Impacts of an Autism-Linked Dopamine Transporter Variant on Brain and Behavior in a Murine Model

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For my mother, Sheryl

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3-MT	3-methoxytyramine
5-HT	Serotonin
AADC	L-amino acid decarboxylase
ABC	Aberrant Behavior Checklist
aCSF	Artificial cerebral spinal fluid
AMPH	Amphetamine
ARJP	Autosomal recessive juvenile parkinsonism
ASD	Autism Spectrum Disorder
ASR	Acoustic startle response
ADHD	Attention deficit hyperactivity disorder
BCA	Bicinchoninic acid
BPD	Bipolar disorder
CaMKII	Ca^{2+}/calmodulin kinase
cAMP	Cyclic adenosine monophosphate
CFM	Carbon fiber microelectrode
CNS	Central nervous system
CNV	Copy number variant
COC	Cocaine
COMT	Catechol-O-methytransferase
CSF	Cerebral spinal fluid
CV	Cyclic voltammogram
DA	Dopamine

DAT T356M	Threonine to methionine substitution at site 356 of the dopamine transporter
DBH	Dopamine-β-hydroxylase
DDC	Dopamine decarboxylase
DGBI	Disorders of gut-brain interaction
DHPA	3,4-dihydroxyphenylacetaldehyde
DZ	Dizygotic
DOPAC	3,4-dihydroxyphenylacetic acid
DSM-V	Diagnostic and statistical manual, fifth edition
E/I	Excitatory/inhibitory
EMG	Electromyography
ENS	Enteric nervous system
ERK1/2	Extracellular signal-releated kinase
FDOPA	Fluorine-18-labelled fluorodopa
fGID	Functional gastrointestinal disorders
fMRI	Functional magnetic resonance imaging
FMRP	Fragile X Mental Retardation Protein
FSCAV	Fast-scan controlled adsorption voltammetry
FXS	Fragile X Syndrome
GABA	γ-aminobutyric acid
GI	Gastrointestinal
GPCR	G-protein-coupled receptor
hDAY	Human dopamine transporter
HPLC	High performance liquid chromatography
hSERT	Human serotonin transporter

HVA	Homovanillic acid
KO	Knockout
L-DOPA	L-3,4-dihydroxyphenylalanine
LC	Locus coeruleus
MAO	Monoamine oxidase
MZ	Monozygotic
MSN	Medium spiny neuron
NAc	Nucleus accumbens
NE	Norepinephrine
ODD	Oppositional defiant disorder
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
pERK1/2	Phosphorylated extracellular signal-releated kinase
PFC	Prefrontal cortex
PI3K	Phosphatidylinositol 3 kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC β	Protein kinase C- β
PET	Positron emission tomography
PPI	Prepulse inhibition
pTH	Phosphorylated tyrosine hydroxylase
RDoC	Research Domain Criteria
SAM	*S*-adenosylmethionine
SEM	Standard error of the mean

SN	Substantia nigra
SNc	Substantia nigra pars compacta
SRU	Startle response unit
TH	Tyrosine hydroxylase
TH	Tyrosine hydroxylase
THB	Tetrahydrobiopterin
VMAT	Vesicular monoamine transporter
VPA	Valproic acid
VTA	Ventral tegmental area
WT	Wild type

CHAPTER I

INTRODUCTION

Preface

The introduction that follows attempts to provide the requisite information for interpreting the original research described in this thesis. This introduction is not meant to serve as a comprehensive review of all the topics discussed herein, but will instead highlight key concepts that form the basis of our hypotheses, experimental design, and interpretation of the data. Specifics of these topics not discussed here will be described within the introduction to each of the following chapters.

We will first consider the neurobiology of the dopamine (DA) system, the key regulators of DA function in the brain, and the role of DA in behavior. To better understand the possible role of DA in Autism Spectrum Disorder (ASD), we will consider the clinical definition and presentations of ASD. The neurobiological theories regarding the interplay between DA dysfunction and ASD will then be discussed in detail. Finally, we will consider therapeutic strategies for individuals with ASD. Specifically, we will discuss therapeutics that target the dopaminergic system and evidence for their efficacies in treating the core symptomatology of ASD as supporting evidence for the role of DA dysfunction in ASD.

Introduction

Autism Spectrum Disorder (ASD) is a highly heritable neurodevelopmental disorder estimated to affect 1 in 59 children[1]. This disorder is characterized by deficits in social communication and by patterns of restricted interests and/or repetitive behaviors that persist throughout life[2]. Since the initial description of autism by Leo Kanner in 1943[3], mounting evidence has suggested there is no single cause of ASD. Rather, the diagnosis of ASD is structured around a core set of behavioral symptoms that serve to unify individuals with a heterogeneous collection of genetic and mechanistic differences.

