, one-way ANOVA).

further studies are needed to examine basal and stimulated excitability of DA neurons in the DAT Val559 mouse model.

The observed changes in serotonin levels in the striatum and cortex are also quite interesting. Caron's research group reported in DAT KO mice that locomotor hyperactivity was attenuated by treatment with selective 5-HT reuptake inhibitor (SSRI) treatment (Gainetdinov et al., 1999) even though locomotor activity of WT animals was unaffected, and that cocaine self-administration remained intact and was mediated by cocaine binding to SERT (Rocha et al., 1998). In addition, DAT KO mice have elevated 5-HT levels in the frontal cortex and hippocampus (Fox et al., 2013), suggesting that 5-HT signaling undergoes compensatory changes in the context of hyperdopaminergia. On the other hand, following loss of DA signaling by 6-OHDA lesions, Avale and coworkers found increased 5-HT axon sprouting and elevated striatal 5-HT levels (Avale et al., 2004b), suggesting that 5-HT signaling can also compensate for a hypodopaminergic state. Investigation of specific 5-HT receptor-mediated modulation of DA signaling revealed that 5-HT_{2A} and 5-HT_{2C} receptors exert excitatory and inhibitory control of DA signaling, respectively (Di Matteo et al., 2002; Porras et al., 2002; reviewed in Esposito et al., 2008), offering a mechanism by which 5-HT signaling can respond to both hypo- and hyperdopaminergic states. In the context of DAT Val559, we expect ADE to cause a mildly hyperdopaminergic state. The observed increases in cortical and striatal 5-HT, therefore, may be indicative of compensatory changes in 5-HT signaling to account for the altered dopaminergic tone due to DAT Val559. Further exploration of serotonergic function, in general, and 5-HT receptor expression and function in

the cortex and striatum, specifically, is needed to better elucidate the impact of DAT Val559.

In contrast to DAT KO mice, we showed that DA receptor density is unchanged in DAT Val559 mice. DAT KO animals show significant reduction in DA receptor mRNA expression (Giros et al., 1996) and D2 receptor protein expression (Jones et al., 1999). It is possible that the increase in extracellular DA concentration due to ADE, partially offset by normal DA uptake, is of a smaller magnitude than that observed with a complete loss of DA reuptake in the DAT KO mice and, as a result, DA receptor levels may not change. Furthermore, changes in D2 receptor expression in DAT KOs were subsequently shown to reflect a reduction in somatodendritic D2 autoreceptor function (Jones et al., 1999). Also, the radioligand binding data presented here do not address potential changes in DA receptor localization (i.e. internalization) or function (i.e. desensitization) that do not necessarily require changes in protein levels. Further studies are warranted to more closely examine DA receptor function and regulation in the context of DAT Val559.

CHAPTER IV

BEHAVIORAL CHARACTERIZATION

Introduction

DA signaling plays an important role in a variety of behaviors that can be readily evaluated in rodent models. For example, locomotor activity in the open field is very sensitive to extracellular DA levels. Also, elevation of extracellular DA via treatment with AMPH (Dews, 1953; Smith, 1965) or cocaine-like psychostimulants (Heise and Boff, 1962; Van Rossum and Hurkmans, 1964; Smith, 1965.) produces significant hyperactivity in open field chambers. Consistent with these findings, DAT KO and KD animals where extracellular levels of DA are elevated 5 and 2 fold, respectively, are extremely hyperactive in a novel environment (Giros et al., 1996; Zhuang et al., 2001), whereas DA depletion by reserpine treatment (Carlsson et al., 1958; Utley and Carlsson, 1965; Almgren et al., 1976; Johnels, 1982), extensive 6-OHDA lesion (Koob et al., 1981), or genetic ablation of TH (Zhou and Palmiter, 1995; Nishii et al., 1998) produces hypokinesis, a characteristic of Parkinson's disease. Assuming that DAT Val559 mice have elevated extracellular DA levels due to ADE, we anticipated that these mice could be spontaneously hyperactive as compared to WT littermates.

As reviewed in Chapter 1, *in vitro* data have shown that DAT Val559 has an abnormal response to AMPH, such that AMPH blocks ADE as opposed to inducing DA release (Mazei-Robison et al., 2008), acting more like a competitive substrate or

inhibitor. In the DAT Val559 mice, therefore, AMPH is expected to lead to a certain degree of locomotor activation, as AMPH will prevent reuptake of released DA (in a mechanism similar to cocaine (Izenwasser et al., 1993) or methylphenidate (Schweri, 1990; Héron et al., 1994; Wall et al., 1995). However, in the absence of AMPH-evoked, nonvesicular DA release, this locomotor activation is expected to be of a smaller magnitude than that seen in WT mice.

It is important to note that both of the children identified with the DAT Val559 each possessed only one mutant allele (Mazei-Robison et al., 2005). We expect, therefore, that DAT Val559 acts dominantly and can exert its effects despite the presence of a WT DAT allele. Accordingly, we may also see blunted responses to AMPH in DAT Val559 heterozyogtes. If DAT Val559 is acting in a dominant manner, then we anticipate that hetero- and homozygotes will display similar phenotypes, rather than intermediate, gene-dosage dependent differences among genotypes.

The impact of DAT Val559 on other behavioral readouts is difficult to predict. In addition to locomotor hyperactivity, DAT KO mice display stereotyped movements such as head weaving (Wong et al., 2012; Fox et al., 2013). It is not unreasonable, then, that DAT Val559 mice could also exhibit stereotyped behaviors, though the extent may be dictated by the size of the elevations in extracellular DA and/or how they are achieved. In this this chapter, I present a characterization of locomotor phenotypes using the DAT Val559 mice, as well as their response to treatment with AMPH.

Methods

Open field Locomotor Assay

All locomotor behavior was assessed using male mice, aged 5-9 weeks, with 7-10 days of acclimation to a reverse light/dark cycle between 3-5 weeks of age, during the animal's active (dark phase of light/dark cycle) period. Locomotor activity in the open field was measured using 27 x 27 x 20.5 chambers (Med Associates, St. Albans, VT) placed within light- and air-controlled, sound-attenuating boxes (64 x 45 x 42 cm). Locomotion was detected by interruption of infrared beams by the body of the mouse (16 photocells in each horizontal axis located 1 cm above the activity chamber floor, as well as 16 photocells elevated 4 cm above the chamber floor to detect rearing and jumping behaviors). Data were collected and quantified by Med Associates Activity Monitor software.

Mice were acclimated to the activity chambers during a 30-minute session 2-3 days before data recording began. On Day 1 of the experimental sequence, mice were weighed then placed into activity chambers and activity was monitored for 30 min. The mice were then removed from the activity chambers and received a 5 mL/kg i.p. injection of 0.9% saline (AMPH experiments) or 0.02% DMSO (SKF 83822 experiments). Mice were returned to the activity chambers and locomotor activity was recorded for 60 min. No manipulations were performed on Day 2. On Day 3, mice were weighed then placed into activity chambers and activity recorded for 30 minutes. Mice were then removed from the activity chambers and injected i.p. with AMPH (in 0.9% saline; 3 mg/kg, 5 mL/kg) or SKF83822 (in 0.02% DMSO, 2 mg/kg, 5 mL/kg). Mice were then returned to the chambers and activity recorded for 60 min.

Time course data were analyzed using two-way ANOVA with Sidak's *post hoc* test for multiple comparisons, and cumulative measures of behavior (i.e. total distance traveled, time in stereotypy, number of stereotyped behaviors, time rearing, and number of rearing behaviors) during the entire 60-minute recording analyzed using one-way ANOVA with Tukey's *post hoc* test for multiple comparisons.

Darting

Mice were moved from group housing and isolated in clean cages for 60-75 minutes. After acclimating to the new cage, darting responses were initiated by a researcher reaching for the base of the mouse's tail, mimicking an attempt to pick up the animal, without making contact. Each mouse was approached 5-7 times, with 20-30 seconds between approaches, to elicit the darting reaction (total number of approaches: 130 WT, 223 het, 103 homo). Escape reactions ("darting") were recorded and then videos were imported into iMovie (Apple, Cupertino, CA) and slowed to 10% of original speed. The beginning and ending video frames of each darting incident were noted with timestamps and then imported into ImageJ (NIH, Bethesda, MD) with the timestamps used to isolate the first frame of each dart. A Z-stack image was then created over the entire darting reaction, and a subtracted image (first frame subtracted from Z-stack) was produced, leaving a negative image where the mouse moved during the dart. A freehand line was then plotted through the center of each mouse's darting path and the distance quantified. To account for variability between videos, the length of one side of each mouse's cage was measured and used to convert from pixels (path length measurement in ImageJ) to centimeters (Fig. 16). Speed from the initiation of the darting movement until 1

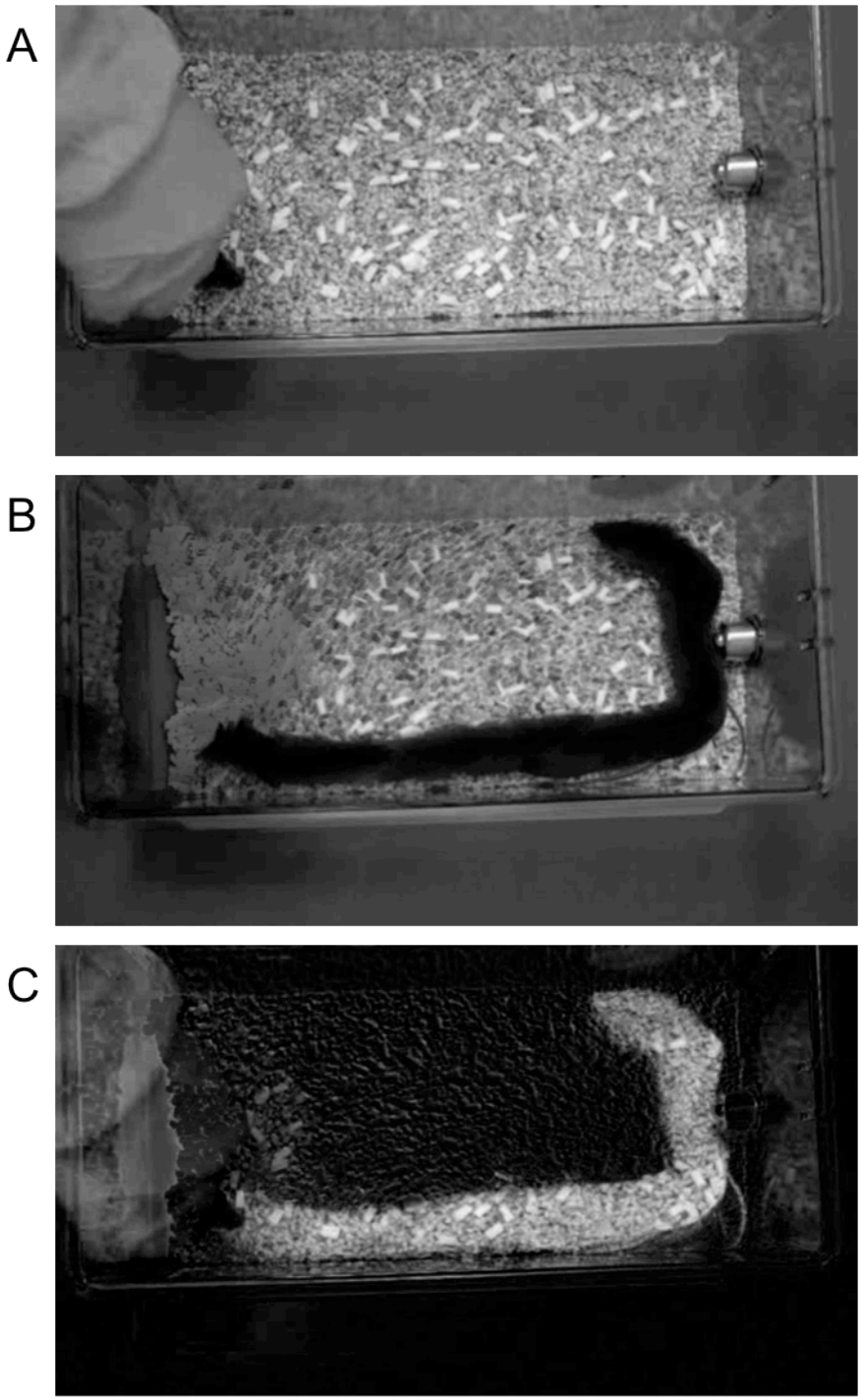

Figure 16. Darting behavior analysis. For each darting event, the first video frame before the mouse starts moving (A) is subtracted from a stacked image of the dart in which the entire video clip is compressed into a single still image (B). The resulting negative image (C) reveals the path of the dart that can be measured.

second elapsed (or the animal stopped moving) was then calculated based on the distance traveled and duration of each dart. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons test and are depicted as a frequency distribution of darting speed, as well as average darting speed.

Elevated Zero Maze

Anxiety behaviors were examined using an elevated zero maze (62.5 cm outer diameter, 5 cm path width, 15 cm wall height in closed segments; maze fabricated by Vanderbilt Kennedy Center Scientific Instrumentation Service) with recordings lasting for 5 min. At the start of each trial, mice were placed onto an open portion of the maze, adjacent to and facing one of the closed segments. Each session was recorded by a ceiling-mounted video camera connected to a computer for digital video acquisition and analysis with ANY-maze software (Stoelting, Wood Dale, IL). Data analyzed include the percent of time spent in the open zone, number of open and closed zones entries, and distance traveled around the maze. Data were analyzed using one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons.

Acoustic Startle

The acoustic startle response was assessed in sound-attenuated acoustic startle chambers (Med-Associates, St. Albans, VT). Mice were gently restrained in transparent acrylic cylinders (3.2 cm inner diameter; Med Associates, Inc. model: ENV-263A) and placed on a startle platform with load cells and a force transducer. Mice were habituated to the chambers for five minutes before the presentation of the first acoustic startle stimulus. Each mouse was recorded for 48 startle trials (70,

80, 90, 100, 110, or 120 dB white noise bursts, each presented 8 times in pseudorandom order). The startle response following each stimulus was recorded and analyzed by Startle Reflex software (MED Associates, version 5). Data were analyzed using two-way ANOVA with Tukey's *post hoc* test for multiple comparisons.

Results

Locomotor Response to a Novel Environment

DAT KO mice display profound locomotor hyperactivity in open field testing (Giros et al., 1996; Speilewoy et al., 2000). In open field assessments of locomotor response to a novel environment, we did not observe locomotor hyperactivity, but rather a modest, but significant, reduction in horizontal locomotor activity between WT and DAT Val559 heterozygous animals (Fig. 17A). Interestingly, rearing behavior was also significantly reduced in DAT Val559 animals (Fig. 17B). No genotype differences were detected in time spent in the center versus total time in the chamber, a measure of anxiety (Fig. 17C). In addition, preliminary evidence obtained using home-cage recordings also indicates a reduction in rearing activity in DAT Val559 mice (see Appendix 3), suggesting that this behavioral change reflects a trait induced by constitutive DAT Val559 expression versus a state induced by a novel environment (or their interaction).

Darting, Elevated Zero Maze, and Acoustic Startle

Although we observed no spontaneous hyperactivity, we did notice early on in our handling of the DAT Val559 mice that when researchers reached to transfer animals between cages, the presence of a DAT Val559 genotype could be fairly

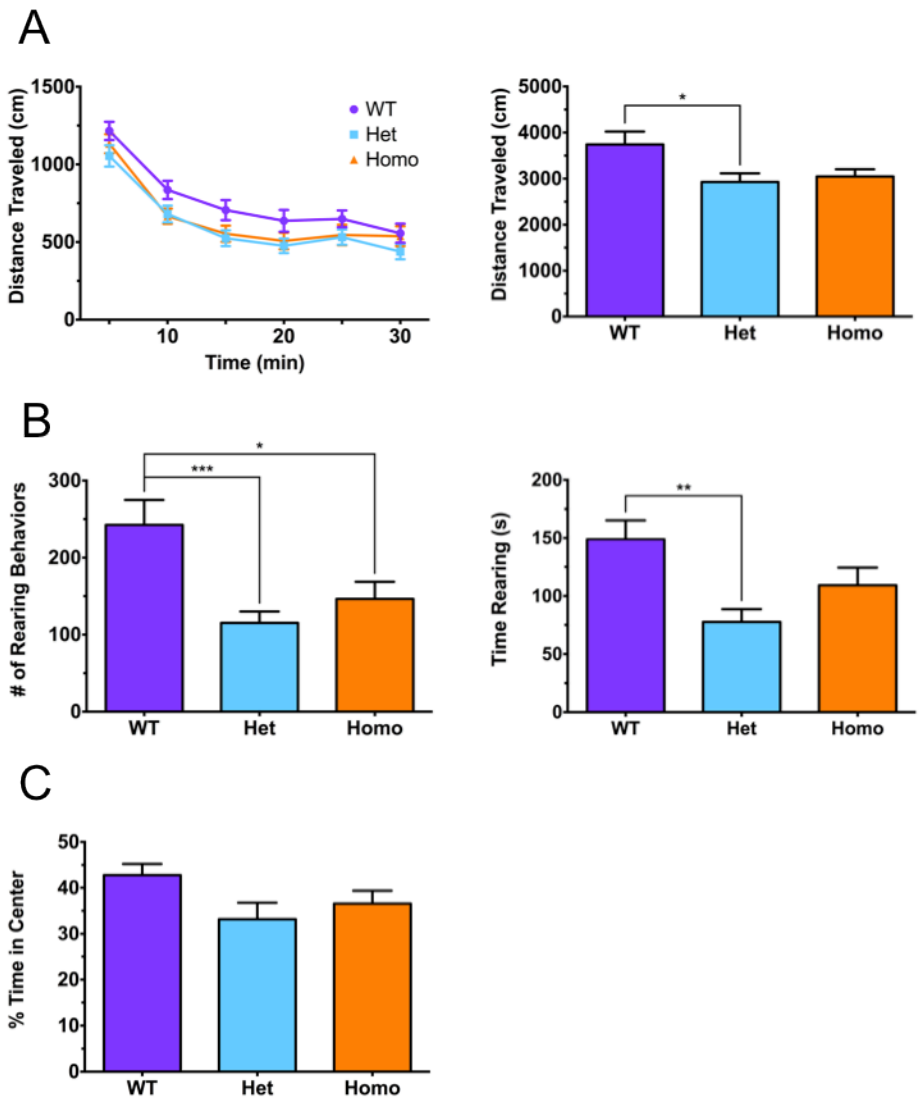

Figure 17. DAT Val559 mice display reduced locomotor activity and rearing behavior in a novel testing environment. (A) Horizontal locomotor activity (n = 28 WT, 30 het, 25 homo; *left*: time course of locomotor behavior, *P*(genotype) > 0.05, two-way RMANOVA; *right*: total +/- SEM distance traveled in 30 minutes in activity chambers, overall $P < 0.05$, post hoc tests (Tukey's multiple comparisons test) reveals * = $P < 0.05$ for WT vs. het, $P = 0.0764$ (n.s.) for WT vs. homo, one-way ANOVA) and (B) rearing activity (*left*: number of rearing behaviors +/- SEM in 30 minutes in activity chambers, overall $P < 0.001$, post hoc tests (Tukey's multiple comparisons test) reveals *** = $P < 0.001$ for WT vs. het, * = $P < 0.05$ for WT vs. homo, one-way ANOVA; *right*: time +/- SEM rearing in 30 minutes in activity chambers, overall $P < 0.01$ for WT vs. het, $P = 0.1334$ for WT vs. homo, one-way ANOVA) are significantly reduced in DAT Val559 mice without changes in (C) time spent in the center versus the edge of the activity chambers (percent of time +/- SEM in center of chambers during 30-minute recording, $P > 0.05$, one-way ANOVA).

reliably assigned based on the presence of a more robust escape response, a behavior that we termed "darting". After rigorous quantification of the speed of each escape response, we identified a significant genotype effect, such that DAT Val559 mice display a faster escape reaction than their WT counterparts, whether considered as a distribution of all darting speeds (Fig. 18A, or average darting speed for each genotype (Fig. 18B). Furthermore, the increase in darting speed for DAT Val559 animals is specific to the darting behavior; average locomotor speed during recordings of basal open field activity do not differ among genotypes (Fig. 18C).

The increased darting speed that we observed in DAT Val559 animals could reflect an increase in the general startle response and/or anxiety. To test these possibilities we assessed DAT Val559 mice and WT littermates for their acoustic startle response and performance on the elevated zero maze (EZM). We observed no impact of genotype on startle latency or peak startle magnitude at any decibel level tested (Fig. 19). We also found no genotype-dependent effect on locomotor activity in the zero maze or in time spent in the open areas of the maze (Fig. 20).

Blunted Locomotor Activation by AMPH

In control experiments, DAT Val559 mice display a small but non-significant reduction in locomotor activity following saline injection (Fig. 21A), similar to the behavioral pattern observed during recording of basal locomotor activity (Fig. 17A). DAT Val559 mice are less active during the first 30 minutes after injection, perhaps reflecting a subdued response to injection stress, as activity levels of DAT Val559 mice normalize to WT levels during the last 30 minutes of the trial.

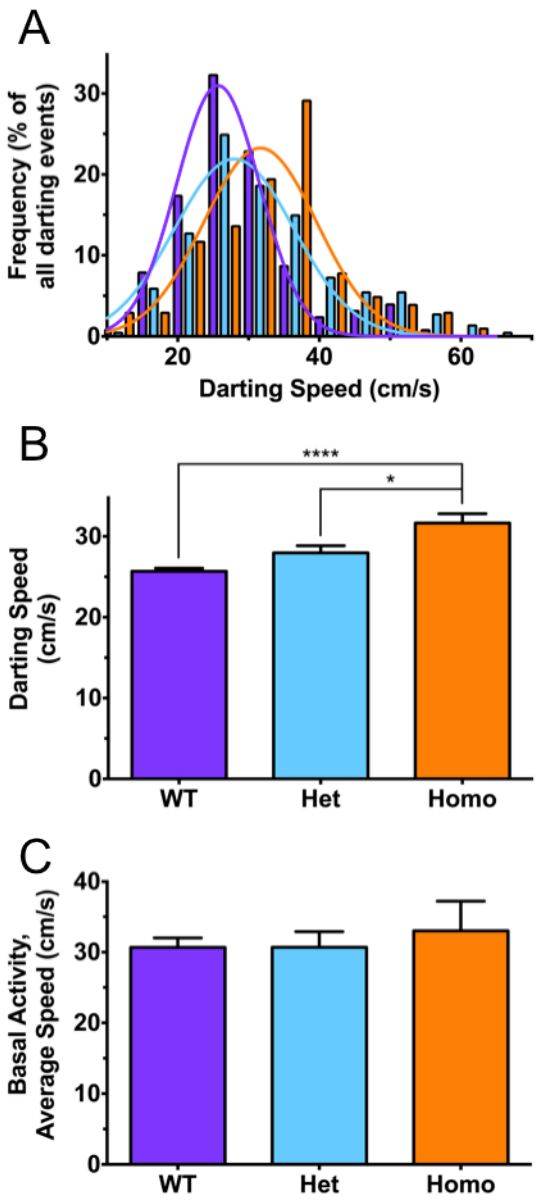

Figure 18. DAT Val559 mice display significantly increased darting speed upon imminent handling. (A) Frequency distribution of darting speeds. (B) Mean +/- SEM darting speed (n = 21 WT, 33 het, 14 homo; overall $P < 0.0001$, post hoc test (Tukey's multiple comparisons test) reveal **** = $P < 0.0001$ for WT vs. homo and * = $P < 0.05$ for het vs. homo comparisons, one-way ANOVA)) is significantly faster for DAT Val559 mice, but (C) mean +/- SEM locomotor speed during basal open field recording is not different among genotypes ($P > 0.05$, one-way ANOVA).

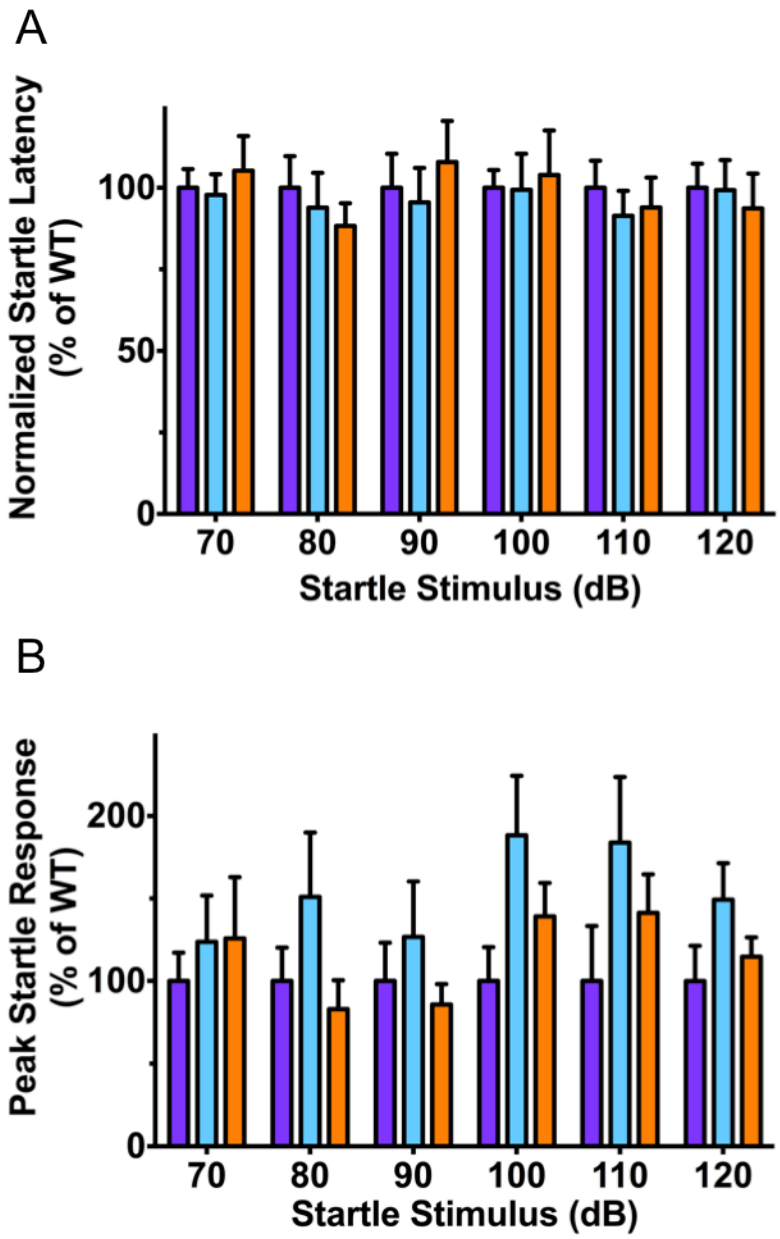

Figure 19. Despite significantly faster darting responses, DAT Val559 mice show no differences in (A) acoustic startle latency or (B) peak startle response (n = 8 per genotype; $P > 0.05$, two-way ANOVAs).

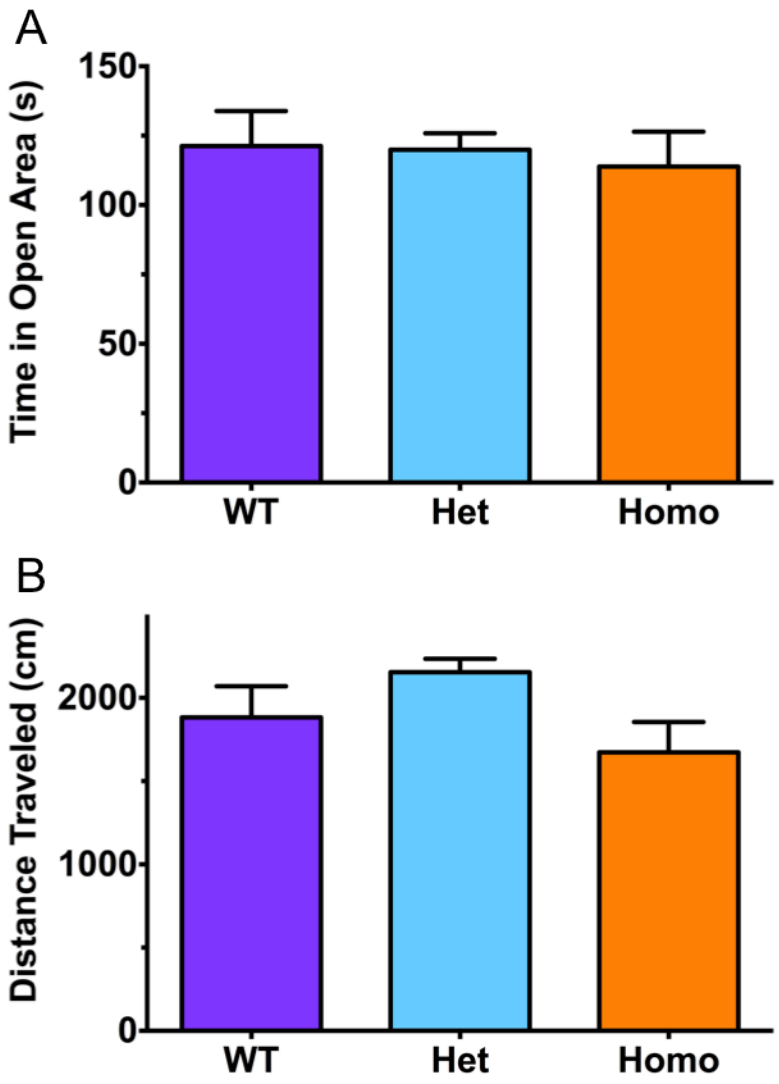

Figure 20. Despite significantly faster darting responses, DAT Val559 mice show no differences in elevated zero maze behavior, including (A) time in the open area and (B) distance traveled on the maze (n = 20 WT, 24 het, 14 homo; $P > 0.05$, one-way ANOVAs).

In vitro studies of AMPH action on DAT Val559 demonstrated that the drug acts only as a competitive substrate, inhibiting both DA uptake and ADE (Mazei-Robison et al., 2008), as opposed to its well-established ability to produce DAT-mediated DA efflux (Sulzer et al., 2005). Consistent with these previous findings, DAT Val559 mice have a significantly blunted (approximately 50%) locomotor response after i.p. administration of 3 mg/kg AMPH (Fig. 21B). Interestingly, the blunted response to AMPH of Val559 mice relative to WT animals was not limited to ambulatory behavior; AMPH also elicited significantly less rearing behavior in DAT Val559 mice (Fig. 22A), but stereotyped movements were equivalent between WT and DAT Val559 animals (Fig. 22B).

D1 receptors have been shown to mediate the locomotor hyperactivity triggered by AMPH treatment *in vivo* (O'Neill and Shaw, 1999; Hall et al., 2009)., After titrating the i.p. dosage (data not shown) of the D1 agonist SKF 83822 (2 mg/kg) to elicit an equivalent locomotor activation as 3 mg/kg AMPH, we observed no genotype differences in the locomotor response (Fig. 23A). DAT Val559 animals display a small but non-significant reduction in rearing behavior following SKF 83822 treatment (Fig. 23B), as well as no change in stereotyped behavior (Fig. 23C).

Across conditions (basal, saline-, AMPH-, or SKF 83822-treated), DAT Val559 animals display reduced locomotor behavior relative to WT mice only after AMPH treatment (Fig. 24A). In contrast, rearing behavior is significantly reduced at baseline, and continues to trend towards reduction across all other conditions (Fig.