Recurrence within families and twin studies have implicated a strong genetic component to ASD[4–7]. However, this disorder is not monogenic – there is not one gene responsible for all causes of autism. A growing number of genetic changes, including *de novo* single nucleotide variants[8–10] as well as both *de novo* and transmitted copy number variants (CNVs)[11–14] have been identified in individuals with autism. Concordance rates and linkage studies suggest a multigenic inheritance pattern, although a subset of ASDs may have a monogenic etiology[15].

Within this context, a key question is how these various genetic risk factors ultimately result in the constellation of behavioral symptoms that are the hallmark of autism. Many have suggested that these seemingly disparate genetic risk factors ultimately converge on downstream mechanisms. However, the high clinical and genetic heterogeneity of ASD instead suggests that rather than considering ASD as a disorder with a singular pathway abnormality, it may be more appropriate to define subgroups of the ASD population based on genetic risk factors, biomarkers, or specific clinical

presentation (e.g. area(s) of greatest impairment(s), existing comorbidities, etc.). These subgroups could be a result of mechanistic commonalities, and thus could be used to delineate the populations that might benefit from differential, targeted therapies.

In this chapter, we will consider evidence for one such possible subgroup: individuals in whom dopaminergic dysfunction may underlie part or all of their autism symptomatology. We will consider evidence for primary dopaminergic system dysfunction (i.e. dysfunction of either dopaminergic brain structures or brain structures receiving dopaminergic input) in both individuals with ASD and in various animal models that demonstrate many of the phenotypic characteristics of ASD, from *Drosophila melanogaster* to the mouse. We will discuss the growing evidence suggesting DA dysfunction as a contributing factor to ASD, the endophenotypes of ASD that may be characteristic of this group, and possible therapeutic strategies for such individuals. We will first discuss the anatomy and physiology of the dopaminergic system to briefly review the requisite knowledge for our discussion of ASD and the possible role of DA in ASD.

The dopaminergic system

Dopamine (DA, 3-hydroxytyramine) is one of several monoaminergic neurotransmitters produced in the brain (the other monoaminergic neurotransmitters being serotonin [5-HT], epinephrine, norepinephrine [NE], and histamine). DA is synthesized from the amino acid tyrosine in the neurons whose cell bodies reside in the substantia nigra (SN) and the ventral tegmental area (VTA; See Figure 1). A small amount of DA is also produced by the hypothalamus. The synthesis of DA (see Figure 2 and Figure 3) is a two-step reaction catalyzed by the enzymes tyrosine hydroxylase (TH) and

L-amino acid decarboxylase (AADC). TH first converts DA to L-DOPA (also known as L-3,4-dihydroxyphenylalanine), which is then converted to DA by AADC. DA can be further metabolized to NE by DA β-hydroxylase (DBH). DA and NE together constitute the class of neurotransmitters called catecholamines.

The dopaminergic neurons of the SN project to the dorsal striatum, a contiguous group of subcortical nuclei that collectively form the input structure of the basal ganglia[16], forming the nigrostriatal pathway. This pathway is thought to mediate motor activity. A large population of neurons that produce GABA (γ-aminobutyric acid) are found in the striatum. These cells, called medium spiny neurons (MSNs), account for 95% of the striatal neuron population. These neurons are the primary target of dopaminergic neurons whose cell bodies reside in the SN. MSNs also receive input from the thalamus, various association areas of cortex, and sensory cortex[17]. MSNs are typically active immediately prior to the initiation of a movement and may encode the decision to make a movement toward a specific target[18].

Dopaminergic neurons of the VTA project to the ventral striatum (which includes the nucleus accumbens, NAc), the bed nucleus of the stria terminalis (BNST), and the amygdala to form the mesolimbic pathway and to the prefrontal cortex to form the mesocortical pathway (see Figure 1). The mesolimbic pathway is thought to mediate the interpretation of potential positive and negative reinforcers and to assess the value of these reinforcers[19]. DA in the NAc is a sensorimotor integrator that modulates the response output of an organism to motivational cues from the environment, thus allowing an organism to overcome response costs for a reward[20]. Much like the dorsal striatum,

the NAc is composed mainly of MSNs. The amygdala is an integrative site for emotion and memory and is involved in memory consolidation for emotionally charged events[19]. The BNST, which is considered a part of the extended amygdala, is involved in the behavioral response to fearful events and stress[19]. Mesocortical DA neuron input to the PFC is neuromodulatory in nature and is thought be involved in working memory, planning, and attention[21]. DA in the cortex modulates ongoing inhibitory and excitatory neurotransmission to modulate how strongly a certain cortical representation is maintained. This pathway is thought to be dysfunctional in the brains of individuals with schizophrenia[22].

Hypothalamic dopaminergic neurons project to the pituitary (forming the tuberoinfundibular pathway) where DA inhibits the lease of prolactin[23,24]. DA in the pituitary binds to D2 receptors to both reduce prolactin exocytosis and to downregulate gene expression involved in the synthesis of prolactin[25]. Prolactin released by the anterior pituitary acts on hypothalamic DA neurons to increase DA synthesis to regulate its own release[25].

Presynaptically, DA is concentrated into vesicles via the vesicular monoamine transporter (VMAT). Neuronal depolarization following excitation results in calcium influx and fusion of these vesicles to the presynaptic membrane, releasing DA into the synapse. DA then signals through post-synaptic D1-like receptors and pre- and post-synaptic D2-like receptors (see Figure 2)[26]. Both D1-like and D2-like receptors are G-protein-coupled receptors (GPCRs). D1-like receptors (which include D1 and D5 receptors) are primarily G_s-coupled GPCRs, meaning that stimulation of D1-like receptors activates stimulatory g-proteins, which stimulate adenylyl cyclase, an enzyme that catalyzes the formation of

cyclic adenosine monophosphate (cAMP). Increases in intracellular cAMP lead to activation of protein kinase A (PKA) and, in turn, phosphorylation and regulation of many intracellular targets including receptors, ion channels, transcription factors, and enzymes[26–28]. Stimulation of D2-like receptors (which include D2, D3, and D4 receptors) by DA leads to inhibition of adenylyl cyclase, stimulation of phospholipases, protein kinases, receptor tyrosine kinases, and regulation of ion channels[26–28]. Via these intracellular signaling cascades, D1- and D2-like receptors act to regulate neuronal excitability and the effects of GABAergic[29] and glutamatergic signaling[27].

The temporal and spatial aspects of the DA signal are regulated, in part, by the dopamine transporter (DAT). The DAT is a Na^+/Cl^--dependent membrane transporter that acts to rapidly clear released DA from the synapse. Both cocaine and amphetamine (AMPH) inhibit DA uptake by the DAT and increase extracellular DA, though through distinct mechanisms. AMPH is a substrate of the DAT that competitively inhibits DA uptake into the presynaptic terminal and into synaptic vesicles via the VMAT isoform VMAT2. AMPH also promotes an inward-facing DAT conformation that favors DA efflux[30]. Cocaine elevates synaptic levels of DA by binding to the DAT in a way that prevents DA uptake[31].

The DAT is highly mobile within the neuron and is constitutively trafficked between the cell surface and intracellular vesicular structures[32]. The trafficking of DAT to and from the plasma membrane and the activity of DAT are dynamically regulated by the concentration of DAT substrate (i.e. DA and AMPH) in the synapse, DAT inhibitors, DAT-interacting proteins, and by a number of intracellular protein kinases[33]. Substrate-induced trafficking of the DAT is regulated by posttranslational modifications (including

phosphorylation and ubiquitination of the DAT). Kinases regulating DAT trafficking include protein kinase C- β (PKC β), protein kinase B (PKB, also known as Akt), phosphatidylinositol 3 kinase (PI3K), Ca^{2+}/calmodulin kinase (CaMKII), and extracellular signal-related kinases (ERK1/2). Evidence suggests PKCβ, PI3K, and PKB promote DAT trafficking to the plasma membrane in response to AMPH[34], while CaMKII promotes trafficking away from the plasma membrane (via inhibition of PKB)[35]. DAT phosphorylation may be induced via signaling cascades initiated by presynaptic D2 autoreceptors. D2/D3 agonists increase surface DAT and DA uptake, an effect thought to be mediated by intracellular ERK1/2 signaling[36]. Surface DAT expression undergoes biphasic trafficking in response to substrate exposure. Short-term exposure to DA or AMPH stimulates ultra-rapid increases in DAT surface expression, while continuous exposure to these substrates causes internalization of DAT[37–39]. Cocaine, a DAT blocker, inhibits AMPH-induced internalization of DAT[37] and, in contrast to the effects of AMPH, long-term exposure to cocaine increases surface DAT[40].

The DA signal is also terminated by the catabolism of DA (see Figure 3)[41]. There are two enzymes that degrade DA: catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO). MAO is located intracellularly in the presynaptic terminal. DA that accumulates in the cytosol via leakage from intracellular vesicles is catabolized by MAO. Oxidative deamination of DA by MAO produces hydrogen peroxide and 3,4-dihydroxyphenylacetaldehyde (DHPA). DHPA is primarily oxidized to produce 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (AD). COMT is located in the synaptic cleft and in surrounding glial cells. COMT transfers methyl groups from *S*-adenosylmethionine (SAM) to DOPAC leading to the production of homovanilic

acid (HVA). This enzyme always directly catabolizes DA to 3-methoxytyramine, which is further catabolized to 3-methoxy-4-hydroxyphenylacetaldehyde and ultimately to HVA.