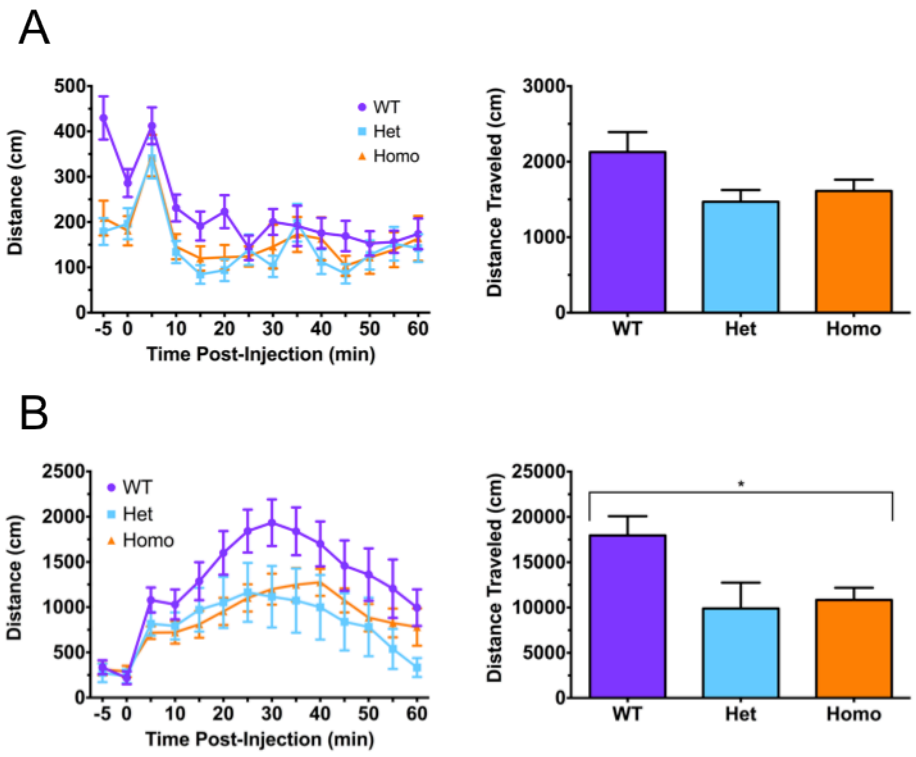

Figure 21. DAT Val559 mice have a significantly blunted locomotor response to AMPH. (A) DAT Val559 mice display a small but insignificant reduction in locomotor activity following saline injection (n = 37 WT, 21 het, 15 homo; *left*: time course of locomotor behavior, *P*(genotype) > 0.05, two-way RMANOVA, *right*: total +/- SEM distance traveled in 60 minutes after saline injection, *P* > 0.05, one-way ANOVA) but (B) an approximately 50% reduction in AMPH-induced locomotor stimulation (n = 9 WT, 9 het, 6 homo; *left*: time course of AMPH response, *P*(genotype) < 0.05, post hoc testing (Tukey's multiple comparisons test) reveals *P* < 0.05 at 20, 25, 40, 45, and 55 min and *P* < 0.01 at 30 and 35 min after AMPH injection for WT vs. het and *P* < 0.05 at 20, 25, 30, and 35 min after AMPH injection for WT vs. homo, two-way RMANOVA; *right*: total +/- SEM distance traveled in 60 minutes after AMPH administration, * = overall *P* < 0.05, one-way ANOVA).

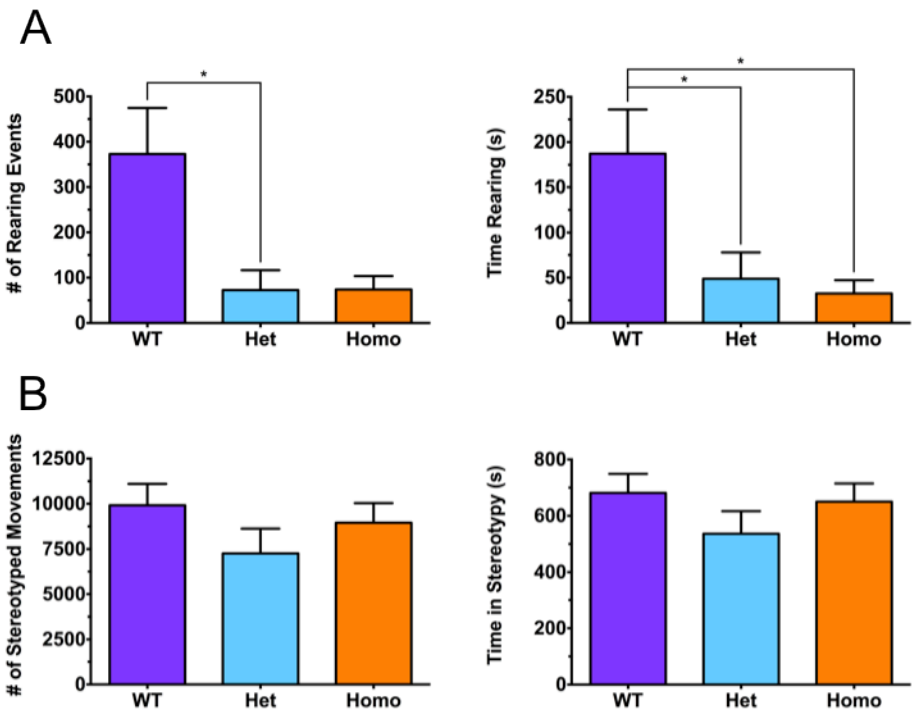

Figure 22. DAT Val559 mice display reduced rearing behavior following AMPH treatment. (A) The number of rearing behaviors and time spent rearing are significantly reduced following 3 mg/kg AMPH treatment (n = 9 WT, 9 het, 6 homo; *left*: number of rearing behaviors +/- SEM in 60 minutes following AMPH, overall $P < 0.05$, post hoc tests (Tukey's multiple comparisons test) reveals * = $P < 0.05$ for WT vs. het, $P = 0.0537$ for WT vs. homo, one-way ANOVA; *right*: time spent rearing +/- SEM after AMPH, overall $P < 0.05$, post hoc tests (Tukey's multiple comparisons test) reveals * = $P < 0.05$ for WT vs. het and WT vs. homo comparisons, one-way ANOVA) but (B) stereotyped behaviors are unaffected (n = 9 WT, 9 het, 6 homo; *left*: number of stereotyped behaviors +/- SEM in 60 minutes following AMPH, $P > 0.05$, one-way ANOVA; *right*: time in stereotypy +/- SEM in 60 minutes after AMPH, $P > 0.05$, one-way ANOVA).

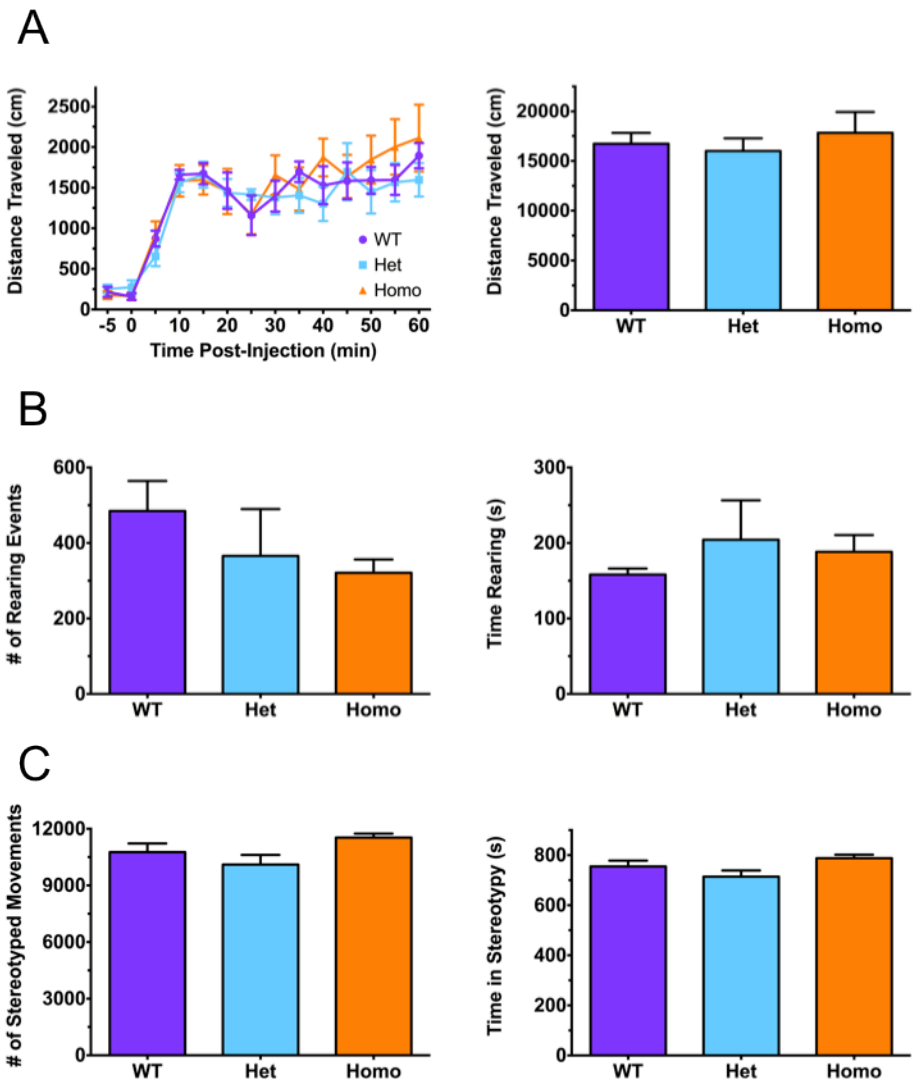

Figure 23. Locomotor activity, stereotypy, and rearing behavior do not differ between DAT Val559 mice and WT controls after treatment with D1 receptor agonist SKF83822. (A) Locomotor activity following treatment with 2 mg/kg SKF83822 (D1R agonist) does not differ between DAT Val559 mice and WT controls (n = 8 per genotype; *left*: time course of SKF83822 response, *P*(genotype) > 0.05, all post hoc comparisons *P* > 0.05, two-way RMANOVA; *right*: total +/- SEM distance traveled in 60 minutes following SKF83822 administration, *P* > 0.05, one-way ANOVA). Similarly, (B) rearing behavior (*left*: number of rearing behaviors +/- SEM following SKF83822 treatment, *P* > 0.05, one-way ANOVA; *right*: time +/- SEM spent rearing after SKF83822 administration, *P* > 0.05, one-way ANOVA) and (C) stereotyped behaviors (*left*: number of stereotyped movements following SKF83822 treatment, *P* > 0.05, one-way ANOVA; *right*: time +/- SEM in stereotypy after SKF83822 administration, *P* > 0.05, one-way ANOVA) do not differ between DAT Val559 mice and WT controls.

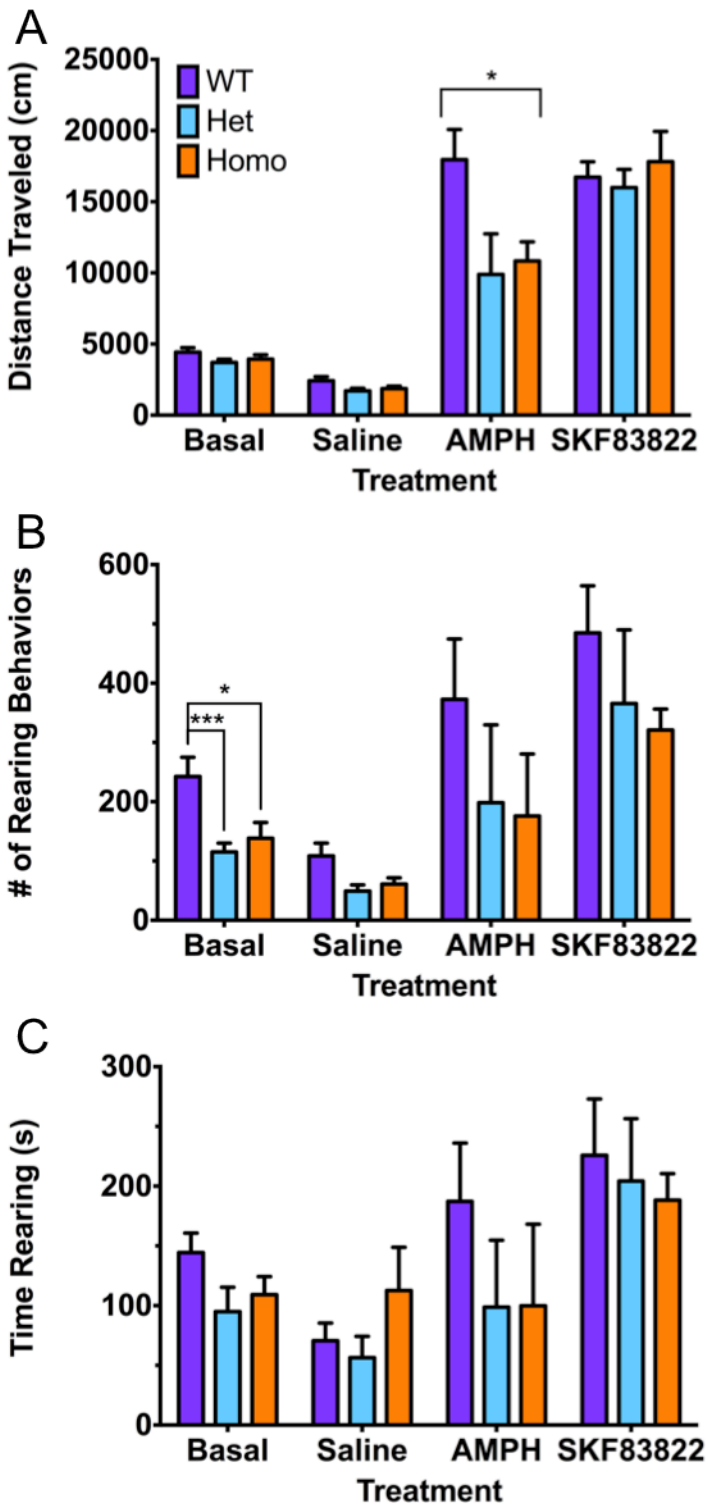

Figure 24. Locomotor and rearing behavior in an open field across all treatments. (A) Total distance traveled +/- SEM, (B) number of rearing behaviors +/- SEM, and (C) time spent rearing +/- SEM during 60 minutes following injection of nothing (basal), saline, AMPH, or D1 agonist SKF 83822.

24B, C). Stereotyped behavior does not differ among genotypes regardless of treatment condition (data not shown).

Discussion

Because ADHD is a childhood disorder (American Psychiatric Association, 1994) and displays a consistent male bias (Gaub and Carlson, 1997; Getahun et al., 2013a), we focused our studies on juvenile/adolescent (aged 5-9 weeks) male mice. All experiments were performed during the dark phase of the light-dark cycle. Mice are generally a nocturnal species, becoming more active during the dark phase (Cambras and Díez-Noguera, 1991; Goulding et al., 2008), and activity levels vary between mouse background strains (Goulding et al., 2008; Gürkan et al., 2008). In my own experience, the DAT Val559 mice were considerably less active during the light phase, such that their lack of activity made basal behavioral analysis difficult. For ease of performing experiments during the dark phase, therefore, mice were maintained on a reverse light-dark cycle. Mice were given at least one week to acclimate to the reverse light cycle, as light cues are known to affect feeding, sleep, and locomotor activity (Ikeda et al., 2000), and 5-6 days is sufficient for mice to re-entrain to the new light-dark cycle (Kopp et al., 1998).

Somewhat surprisingly, given the prediction of *in vivo* ADE for DAT Val559, no basal locomotor hyperactivity was observed in the open field test, and, in fact, the animals were slightly hypoactive, suggesting less arousal by the novel environment. The lack of hyperactivity relative to WT littermates suggests either that 1) ADE is not occurring *in vivo* or 2) that adaptive changes in response to ADE may have occurred that have diminished environmental arousal or overall exploratory

interest. In support of the latter hypothesis, DAT Val559 animals displayed reductions in rearing behavior in both open field chambers and in home cage recordings (see Appendix 3). Arguing against a perturbation of exploratory behavior, our preliminary studies using the novel object recognition paradigm reveal no impact of the DAT Val559 variant (see Appendix 3). At a molecular level, the observed change in rearing behavior could reflect a change in D2R function, as Kelly and colleagues demonstrated that D2R deficient mice display reduced locomotor activity and rearing behavior (Kelly et al., 1998). In addition, D2R KO mice have drastically reduced locomotor responses upon amphetamine treatment (Kelly et al., 2008; Neve et al., 2013), a finding attributed primarily to loss of the postsynaptic isoform, D2L. Since we observed no change in D2 protein levels in striatum and midbrain, we suspect that if changes in D2R do occur, they will be at the level of surface expression or functional coupling to signaling pathways. In light of the loss of AMPH sensitivity in D2R KO mice, downregulation of D2R functionality (likely desensitization arising from constant DA stimulation due to ADE) provides a reasonable mechanism for the blunted rearing behavior that we observe in DAT Val559 mice following AMPH treatment. Experiments to determine the underlying mechanism of the reduced rearing behavior observed in DAT Val559 mice are certainly warranted.

Although we observed no hyperactivity in the DAT Val559 mice on open field testing or in our preliminary studies with home cage monitoring, we did identify a novel hyperactive phenotype that we termed “darting”. We noticed that when we reached to transfer the mice between cages, the presence of a DAT Val559 allele

could be fairly reliably determined based only on a more robust escape reaction. As we found no genotype differences in tests of anxiety behavior or startle responses, we believe darting behavior to be a behavioral phenotype linked to a response to imminent handling. Interestingly, spontaneous darting behavior has been reported as a phenotype that reliably differentiates C57BL6J and DBA/2J inbred mouse strains (Kafkafi et al., 2003). Additionally, spontaneous darting behavior mediated by glutamatergic corticostriatal projections (Fonnum et al., 1981) has been reported following administration of the NMDA receptor antagonist CPP (3(2-carboxypiperazin-4-yl)-propyl-l-phosphonic acid) in the prefrontal cortex of rats (O'Neill and Liebman, 1987; Crawley et al., 1992). Together, these findings raise the possibility that alterations in DA signaling in the cortex or striatum of DAT Val559 mice could lead to alterations in glutamatergic signaling that contribute to darting.

The darting phenotype that we observed may derive from a deficit in cortical inhibition of DA release (Lodge, 2011). Studies in humans with ADHD have demonstrated deficits in response inhibition (reviewed in Aron and Poldrack, 2005; and Barnes et al., 2011) that has been explained using the "activation-suppression model" (van den Wildenberg et al., 2010). According to this model, the motor response to a salient but irrelevant stimulus must be suppressed in order to respond to a relevant stimulus. In the context of this model, the darting behavior exhibited by DAT Val559 mice may reflect an inability to suppress an escape response to imminent handling, a harmless stimulus that the animals experience regularly during cage transfers. Thus far, we have been unable to pharmacologically manipulate the darting response of DAT Val559 mice, likely due to associations of

approach linked to a negative stimulus that overwhelms genotype differences in darting behavior. Clearly, further studies are needed to define the nature of brain circuits and signaling alterations that drive the darting behavior we report.

AMPH is well known to elicit DAT-mediated DA efflux (Sulzer et al., 2005) and induce locomotor hyperactivity (Dews, 1953; Smith, 1965). Based on our *in vitro* findings with DAT Val559 that AMPH lacks DA releasing properties but remains a competitive substrate (Mazei-Robison et al., 2008), we predicted a blunted, locomotor stimulatory effect of AMPH though not a full insensitivity to the stimulant. Consistent with the *in vitro* data, DAT Val559 mice display an approximately 50% reduction in AMPH-induced locomotor activity. Interestingly, DAT Val559 mice exhibited reduced rearing behavior, both during acclimation to the open field, after saline injections, and after AMPH injections. D1Rs have been implicated in the locomotor hyperactivity triggered by AMPH *in vivo* (O'Neill and Shaw, 1999; Hall et al., 2009). Upon stimulation with the D1 agonist SKF83822 at a dose titrated to achieve stimulation at levels seen with 3 mg/kg i.p. AMPH, we observed no genotype differences in locomotor stimulation. This finding suggests that striatal D1 signaling is intact in DAT Val559 mice, and that the differences in locomotor response between WT and DAT Val559 mice observed with AMPH likely arise from presynaptic mechanisms and/or network-level plasticities.

As mentioned above, DAT Val559 mice were also recorded in a home cage monitoring (HCM) system (see Appendix 3). For HCM experiments, mice are housed individually, then video-recorded over the course of several days, and all behaviors documented by an automated system. While open field testing is informative,

especially for measuring drug effects, HCM analysis allows for detection of changes in basal behaviors, and removes potential confounds arising from the novelty of the testing apparatus. HCM of DAT Val559 mice confirms the reduction in rearing behavior observed in open field tests, and may prove useful for detecting spontaneous darting behavior.

Interestingly, Val559 hetero- and homozygotes displayed remarkably similar phenotypes on all behaviors measured. Such findings suggest that DAT Val559 may act in a dominant fashion in the heterozygous animals, such that one mutant allele drives the mutant phenotype despite the presence of a WT allele. Several studies have provided evidence that DAT forms dimers (Hastrup et al., 2001; Torres et al., 2003; Sorkina et al., 2003), providing a possible mechanism by which DAT Val559 might act in a dominant manner. Further studies are required to formally demonstrate DAT Val559's dominant effect.

CHAPTER V

IN VIVO AND *EX VIVO* CHARACTERIZATION

Introduction

As discussed previously, *in vitro* characterization of DAT Val559 revealed that the mutant transporter exhibits normal DA transport, but supports a spontaneous outward DA leak, a phenomenon termed anomalous DA efflux (ADE) (Mazei-Robison et al., 2008). Furthermore, DAT Val559 displays an inappropriate response to the psychostimulant AMPH – instead of inducing DAT-mediated DA efflux, AMPH blocks both DA uptake and ADE. Based on these phenotypes, we made several predictions and performed several experiments regarding the biochemical (Chapter 3) and behavioral (Chapter 4) ramifications of the DAT Val559 variant.

Despite a strong *in vitro* phenotype, there remains a possibility that adaptive changes can occur during development that might change how DAT Val559 functions *in vivo*. For example, compensatory changes in DA synthesis, vesicle pools, DAT regulation, or alternative inactivation mechanisms could be engaged to nullify any extracellular changes in DA that are expected from DAT-mediated ADE or AMPH blockade. Additionally, we certainly cannot disregard the possibility that the Val559 allele produces other effects on DAT *in vivo* that could alter the basal efflux observed *in vitro*. Thus, in order to provide direct evidence of basal DA efflux and an altered ability of AMPH to produce transporter-mediated DA efflux, we performed *in vivo* microdialysis and *ex vivo* [^{3}H]-DA release studies. These techniques allow for

measurement of basal and evoked DAT Val559 function in native tissue, and provide critical evidence regarding the ongoing potential for altered DAT function to modify synaptic events.

Methods

Microdialysis

Mice were anesthetized with isoflurane (5% for induction, 2% for maintenance during surgery) and placed in a stereotaxic frame using mouse-specific ear bars (Kopf Instruments, Tujunga, CA). 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 using glass ionomer cement (Instech Solomon, Plymouth Meeting, PA). After recovery from surgery (18-24 hours), animals were placed in individual dialysis chambers (clear cylindrical enclosure, 14 cm diameter, 22 cm high; Instech Solomon, Plymouth Meeting, PA). A microdialysis probe (CMA Microdialysis, USA) 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 attached to a liquid swivel (Instech Solomon, Plymouth Meeting, PA) that was mounted on a counterbalanced arm above the dialysis chamber. The probe was perfused with artificial cerebral spinal fluid (aCSF; 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.4 mM d-glucose, pH 7.2) at a flow rate of 1.0 uL/minute overnight. After the fourth baseline sample, the aCSF was switched to aCSF containing 0.1 uM D-amphetamine for the remaining 9 samples. Dialysate samples were stored at -80°C and analyzed by HPLC-EC for DA and serotonin levels, as described above. After the dialysis session, animals were overdosed with sodium

pentobarbital, brains removed and post-fixed in 4% paraformaldehyde in 100 mM phosphate buffer, sectioned, stained for Nissl substance, and then inspected for acceptable probe placement. Data were analyzed using two-way ANOVA with Sidak's *post hoc* test for multiple comparisons.

DA Release in Striatal Slices

Brains were harvested from mice following rapid decapitation. Brains were immediately placed on an ice-cold metal platform and the striatum quickly dissected. Striatal slices were then incubated for 30 min. with 150 nM [^{3}H]-DA at 37^{o}C and loaded into the perfusion chambers of a Brandel SF-12 superfusion system (Brandel, Inc., Gaithersburg, MD), sandwiched between GF/B glass fiber filter discs (Whatman, Clifton, NJ). The chambers were perfused at a flow rate of 0.4 mL/min. with oxygenated Krebs'-Ringer's-HEPES (KRH) buffer (130 mM NaCl, 3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM D-glucose, 10 mM HEPES pH 7.4) at 37°C for 1 hr prior to the start of the experiment to achieve a steady baseline of [^{3}H]-DA release. For studies of K^+- or AMPH-evoked [^{3}H]-DA release, chambers were perfused with KRH at a flow rate of 0.4 mL/min and samples were collected every 5 minutes. After collection of 3 baseline samples, perfusate was switched to KRH buffer supplemented with 20 mM KCl to evoke vesicular [^{3}H]-DA release or 1 uM D-AMPH to evoke non-vesicular [^{3}H]-DA release for 15 min and then returned to perfusion with KRH for an additional 15 min. For studies of quinpirole modulation of DA release, two 5 min baseline KRH perfusates were collected and then the perfusion buffer was switched to KRH supplemented with 5 uM quinpirole for 5 min. Then the perfusate was switched to KRH containing 5 uM quinpirole and 1 uM

D-AMPH and samples were collected for the next 15 minutes, followed by a return to perfusion for 15 minutes with KRH. Five mL of EcoScint XR scintillation fluid (National Diagnostics, Atlanta, GA) was added to all samples and radioactivity was counted using a TriCarb 2900TR scintillation counter (Perkin Elmer, Waltham, MA). Data for [^{3}H]-DA released are presented as the fraction of the total [^{3}H]-DA loaded into each sample (amount released + amount remaining in the tissue). Data were analyzed using either one-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons (for total [^{3}H]-DA release) or two-way ANOVA with Sidak's *post hoc* test for multiple comparisons (for time course of [^{3}H]-DA release).

Results

In vivo *Microdialysis*

In order to determine whether the ADE, and loss of AMPH-induced DA efflux, observed in our *in vitro* studies of transfected DAT Val559 could account for the blunted locomotor response to AMPH we observed in the DAT Val559 mice, we performed *in vivo* microdialysis studies in unanesthetized, freely moving animals and used HPLC to measure endogenous extracellular levels of DA and 5-HT. Although basal extracellular 5-HT levels in the striata of WT and DAT Val559 animals exhibited no significant genotype effect (Fig. 25A), such an effect was evident for extracellular DA levels where we observed an ~10-fold increased in DA levels in the DAT Val559 homozygotes over that of WT control animals (Fig. 25B). Although variability in the samples from heterozygous DAT Val559 mice precluded a conclusion of significant changes, our mean values for these animals were also much higher than seen for WT animals.

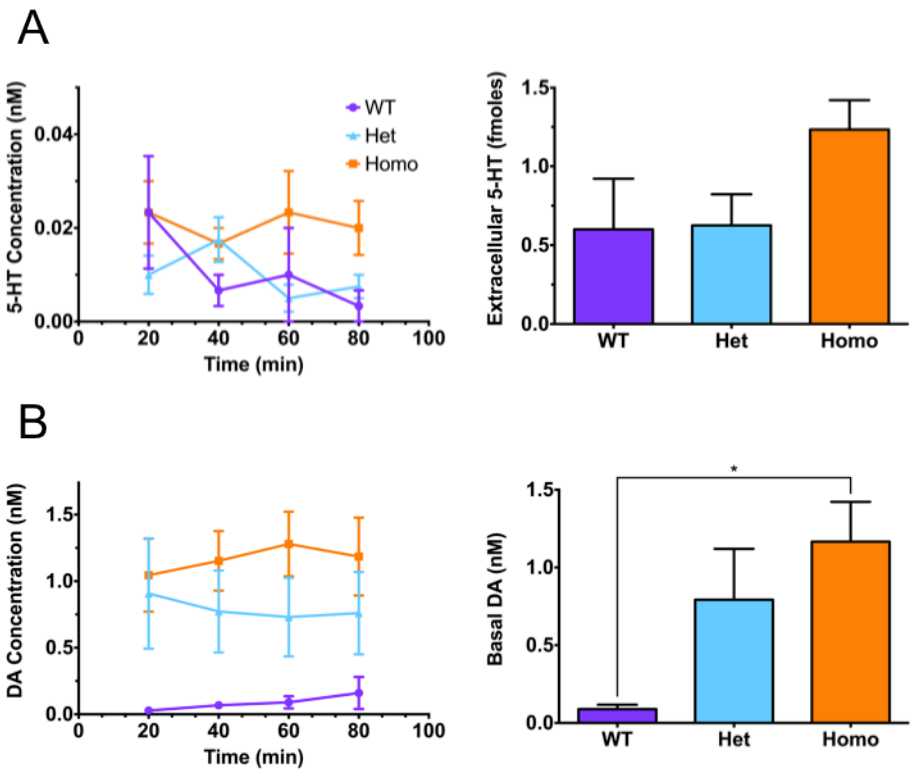

Figure 25. DAT Val559 mice have elevated basal extracellular DA, but 5-HT levels are unaffected. (A) Basal extracellular 5-HT levels do not differ between genotypes (n = 4 per genotype; *left*: time course of basal 5-HT release, *P*(genotype) > 0.05, two-way RMANOVA; *right*: mean +/- SEM extracellular 5-HT concentration prior to AMPH stimulation, *P* > 0.05, one-way ANOVA) but (B) basal extracellular DA levels are significantly elevated in DAT Val559 mice (n = 4 per genotype; *left*: time course of basal DA release, *P*(genotype) < 0.01, post hoc tests (Tukey's multiple comparisons test) reveal *P* < 0.05 at 20 min for WT vs. het and *P* < 0.05 at 20, 40, and 80 min and *P* < 0.01 at 60 min for WT vs. homo, two-way RMANOVA; *right*: mean +/- SEM extracellular DA concentration prior to AMPH stimulation, * = *P* < 0.05, one-way ANOVA).

Next, to probe the capacity of AMPH to induce DA efflux by DA terminals, we infused AMPH (0.1 μM) into the striatum of cannulated mice, sampling to monitor extracellular DA levels before and after infusion. We chose this route of administration as systemic AMPH would have more complex effects and possibly cloud interpretations as compared to direct application of the drug onto DA terminals. Whereas intrastriatal administration of AMPH induced genotype-independent 5-HT release (Fig. 26A), both heterozygous and homozygous DAT A559V animals exhibited a significantly lower release of DA as compared to WT littermates. Indeed, DAT Val559 heterozygous and homozygous animals released ~10 times less DA than WT controls (Fig. 26B).

Ex vivo *[^{3}H]-DA Release from Striatal Slices*

AMPH-evoked DA release *in vivo* occurs in the context of DA neuron firing that could differ between genotypes. To determine whether DA terminals of DAT Val559 animals exhibit a diminished capacity for AMPH-evoked DA release independent of afferent activity (i.e. regardless of neuron activity, DAT Val559 does not respond to AMPH), we monitored the release of preloaded [^{3}H]-DA from striatal slices *ex vivo*. Striatal slices from WT and DAT Val559V animals loaded with [^{3}H]DA to the same extent (Fig. 27A). Next, using WT slices, we established an AMPH concentration (1 uM) that generated an intermediate capacity for DA release, such that neither ceiling nor floor effects would obscure genotype effects. As predicted by heterologous expression and *in vivo* microdialysis studies, DAT Val559 slices demonstrated a significantly reduced capacity for evoked DA efflux as compared to slices from WT controls (Fig. 27B).

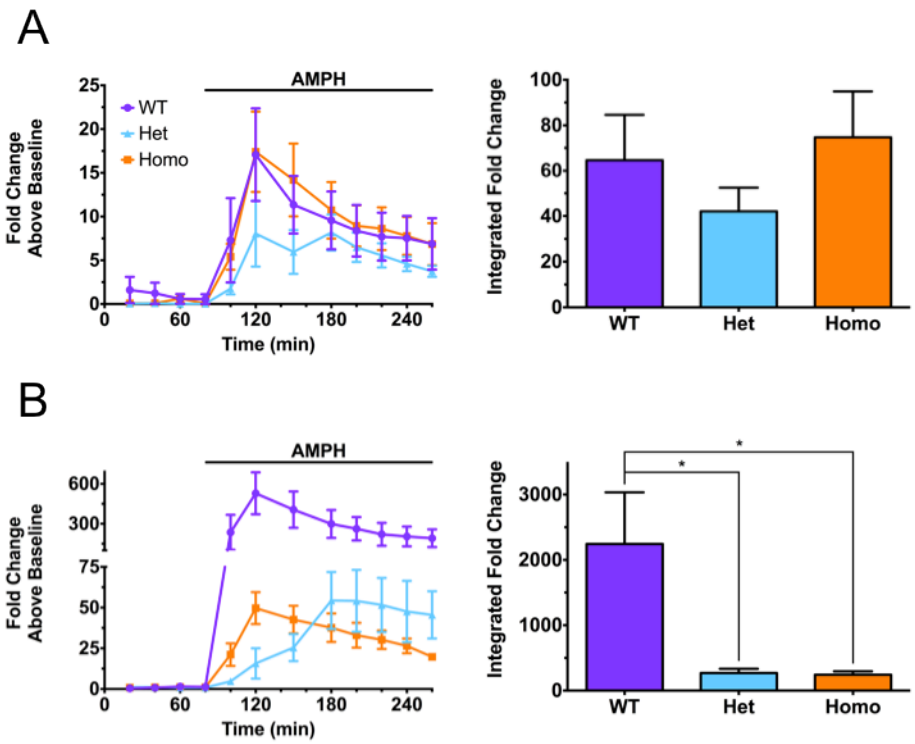

Figure 26. DAT Val559 mice display significantly blunted AMPH-evoked DA release, but 5-HT release is unaffected. Upon AMPH stimulation, (A) evoked 5-HT does not differ between genotypes (n = 4 per genotype; *left*: time course of evoked 5-HT release, expressed as fold change above baseline +/- SEM, *P*(genotype) > 0.05, two-way RMANOVA; *right*: mean +/- SEM integrated fold change of 5-HT relative to baseline, *P* > 0.05, one-way ANOVA), but (D) AMPH-evoked DA is reduced ~10-fold in DAT Val559 mice (n = 4 per genotype; *left*: time course of evoked DA release, expressed as fold change above baseline +/- SEM, *P*(genotype) < 0.05, post hoc tests (Tukey's multiple comparisons test) reveal *P* < 0.05 at 180 min, *P* < 0.01 at 100 and 160 min, and *P* < 0.0001 at 120 and 140 min for WT vs. het and *P* < 0.05 at 100, 200, and 220 min, *P* < 0.01 at 160 and 180 min, and *P* < 0.0001 at 120 and 140 min for WT vs. homo, two-way RMANOVA; *right*: mean +/- SEM integrated fold change of DA relative to baseline, overall *P* < 0.05, post hoc tests (Tukey's multiple comparisons test) reveal * = *P* < 0.05 for WT vs. het and WT vs. homo, one-way ANOVA).

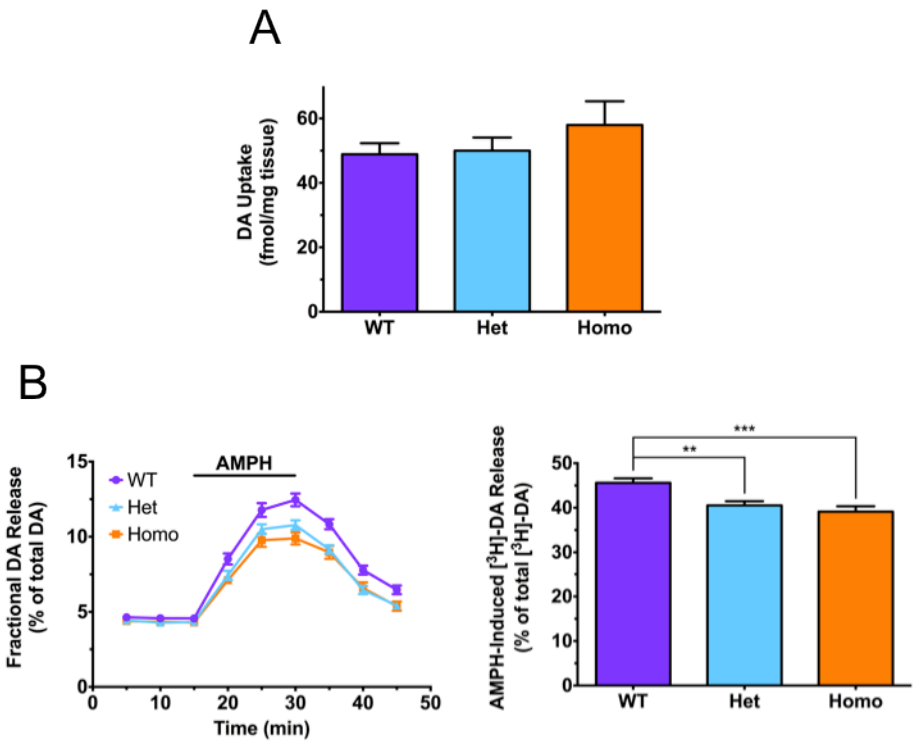

Figure 27. Striatal slices from DAT Val559 animals show blunted [^{3}H]-DA release. (A) Striatal slices from WT and DAT Val559 mice accumulate similar levels of [^{3}H]-DA (n = 11 WT, 10 het, 11 homo; $P > 0.05$, one-way ANOVA), but upon application of 1 uM AMPH (B) DAT Val550 tissue releases significantly less [^{3}H]-DA than WT tissue (n = 11 WT, 10 het, 11 homo; *left*: time course of AMPH-evoked [^{3}H]-DA release, expressed as percentage of available DA released +/- SEM, P(genotype) < 0.001, post hoc tests (Tukey's multiple comparisons test) reveal $P < 0.05$ at 20 and 45 min, $P < 0.01$ at 25 and 40 min, and $P < 0.001$ at 30 and 35 min for WT vs. het, and $P < 0.01$ at 40 and 45 min, $P < 0.01$ at 20 min, and $P < 0.001$ at 25, 30, and 35 min, two-way RMANOVA; *right*: total percentage +/- SEM of AMPH-evoked [^{3}H]-DA released, overall $P < 0.001$, post hoc tests (Tukey's multiple comparisons test) reveal ** = $P < 0.01$ for WT vs. het and *** = $P < 0.001$ for WT vs. homo comparisons, one-way ANOVA).

Discussion

Two key *in vitro* phenotypes of the DAT Val559 variant are ADE and a loss of AMPH-induced DA release (Mazei-Robison et al., 2008). Our findings of significant elevations of basal extracellular DA levels in the striatum of DAT Val559 knock-in mice provide supportive evidence of ADE *in vivo*. However, AMPH's effects *in vivo* are less straightforward. In a heterologous expression system, AMPH blocks DAT-mediated uptake and efflux, but *in vivo*, AMPH does not block efflux, and actually elicits some DA release. It is unlikely that DAT Val559 fails to recognize AMPH, as *in vitro* studies demonstrated equivalent inhibitory potency in blocking DA transport through WT or DAT Val559 transporters (Mazei-Robison et al., 2008). It remains unclear if some AMPH is finding a way into dopaminergic terminals despite DAT Val559's inability to transport it. A two-mechanism model for AMPH entry into neurons has been proposed (Liang and Rutledge, 1982); at low concentrations, AMPH is transported as a DAT substrate and may be responsible for increased locomotor activity, but at higher concentrations, AMPH can passively diffuse across plasma membranes and induce DA release (Zaczek et al., 1991a, b). Assuming that DAT Val559 does not transport AMPH *in vivo*, some AMPH may still enter dopaminergic neurons by passive diffusion, at which point AMPH's ability to induce DA release is still intact.

Alternatively, presynaptic compensations could explain the observed response to AMPH. DAT KO mice display an approximately 90% reduction in D2 autoreceptor function (Jones et al., 1999), a finding attributed to receptor downregulation resulting from constant DA exposure. DAT Val559 mice are also

hyperdopaminergic due to ADE, thus autoreceptors are likely downregulated. Furthermore, in D2R KO mice, the locomotor response to cocaine is significantly reduced relative to WT mice, an effect mediated in large part by presynaptic D2 autoreceptors, as D2L (postsynaptic D2R) KO mice show only a slight reduction in the locomotor response to cocaine (Welter et al., 2007). If D2 autoreceptors are downregulated in DAT Val559 mice and AMPH is acting only as a DAT inhibitor (like cocaine), then we would expect a blunted response to AMPH. In addition, we have not yet tested the impact of a DAT inhibitor such as methylphenidate via microdialysis. Such experiments are necessary to demonstrate that AMPH acts as only as a DAT blocker for DAT Val559.

At this point, however, we cannot rule out the possibility that elevated tonic firing rates of DA neurons in DAT Val559 mice could account, to some degree, for the elevated basal extracellular DA levels. Somatodendritic and presynaptic D2 DA receptors exert powerful negative feedback control over DA neuron firing (Lacey et al., 1987; Beckstead et al., 2007) and vesicular DA release (Anzalone et al., 2012; Zhang and Sulzer, 2012), respectively. Autoinhibitory mechanisms would be expected to oppose local or circuit-level compensations that could drive excessive firing and release of DA. However, under sustained receptor activation, as occurs with chronic DAT blockade, D2 DA receptor-mediated inhibition of firing and release can be desensitized (Jones et al., 1996; Katz et al., 2010). DAT Val559-mediated ADE could result in a similar state of chronic receptor activation, receptor desensitization, and a failure to exert autoinhibitory control over DA neuron firing and excitation-secretion coupling. Although we detected no changes in the levels of

D2 receptors in the midbrain or striatum (see Chapter 3), radioligand binding assays as performed in our studies do not provide information on receptor surface levels or receptor coupling to effectors. Thus, further studies are needed to examine whether DA autoreceptors retain their signaling capacity in the face of constitutive elevation of extracellular DA and to compare resting and modulated DA neuron firing rates *in vivo*.

In addition to tonically elevated extracellular DA in the striatum of DAT Val559 mice, we also observed a diminished capacity for AMPH-evoked DA release in striatal slices *ex vivo* and in the striatum *in vivo*. The blunted ability of AMPH to induce DA release in striatal slices also supports the presence of presynaptic changes independent of any possible changes in DA neuron excitability since AMPH-induced DA release is not dependent on impulse flow. The more substantial loss of AMPH's ability to induce DA release *in vivo* versus *ex vivo* in brain slices may be due to the engagement of other signaling pathways that facilitate AMPH induced DA release *in vivo*. Alternatively, the greater AMPH effect *in vivo* may arise from a measurement of release in this context from endogenous DA stores, whereas our *ex vivo* studies measure the release of preloaded, radiolabeled DA. AMPH treatments lead to liberation of endogenous DA from vesicle stores, providing high cytoplasmic levels that serve as a substrate for DAT-mediated export. Pre-labeling of DA terminals with [^{3}H]-DA is known to largely load a small population of rapidly recycling, readily-releasable vesicles (Herdon et al., 1985; Covey et al., 2013), as opposed to the loading of reserve pool vesicles that are already filled with unlabeled DA. Although it is assumed that radiolabeled neurotransmitter is liberated

equivalently by AMPH from these pools, but if this is not the case, differences between the slice and dialysis measures could arise. Additionally, there may simply be ceiling effects on the amount of radiolabeled neurotransmitter that can be released due to a continual loss of neurotransmitters from the slices that is not resupplied by synthesis, unlike endogenous stores. As our studies progress, incorporation of such distinctions between vesicle pools will likely be necessary, and informative, in understanding the full impact of the DAT Val559 variant on DA signaling. Further studies are needed to examine the effects of AMPH on endogenous vs. prelabeled DA release both *in vitro* and *in vivo*.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

DA signaling is critically important for several behaviors including motor function, reward and motivation, and working memory and executive function (Barnes et al., 2011; Mitaki et al., 2013). Several neuropsychiatric disorders, including drug abuse and addiction (Kalivas and Volkow, 2005), bipolar disorder (Manji and Lenox, 2000; Jones and Craddock, 2001), schizophrenia (Ross et al., 2006; Eyles et al., 2012), Parkinson's disease (Temlett, 1996), and ADHD (Faraone and Biederman, 1998; Mazei-Robison and Blakely, 2005) all involve dopaminergic dysfunction. A key regulator of maintenance of dopamine signaling in the brain is DAT. The DAT KO mouse was initially proposed as a model for ADHD due to its early hyperkinetic phenotype (Giros et al., 1996), however humans with homozygous loss-of-function mutations in the DAT gene develop a complex motor disorder described as infantile parkinsonism-dystonia (Kurian et al., 2009, 2011). Prior to the Blakely lab's efforts to study DAT variation in ADHD, there was no direct link between DAT dysfunction and disease.

DAT is the primary site of action for the most commonly prescribed therapeutic agents used the treat ADHD, suggesting that it likely has a role in ADHD's underlying pathophysiology. Several DAT coding variants had been reported in large-scale genetic screens (Grünhage et al., 2000; Vandenbergh et al., 2000), however it was clear that common coding polymorphisms in DAT that could

confer risk for ADHD were unlikely to exist. However, pursuing the hypothesis that ADHD subjects represented a clinical population enriched for DAT variation, efforts to identify rare, highly penetrant DAT variants were pursued (Mazei-Robison et al. 2005; Mergy MA and Blakely RD, unpublished findings (see Appendix 1)).

Using a high-throughput single nucleotide polymorphism (SNP) discovery system, our lab identified four DAT variants – Val24Met, Leu167Phe, Ala559Val, and Arg615Cys – in ADHD subjects. Of particular interest was Ala559Val, as it had been previously reported in a subject diagnosed with bipolar disorder (Grünhage et al., 2000), then re-discovered in two brothers with ADHD (Mazei-Robison et al., 2005). ADHD and bipolar disorder are highly comorbid (Bernardi et al., 2010; Karaahmet et al., 2013;Pataki and Carlson, 2013), suggesting that DAT Val559 may contribute to both disorders. Furthermore, the small pedigree known for DAT Val559 reveals transmission to the two affected boys from the mother and maternal grandmother. Unfortunately, no other males could be identified as carriers of the variant. The female carriers did not meet diagnostic criteria for ADHD, however the mother reported a mild learning disability and the grandmother rated above average for impulsivity/emotional lability traits on the Conner's Adult ADHD rating scale (CAARS-S:L) (Mazei-Robison et al., 2008).

In vitro characterization revealed that DAT Val559 supports anomalous DA efflux (ADE) that can be blocked by cocaine, methylphenidate, and AMPH (Mazei-Robison et al., 2008). Subsequent work demonstrated that ADE is supported by tonic D2 receptor signaling in a CaMKII-dependent mechanism (Bowton et al., 2010). CaMKII interacts with the DAT C-terminus and is thought to phosphorylate

N-terminal serine residues that are required for AMPH-induced DA efflux (Khoshbouei et al., 2004; Fog et al., 2006). DAT Val559 has been show to be hyperphosphorylated relative to WT DAT (Sakrikar, 2012), and it was suggested that the mutation in DAT Val559 pushes DAT into an efflux-willing conformation that occur after AMPH treatment.

In light of the functional perturbations associated with DAT Val559 *in vitro*, we generated the DAT Val559 knock-in mouse in order to study the biochemical and behavioral effects of this variant *in vivo*. Using traditional transgenesis methods, we successfully created the DAT Val559 mouse. These mice display normal growth, development, and basic sensorimotor function, suggesting that our manipulation of the DAT gene and the presence of the Val559 mutation are well tolerated. Interestingly, we observed a small but significant under-representation of the Val559 allele, particularly in male mice. This suggests that DAT function has a neurodevelopmental role. The male bias of the DAT Val559 under-representation is also interesting in light of the male predominance of ADHD, suggesting a role for DAT in a common pathway between ADHD and neurodevelopment. In order to fully understand how DAT Val559 expression during development imparts long-lasting effects on behavior, future efforts may involve creation of a conditional DAT Val559 knock-in mouse in which the temporal expression of DAT Val559 can be controlled. Alternatively, we could use a viral approach in adult animals to examine the effects of DAT Val559 in the adult animal.

To date, DAT Val559 mice are maintained on a hybrid background that is ~75% 129S6 and ~25% C57BL/6. Backcross to a congenic C57 line has been

completed and certainly warrants further study. Pure inbred strains provide a stable genetic background, and C57BL/6 mice are particularly useful for behavioral and pharmacological analysis (reviewed in Puglisi-Allegra and Cabib, 1997; and Cabib et al., 2002). Furthermore, some phenotypes are affected by strain background. O'Neill and Gu reported that the amphetamine response of mice expressing a cocaine-insensitive DAT was altered upon completion of backcrossing to a pure C57BL/6 background (O'Neill and Gu, 2013). Kerr and colleagues report similar findings in the Gly56Ala SERT knock-in mouse model of autism; when backcrossed to the C57 strain, several phenotypes including hyperserotonemia, 5-HT receptor sensitivity, and repetitive behaviors were lost (Kerr et al., 2013). Studies of DAT Val559 on a pure C57BL/6 background may confirm the impact of DAT Val559 (i.e. phenotypes will not change), or indicate that other genetic factors (i.e. modifier genes, epigenetic regulation, etc.) may be contributing to the observed phenotypes.

In vitro studies demonstrated that WT DAT and DAT Val559 express equivalently (total and surface) and did not differ in their DA transport capabilities. Consistent with these findings, DAT Val559 and WT mice express equal levels of DAT protein and, in striatal synaptosomes, show equivalent DA transport kinetics. Further studies involving cell-surface biotinylation in brain slices are needed to confirm that surface levels of DAT Val559 are normal, as well.

We also examined DA receptor levels. Due to the high degree of homology between DA receptor subtypes, antibodies that differentiate receptor subtypes are not consistent and may not accurately reflect receptor expression levels. Therefore,

we performed radioligand binding studies on membranes prepared from WT and DAT Val559 mice. Unfortunately, pharmacological agents only discriminate between D1-like and D2-like DA receptors, but that, at least, separates receptors by functional class. Binding studies reveal no differences in receptor density between WT and DAT Val559 animals in cortex, midbrain, or striatum. Receptor density, however, may be misleading: receptor function and expression can be uncoupled, so further studies are needed to assay receptor function.

In addition to studying receptor function directly, it is also worthwhile to examine signaling pathways downstream of DA receptor activation. As reviewed in Chapter 1, DA receptors signal through several pathways, including DARPP-32 and GSK3β. If DA receptor signaling is altered in response to DAT Val559-mediated ADE, then downstream pathways may be differentially activated to compensate for the DA receptor dysregulation. On the other hand, DA receptor function may be altered and, as a result, downstream signaling may be dysregulated, as well, and the impact of DAT Val559 ADE can spread far beyond the dopaminergic synapse. Studies are currently underway to probe the impact of DAT Val559 on DARPP-32 phosphorylation (GL Davis, personal communication).

Alteration of striatal and cortical serotonin was, perhaps, the most surprising biochemical alteration observed in DAT Val559 mice, thus far. We observed significant increases in tissue 5-HT levels, suggesting that 5-HT circuits are compensating for dysfunctional DA circuits. Serotonergic adaptation to altered DA signaling is not unprecedented – DAT KO mice respond to psychostimulants in a SERT-dependent manner (Gainetdinov et al., 1999) and loss of DA neurons via 6-

OHDA lesion induces serotonergic axon sprouting (Avale et al., 2004b). The two primary 5-HT receptors that impinge upon DA circuits are 5-HT_{2A} (excitatory) and 5-HT_{2C} (inhibitory) (Di Matteo et al., 2002; Porras et al., 20202; reviewed in Esposito et al., 2008). Future efforts should explore changes in 5-HT receptor expression and 5-HT receptor-dependent behaviors. For instance, the 5-HT_{2A} agonist DOI induces a robust head twitch response (HTR) and is a straightforward behavioral assay for $5HT_{2A}$ receptor activation (Halberstadt and Geyer, 2013).

Behavioral characterization of DAT Val559 mice revealed a surprising small but significant reduction in basal locomotor activity. In addition, DAT Val559 mice had a significantly blunted response to the psychostimulant AMPH, but the locomotor response to the D1 agonist SKF 83822 was intact. Based on the *in vitro* finding that AMPH acts to block transport and efflux through DAT Val559 (Mazei-Robison et al., 2008), the observed locomotor behaviors are remarkably similar to those of D2R KO mice. In D2R KO mice, basal locomotor activity is significantly reduced, as is locomotor activity following treatment with cocaine (Welter et al., 2007). The locomotor response to cocaine, however, was restored when only postsynaptic D2Rs were knocked out, indicating that presynaptic D2 autoreceptor function is responsible for cocaine-induce hyperlocomotion. In DAT Val559 mice, ADE means that D2 autoreceptors are chronically exposed to DA and, therefore, may be desensitized. Thus, even though D2Rs have not been experimentally manipulated in DAT Val559 mice, they may be functionally "knocked out" and AMPH's induction of locomotor activity is severely blunted.

It is important to note that we have not yet tested the behavioral effects of DAT inhibitors such as cocaine or methylphenidate on DAT Val559 mice. In the event that the locomotor response to methylphenidate or cocaine resembles the response to AMPH, such findings would provide evidence that AMPH is merely blocking DAT and support the hypothesis that D2 autoreceptors are desensitized. These behavioral experiments would dovetail nicely with biochemical assays of DA receptor function, as well.

Despite no spontaneous hyperactivity, we noticed early on in our handling of DAT Val559 animals that the DAT Val559 genotype could be fairly reliably assigned based on the presence of a robust escape response to imminent handling, a behavior that we termed "darting". Analysis of darting speed revealed that DAT Val559 mice display faster darting speeds than their WT counterparts. We also considered that darting may be a locomotor manifestation of a startle response or an anxiety response. Acoustic startle testing revealed no difference in startle response at any decibel level test. Testing on the elevated zero maze revealed no anxiety-like behavior. It appears that darting is a phenotype unique to DAT Val559 mice.

In an attempt to ameliorate the darting response, I injected mice with AMPH. The results were uninterpretable, however, as injection with saline affected the distribution of darting speeds among genotypes. It appears that a stressor more substantial than handling (i.e. an injection) may affect darting behavior. In order to test the effects of AMPH on darting behavior, AMPH will need to be administered orally (i.e. mixed into a highly palatable food substance such as peanut butter or a chocolate drink such as Ensure). This finding also raises the possibility that DAT

Val559 mice are more sensitive to stress than their WT counterparts; testing DAT Val559 mice in acute and chronic stress paradigms may provide a fruitful line of research.

It is worth noting that all behavior experiments were performed in juvenile male mice. Despite the under-representation of DAT Val559 alleles predominantly in male mice, as well as the consistently documented 3:1 male bias in ADHD diagnosis (Gaub and Carlson, 1997; Getahun et al., 2013a), the DAT Val559 allele was transmitted from the maternal grandmother (impulsive symptoms) and mother (learning disability, but no ADHD symptoms) to the ADHD subjects where the variant was identified (Mazei-Robison et al., 2008). Future studies may focus on the effect of DAT Val559 in female mice. If DAT Val559 maintains its biochemical characteristics in females (preliminary data suggests that it does), but behavior differs between males and females, then DAT Val559 mice may allow for identification of female-specific factors that confer protection from ADHD.

In order to directly test for ADE and loss of AMPH-induced efflux that were observed *in vitro*, we performed *in vivo* microdialysis studies in unanesthetized, freely moving animals and HPLC to measure endogenous levels of extracellular DA and 5-HT. We observed no difference in basal 5-HT levels, but basal DA was elevated ~10-fold in DAT Val559 homozygous mice. We then applied AMPH directly to the striatum and measured AMPH-induced changes in DA and 5-HT levels. AMPH-induced 5-HT release was similar for all genotypes, but DA release was ~10-fold less in DAT Val559 homozygotes than in WT animals. Taken together, these data indicate that DAT Val559 functions *in vivo* as it did *in vitro*.

We also examined [^{3}H]-DA release in an *ex vivo* slice preparation. Striatal slices were pre-loaded with [^{3}H]-DA, and we then measured AMPH-induced DA release. Since *in vivo* DA release occurs in the context of neuron firing that could be different among genotypes, the de-afferented *ex vivo* slice preparation allows us to assay AMPH-induced DA release as an inherent property of DAT Val559. In this paradigm, we observe equal [^{3}H]-DA loading into slices, but significantly reduced AMPH-induced release.

Microdialysis and slice release studies are still ongoing, as these paradigms allow us to probe the contributions of vesicular and non-vesicular DA release, as well as possible alterations in distribution of vesicle pools. Current efforts are focused on assaying vesicular release using either K^+ or 4-aminopyridine (4-AP) stimulation (R Gowrishankar and PJ Gresch, personal communications). These experiments will offer insight regarding the possible redistribution of DA among vesicle pools in DAT Val559 mice.

Since we have evidence that DAT Val559 supports ADE *in vivo* and behavioral and pharmacological evidence that DA signaling is disrupted as a result, we might predict that larger networks of genes are affected as a result of DAT Val559. Thus, transcriptome analysis would be a worthwhile pursuit, as it would provide an unbiased assessment of gene expression changes that accompany DAT Val559 expression. Such studies may identify new genes or gene networks beyond those involved with DA signaling that contribute more broadly to risk for ADHD.

In addition to the experiments suggested above, several other studies are also underway. In collaboration with Dr. Danny Winder's lab, DAT Val559 mice

have been crossed with TH-EGFP reporter mice. The resulting animals express DAT Val559 and EGFP in dopaminergic neurons, thus allowing for visualization of DA neurons to be used for electrophysiological characterization. Preliminary data suggest that DAT Val559 mice have a reduced (hyperpolarized) resting membrane potential relative to WT mice, a finding that could be attributed to tonic D2-induced hyperpolarization (M Fettig, personal communication).

Further electrophysiological characterization is necessary to examine alterations to patterns of DA signaling. Dopaminergic neurons typically display two activity patterns – tonic signaling, characterized by spontaneous, irregular, single spike events, and phasic signaling, which is depolarization-dependent and displays a burst-firing activity pattern (Grace and Bunney, 1983). Tonic firing is established by an intrinsic pacemaker mechanism that is dependent on a hyperpolarization-activated cation conductance (Grace and Bunney, 1984b). In contrast, phasic or burst firing is depolarization-dependent, and initiated by incoming cortical and brainstem afferents (Grace and Bunney, 1984a; Charara et al., 1996; Lodge and Grace, 2006a, b). Studies have demonstrated that phasic DA neuron activity requires NMDA receptors, as NMDA receptor antagonism inhibits DA neuron firing (Chergui et al., 1993). In light of ADE, DA neurons may alter their firing patterns to account for the constant presence of DA in the synapse. Assessment of tonic and phasic DA neuron activity in DAT Val559 mice will add to our understanding of the full impact of DAT Val559 on neuron function.

Thus far, behavioral characterization has focused on locomotor behavior and AMPH response. However, impulsivity and attention are distinct realms of behavior

that have not yet been explored. In collaboration with Dr. Martin Sarter at the University of Michigan, mice will be tested on attention tasks such as the 5-choice serial reaction time task (reviewed in Robbins, 2002), and impulsivity tasks such as the stop signal or go/no-go task (reviewed in Eagle et al., 2008).

It is also necessary to remain careful not to overstate the impact of our findings surrounding DAT Val559 mice. The DAT KO mouse is a fitting example – DAT KO mice were originally proposed as a model for ADHD as they are extremely hyperactive (Giros et al., 1996). However, DAT KO mice mirror the human loss-of-function syndrome (dopamine transporter deficiency syndrome (DTDS)) almost exactly (Kurian et al., 2009, 2011). For instance, DAT KO mice display early hyperkinesis (that looks similar to ADHD), but later become immobile and dystonic. Furthermore, DAT KO mice fail to develop normally and show significantly reduced survival, reminiscent of the failure to thrive observed in patients with IPD. In fact, Caron's research group has recently acknowledged that the DAT KO mouse is not the most appropriate model for ADHD, and are perhaps most useful for studying IPD (MG Caron, personal communication). The DAT KO is merely an example, however, it serves as a useful reminder to keep an open mind regarding the disease-applicability of our findings.

In conclusion, we have successfully modeled a rare, highly penetrant DAT coding variant derived from human ADHD subjects. DAT Val559 mice display several phenotypes predicted by previous *in vitro* characterization of the mutant transporter, including spontaneous, basal DA efflux and altered response to AMPH. This new model has also allowed us to begin characterizing the impact of DAT

Val559 on animal behavior. DAT Val559 mice show reduced AMPH-induced locomotor stimulation, as well as an as yet unexplained hyper-reactive darting phenotype. Our findings also point towards alterations in DA signaling, most likely dysregulation of D2 autoreceptor signaling. The DAT Val559 mouse represents the first construct-valid mouse model for ADHD and has promise as a useful tool for studying dopaminergic dysfunction relevant to ADHD.

APPENDICES

In addition to the data presented in Chapters 2-5, which has been submitted for publication, I have pursued several other studies. Although these findings are not published, they have informed previous and ongoing efforts.

Appendix 1: Identification of New DAT Coding Variants

Appendix 2: *In Vitro* Characterization of V24M hDAT

Appendix 3: Preliminary Behavior Experiments on DAT Val559 Mice

APPENDIX 1

IDENTIFICATION OF NEW DAT CODING VARIANTS

Past efforts in the Blakely lab have involved screening of the coding sequence and exon-adjacent regions of human subjects diagnosed with ADHD (Mazei-Robison et al., 2005). In a continuation of those efforts, we continued screening human subjects diagnosed with either ADHD or bipolar disorder.

ADHD and Bipolar Disorder Screening

Our collaborators, Dr. Irwin Waldman at Emory University and Dr. John Kelsoe at University of California, San Diego, provided us with collections of genomic DNA samples from 192 ADHD (Waldman) and 418 bipolar disorder (Kelsoe) probands. Using the methods described previously (Mazei-Robison et al., 2005), and with the aid of a postdoctoral fellow in the lab, Dr. Leah Miller, I screened the 14 coding exons of the DAT gene. The results of these efforts are summarized in Tables 6 and 7.

It is important to note that our initial screening step merely identifies the presence of a single nucleotide polymorphism (SNP), and the identity of the SNP is subsequently determined by sequencing. Therefore, we detect synonymous (non-coding) variants as well.

As the tables indicate, the frequency of most of these SNPs is quite low (less than 1%). As these are non-coding SNPs, it is unlikely that these rare, synonymous mutations contribute to any great degree to disease risk. However, several non-

Exon	SNP	Base Pair	Amino Acid	Allele Freq.	Subject Freq.
2	G/A	198	V24M	0.26%	0.52%
	C/T	242	--	0.26%	0.52%
	G/T	278	--	1.04%	1.56%
4	C/A	-12	--	7.03%	11.46%
6	C/T	938	--	0.78%	1.56%
9	A/G	1343	--	8.33%	13.54%
10	G/A	-21	--	6.25%	9.90%
13	C/T	1859	--	0.78%	1.56%

Table 6. Summary of SNPs in DAT coding region identified in 192 probands diagnosed with ADHD.

Exon	SNP	Base Pair	Amino Acid	Allele Freq.	Subject Freq.
2	C/T	242	--	4.78%	2.51%
	G/T	278	--	0.48%	0.24%
	C/T	290	--	0.24%	0.12%
4	C/A	-12	--	27.75%	16.63%
	C/T	674	--	0.24%	0.12%
6	C/T	938	--	0.24%	0.12%
8	C/T	+33	--	0.24%	0.12%
9	A/G	1343	--	28.71%	17.34%
10	G/A	-21	--	23.21%	13.04%
11	G/A	+14	--	0.96%	0.84%
13	C/T	1820	--	0.24%	0.12%
	C/T	1859	--	0.72%	0.36%
14	A/T	-18	--	0.24%	0.12%

Table 7. Summary of SNPs in DAT coding region identified in 418 probands diagnosed with bipolar disorder.

coding variants occur at a much higher frequency and differ between the ADHD and bipolar subject populations. To date, however, there is no indication that these variants contribute lead to any perturbation in DAT expression or function. One new coding SNP, Val24Met, was identified in this screen as well. Further information regarding Val24Met hDAT follows.

Identification of Val24Met hDAT

One new coding variant was identified and confirmed during screening of ADHD probands. This particular SNP generated a new restriction fragment length polymorphism (RFLP) that allowed for the creation of a small, one-generation pedigree (Fig. 28). I demonstrated that Val24Met is an inherited mutation, being transmitted by the father. Further clinical information regarding the ADHD-like characteristics of the father was unavailable.

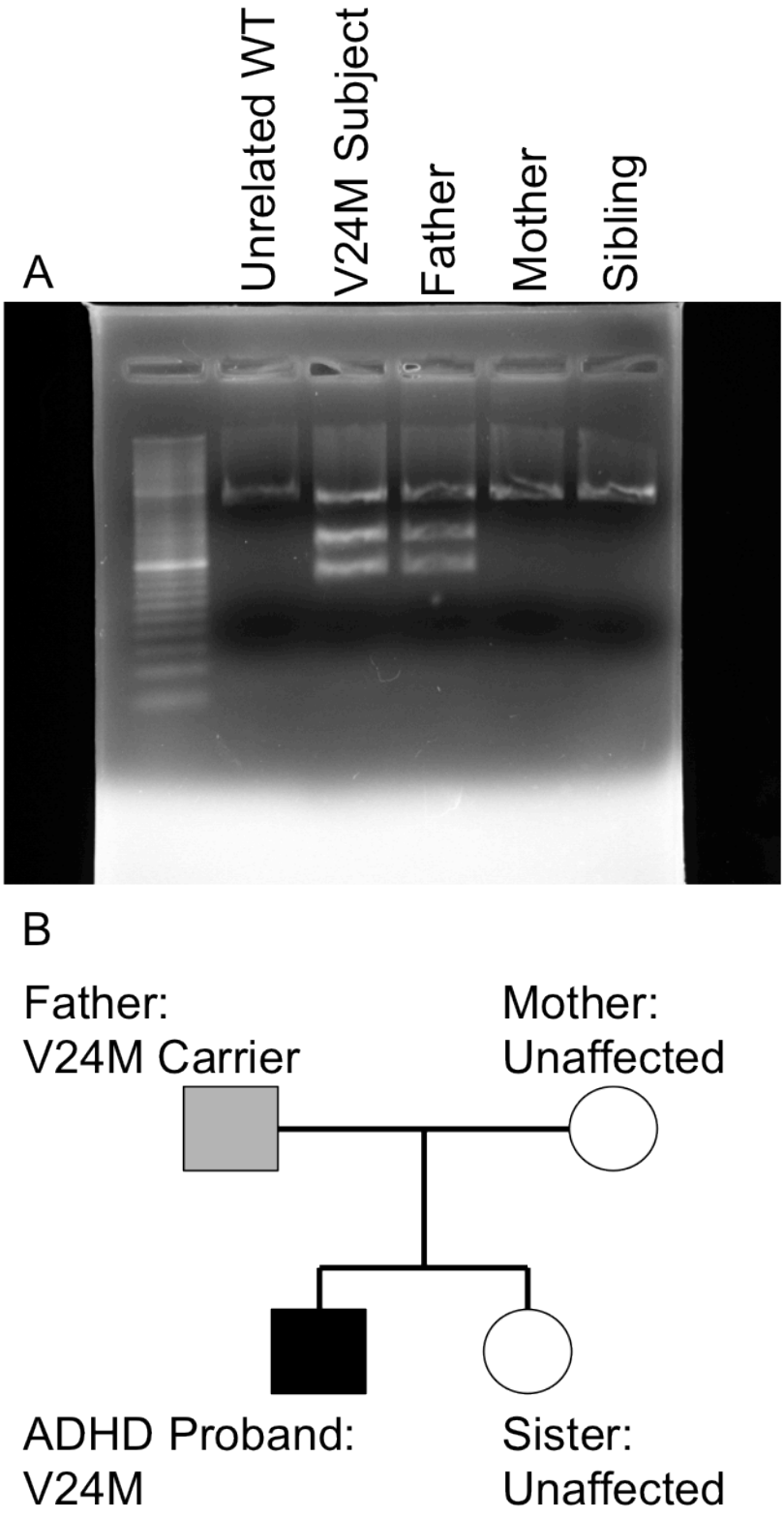

Figure 28. V24M DAT is transmitted from the father to the affected subject. (A) V24M introduces a new restriction enzyme site into exon 2. RFLP analysis reveals the presence of the novel restriction site, and therefore the presence of V24M hDAT, in the affected proband and his father, but not in his mother or unaffected sibling. RFLP analysis allows for the generation of a small pedigree (B) demonstrating this transmission pattern.

APPENDIX 2

IN VITRO CHARACTERIZATION OF V24M HDAT

Having identified a novel coding variant, V24M, in an ADHD proband (Appendix 1), efforts turned to characterizing the functional implications of the mutation. Initial characterization of V24M hDAT transfected in cultured cells revealed that total and surface protein expression (assayed via cell-surface biotinylation and subsequent immunoblotting) were unaffected by the mutation (Fig. 29A). Furthermore, DA transport was equivalent in the mutant (Fig. 29B). These findings suggested that V24M is a tolerated mutation and does not affect DAT function.

SIFT Analysis and Systematic Mutation of Val24

The N-terminal tail of DAT contains several phosphorylation sites that regulate transporter function. It is also the site of interaction between DAT and several other proteins (reviewed in Chapter 1). Although V24M appears to have little impact on DAT function, I wanted to explore the significance of Val24 for N-terminal functionality.

In order to inform my experimental design, I used the computer algorithm SIFT (Sorting Intolerant From Tolerant) to predict the effect of various mutations (Ng and Henikoff, 2001, 2006; Kumar et al., 2009). Briefly, SIFT performs an iterative BLAST search for all protein sequences homologous to the target protein, DAT. Based on the frequency of variation at specific amino acid locations in

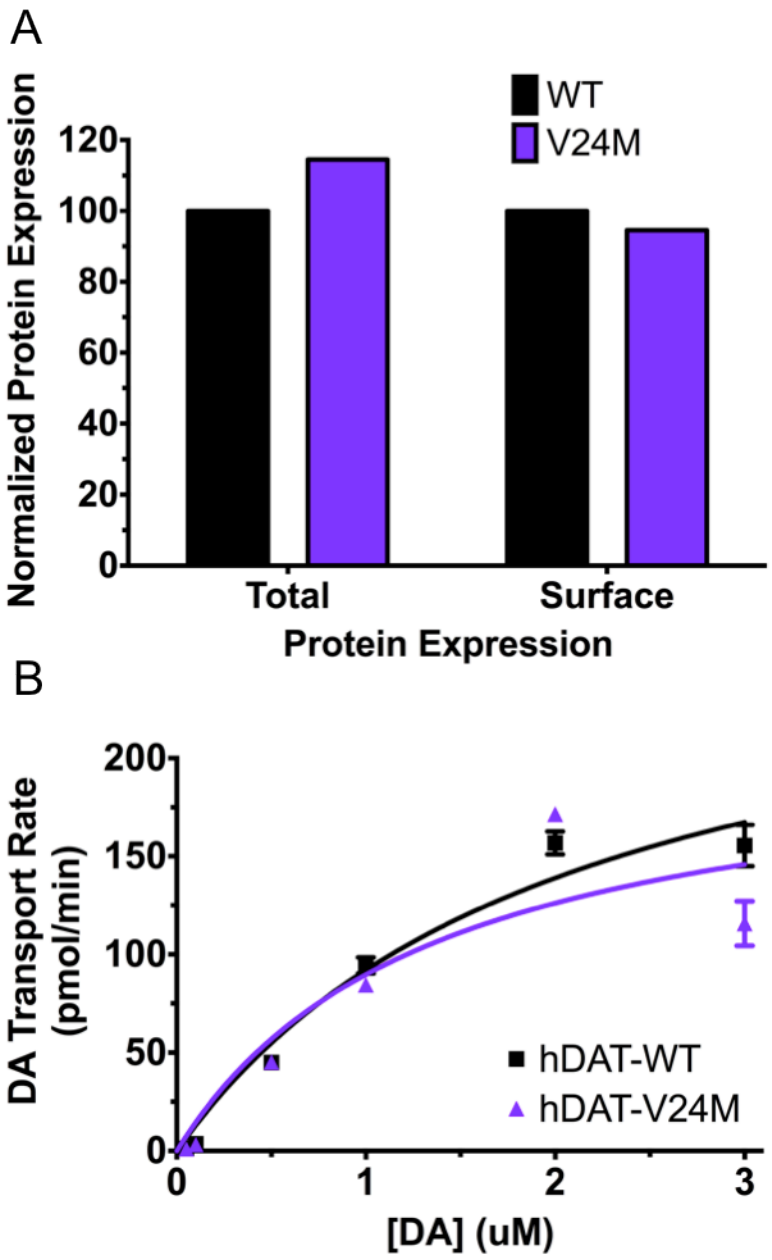

Figure 29. V24M displays normal (A) total and surface protein expression (n = 3 independent experiments; $P > 0.05$, two-tailed t-test) and (B) transport kinetics (n = 3 independent experiments; $P > 0.05$, two-way RMANOVA).

homologous proteins, SIFT calculates the probability that a given amino acid substitution will have a deleterious effect on protein function. SIFT analysis predicted that amino acid position 24 could accommodate any amino acid substitution except for tryptophan.

Although most residues were predicted to be tolerable, I proceeded to use site-directed mutagenesis to introduce non-conservative mutations into amino acid position 24. I generated a series of V24 mutants by mutating the native valine to cysteine, aspartate, phenylalanine, lysine, threonine, and tryptophan. DA transport in all of the mutants except for V24C and V24K was significantly elevated relative to WT hDAT, with V24W showing the greatest increase in DA transport (Fig. 30). It seems that SIFT's prediction of "deleterious" effect on protein function is misleading; V24W hDAT appears to have increased transport function.

Effect of V24 Variation on DAT Regulation

After demonstrating that mutation of Val24 increases transport, I wanted to see if DAT regulation was affected. In cultured cells, treatment with AMPH or PMA induces DAT internalization and, as a result, reduced transport function. I tested the effects of AMPH and PMA on DA transport in DAT Val24 mutants transfected and expressed in cultured cells. Upon AMPH treatment, all V24 variants except for V24D were significantly down-regulated, similar to WT DAT (Fig. 31A). It is thought that DAT regulation by AMPH requires PKC activity (Kantor and Gnegy, 1998; Saunders et al.. 2000). Interestingly, aspartate substitutions are frequently used to mimic phosphorylation. My findings support the notion that addition of the phosphate group at the N-terminal tail is required for DAT internalization.

Since AMPH-induced internalization of the V24 hDATs is mostly intact, I was surprised to find that PKC activation by PMA did not induce downregulation, except for V24C (Fig. 31B). This finding suggests that Val24 may be important for PMA activation of PKC, as replacement of V24 abolishes PKC-mediated downregulation. It must be noted that AMPH and PMA exert their effects primarily through DAT internalization; reduction of DA transport is merely a proxy for DAT internalization. Further studies that directly look at DAT trafficking (i.e. biotinylation studies), may offer more insight as to the effects of mutation of Val24.

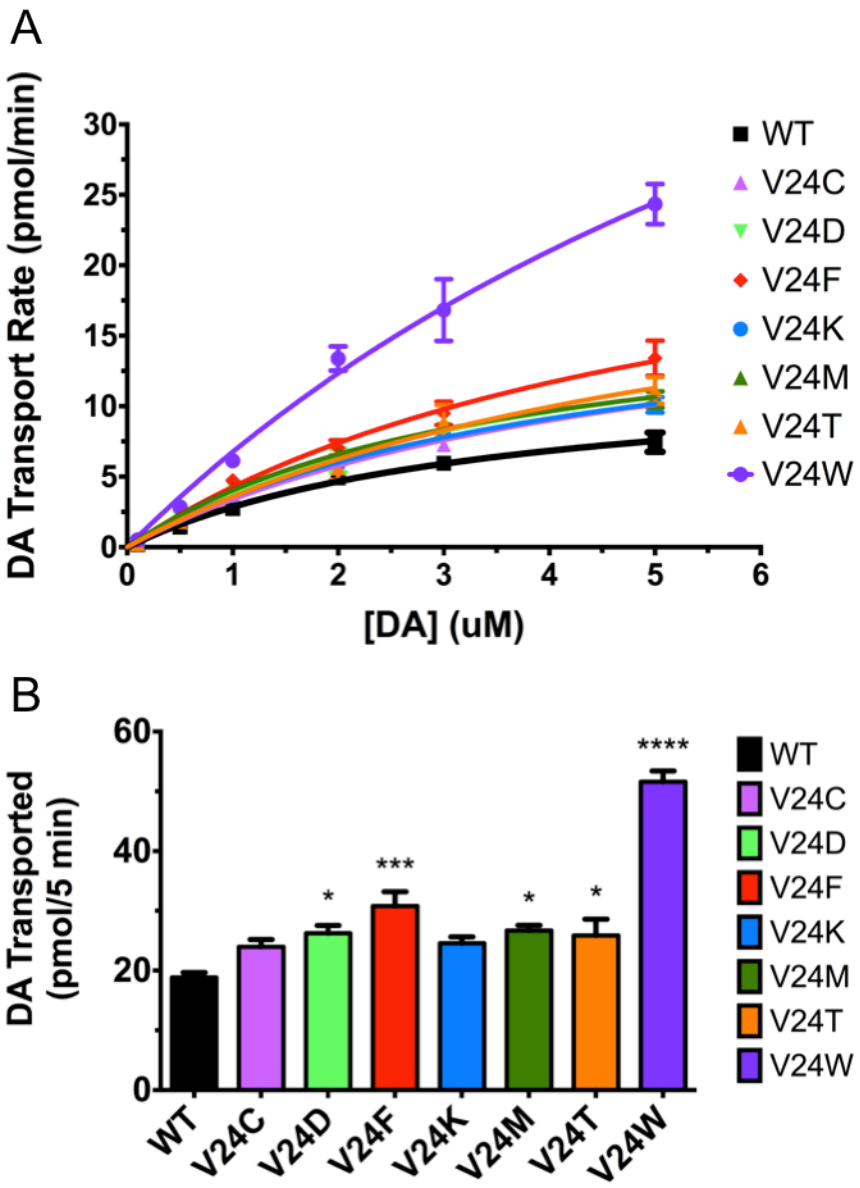

Figure 30. V24 mutants display significantly elevated DA transport relative to WT. (A) Saturation analysis reveals altered transport kinetics, and (B) all V24 mutants except for V24C and V24K transport significantly more DA than WT DAT (n = 3-4 independent experiments per V24 mutant; overall $P < 0.05$, post hoc tests (Tukey's multiple comparisons test) reveals * = $P < 0.05$, *** = $P < 0.001$, and **** = $P < 0.0001$ for V24 mutant vs. WT DAT comparison; one-way ANOVA).

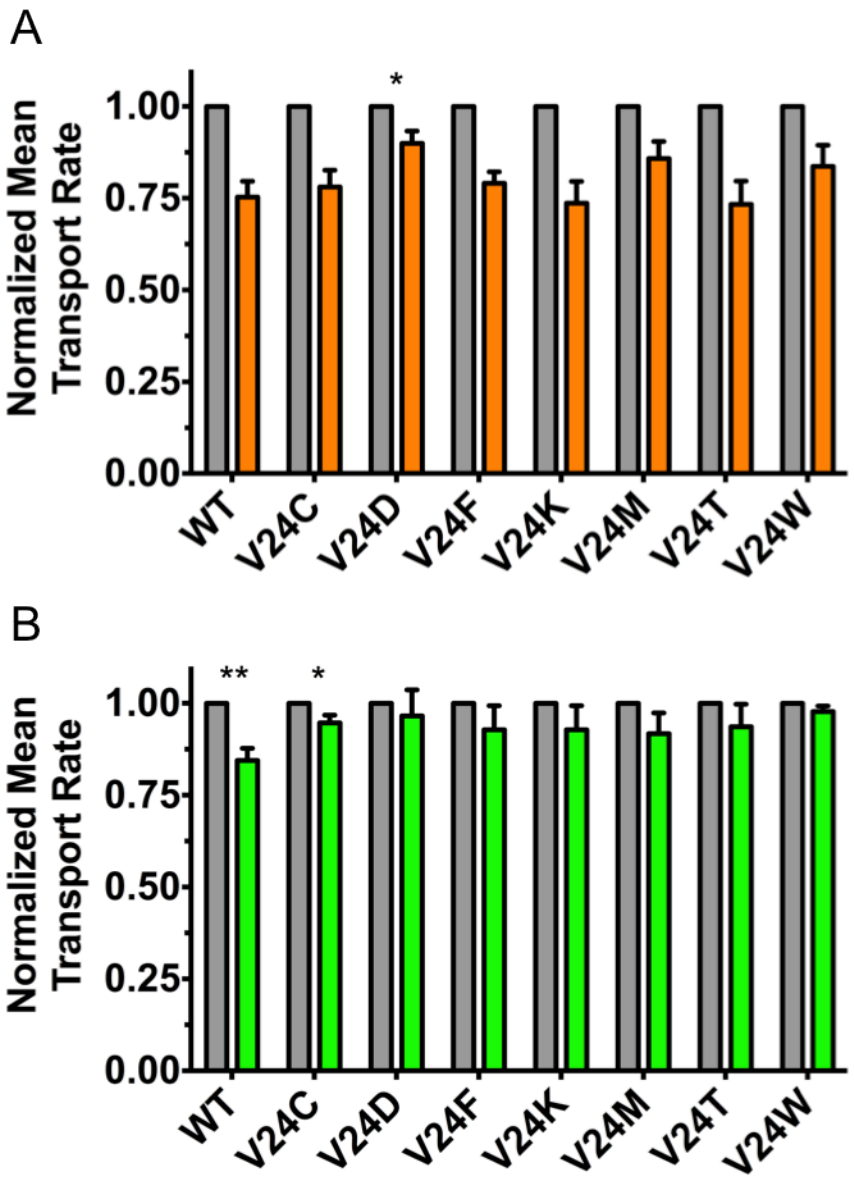

Figure 31. Regulation of V24 DAT mutants by AMPH and PMA. (A) All DATs except for V24D are appropriately downregulated by AMPH treatment (n = 3-4 independent experiments per V24 mutant; overall $P > 0.05$, post-hoc tests reveal that * = $P < 0.05$ for WT vs. V24D comparison; two-way ANOVA).

APPENDIX 3

PRELIMINARY BEHAVIOR EXPERIMENTS ON DAT VAL559 MICE

Throughout the course of behavioral characterization of the DAT Val559 mice, I preformed preliminary experiments on a number of different paradigms, then conducted more thorough experiments on those that were most relevant. This appendix will present those preliminary behavior findings.

Cliff Avoidance Reaction

Impulsivity is a key feature of ADHD, and has been defined as a lack of behavioral inhibition. In animal testing paradigms, premature, mistimed, and/or difficult to suppress behaviors are considered to reflect impulsivity (Dalley et al., 2008; Eagle and Baunez, 2010). A simple test of impulsivity involves placing a mouse atop a raised platform and assessing how mice explore the edge of the platform (Yamashita et al., 2013). Mice typically balance their exploratory tendencies with a desire to remain safe and therefore avoid the edge of the elevated platform. This edge avoidance behavior has been termed the cliff avoidance reaction. I performed this assay with DAT Val559 mice to test for obvious signs of impulsivity and observed no differences in latency to fall (Fig. 32A) or the rate of head dips over the edge of the cliff (Fig. 32B), suggesting that DAT Val559 mice do not impulsively explore the edge of the cliff.

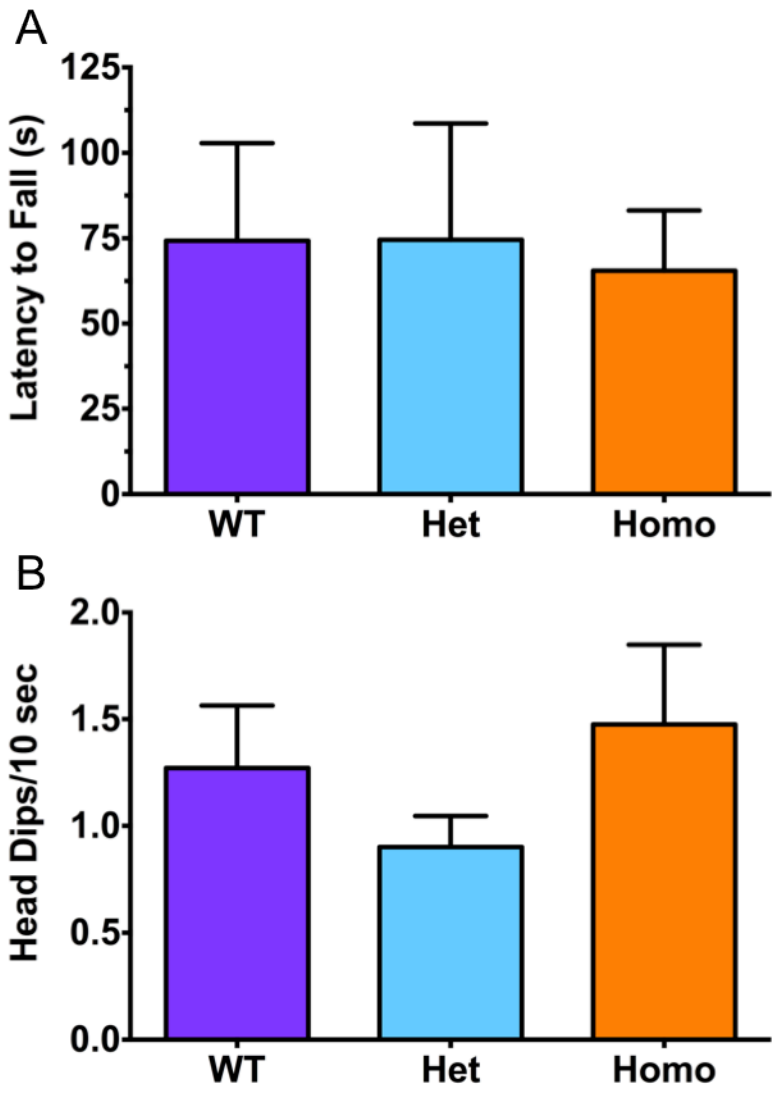

Figure 32. DAT Val559 mice do not differ from WT controls in either (A) latency to fall +/- SEM (n = 13 WT, 7 het, 14 homo; $P > 0.05$, one-way ANOVA) or (B) number of head dips per 10 seconds +/- SEM (n = 15 WT, 16 het, 16 homo; $P > 0.05$, one-way ANOVA) in the cliff avoidance test.

Novel Object Recognition

The novel object recognition (NOR) paradigm is a test of cognitive function that addresses learning and memory and preference for novelty (reviewed in Antunes and Biala, 2012). Briefly, the test involves exposing mice to two identical objects for a brief period, then after a delay, replacing one of the familiar objects with a novel object. Mice can then explore both objects; normal mice typically explore the new object, as they have learned that the familiar object does not offer any particular advantage. DAT Val559 mice display a small but non-significant increase in preference for the novel object (Fig. 33), however, WT mice do not show an object preference, so interpretation of these data is not clear. It is important to note that NOR was tested during the light phase of the light/dark cycle, so the lack of novelty preference may be partly attributable to reduced activity of the animals. Performing NOR during the dark phase and/or using DAT Val559 animals that have been backcrossed to the more behaviorally robust C57BL/6J background strain is a worthwhile experiment to pursue.

Prepulse Inhibition

Prepulse inhibition (PPI) of acoustic startle is a dopamine-sensitive behavior (Koch, 1999; Ralph et al., 2001; Powell et al., 2008; Wong et al., 2012) in which the acoustic startle response is reduced by a lower-intensity stimulus preceding the startle stimulus. DAT Val559 mice show no PPI deficits at any of the prepulse intensities tested (Fig. 34), suggesting that startle circuitry through the VTA is intact. However, AMPH is known to disrupt PPI (Swerdlow et al., 1990). Since DAT

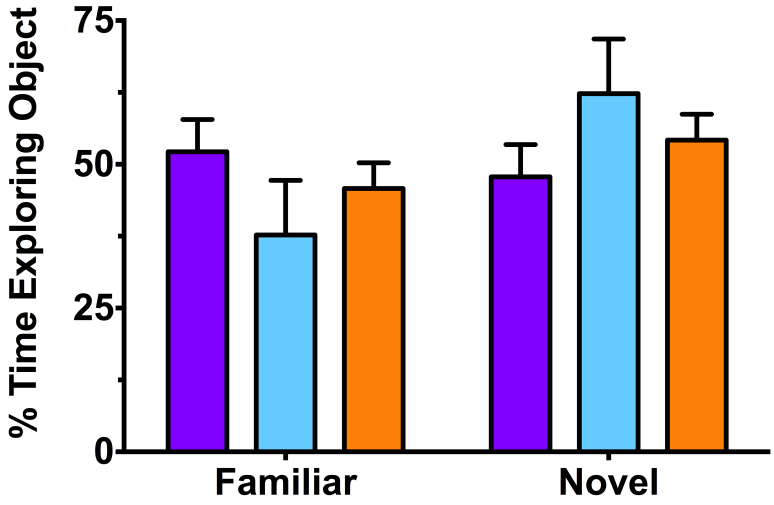

Figure 33. DAT Val559 mice do not differ from WT mice in their performance on the novel object recognition task. NOR performance is indexed as the percent of trial time (10 minutes) +/- SEM spend exploring the novel and familiar objects n = 8 per genotype, $P > 0.05$, two-way ANOVA).

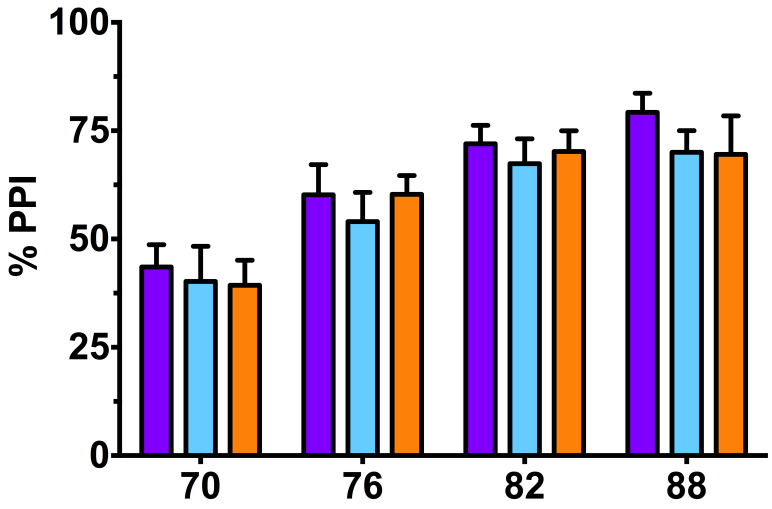

Figure 34. DAT Val559 mice display no deficits in prepulse inhibition compared to WT controls. PPI is displayed as the percentage +/- SEM of the startle response that can be inhibited by the presence of a prepulse (n = 16 per genotype, $P > 0.05$, two-way ANOVA).

Val559 mice show significantly altered response to AMPH, examining its effects on PPI is certainly warranted.

Home Cage Monitoring

Although we did not observe locomotor hyperactivity during open field testing, we still wanted to analyze the behavior of DAT Val559 mice in a familiar environment to remove any potential caveats of apparatus novelty. I used the home cage monitoring system (HCM) to record and annotate the behavior of individual mice over a period of three days. Mice were singly housed to establish a "home cage", then recorded within a climate-controlled incubator to limit the effect of any experimenter intervention. HCM returns an enormous volume of data, including locomotor activity, rearing behavior, hanging from the wire cage insert, sleeping, eating, drinking, and grooming. Over the course of three days, DAT Val559 mice do display a slight increase in total locomotor behavior (Fig. 35A), driven largely by increased locomotion during the dark (active) phase (Fig. 35B). Locomotor activity during the light period did not differ among genotypes (Fig. 35C).

Throughout our various open field activity recordings, we observed a significant reduction in rearing behavior in DAT Val559 mice. HCM recordings reveal a similar phenotype – rearing activity is consistently reduced in DAT Val559 animals, regardless of light/dark phase (Fig. 36). As discussed in Chapter 4, downregulation of D2 receptors offers a potential mechanism for this behavioral finding. These HCM findings confirm a reduction of rearing activity and lend support to the notion that DAT Val559 leads to desensitization of D2 receptors in the brain.

It is important to note that this experiment is extremely underpowered – only three homozygous animals were included in the experiment. Future experiments must first focus on replicating the current findings in additional homozygous animals.

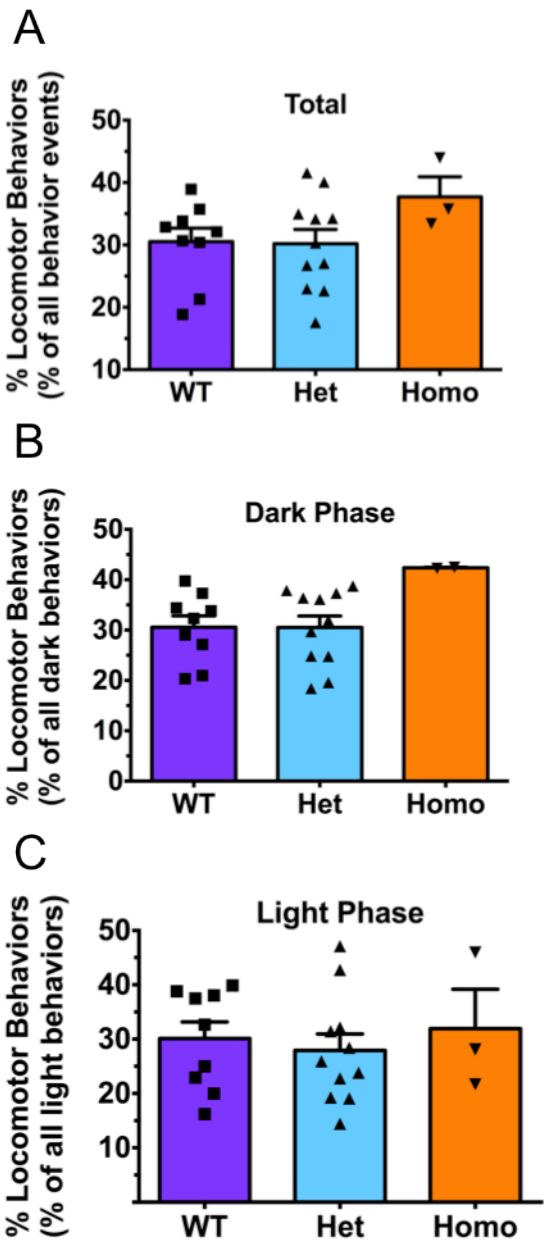

Figure 35. DAT Val559 mice display small, insignificant increases in locomotor behavior during (A) the total 3-day recording, or (B) dark and (C) light phases. Due to the low number of homozygous animals, differences in locomotor behavior are not statistically significant (n = 10 WT, 11 het, 3 homo; $P > 0.05$, one-way ANOVAs).

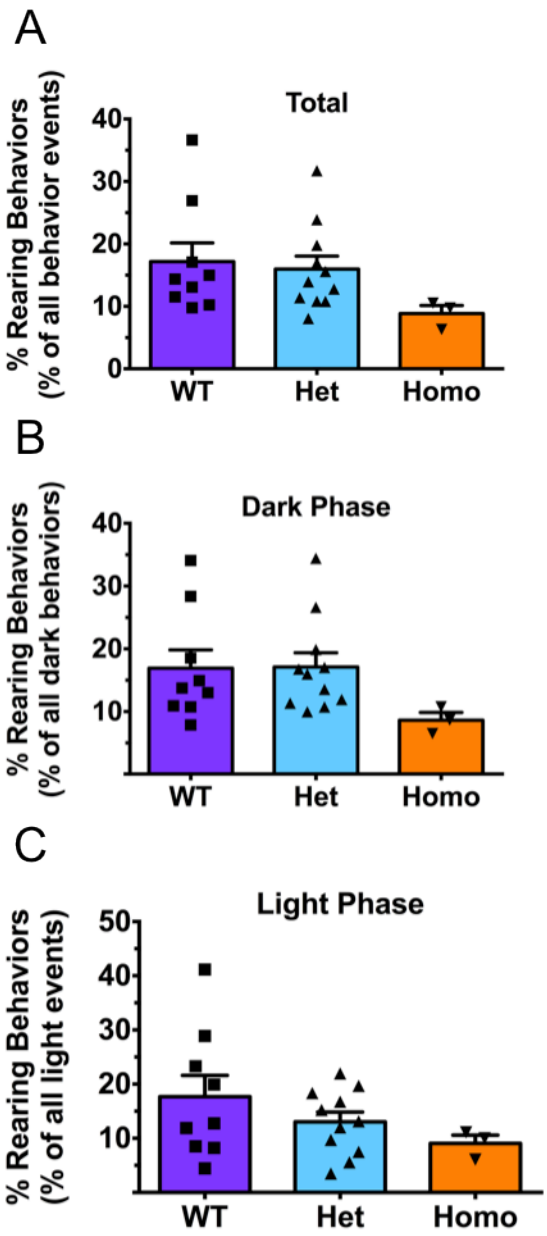

Figure 36. DAT Val559 mice display reduced rearing behavior in the home cage environment during (A) the total 3-day recording period, or (B) dark and (C) light phases. Due to the low number of homozygous animals, the difference in rearing behavior is not statistically significant (n = 10 WT, 11 het, 3 homo; $P > 0.05$, one-way ANOVAs).

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