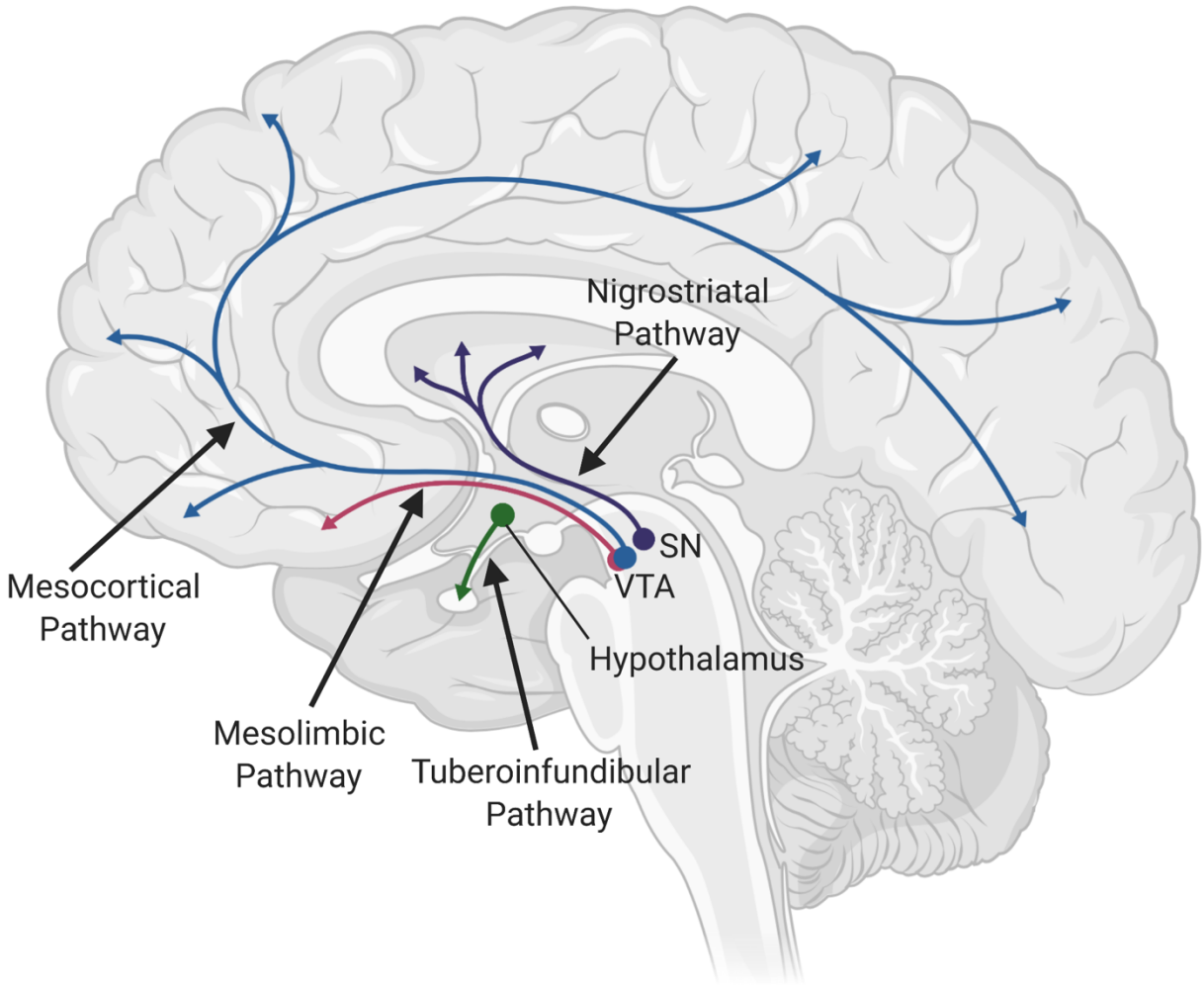

Figure 1. *Sites of DA synthesis and dopaminergic projection patterns in the brain.*

DA is primarily synthesized in the substantia nigra (SN), the ventral tegmental area (VTA), and the hypothalamus. Projections from the SN to the striatum form the nigrostriatal pathway (here in purple). Projections from the VTA to the cortex form the mesocortical pathway (here in blue). The VTA also projects to limbic structures in the brain, forming the mesolimbic pathway (pink). The hypothalamus projects to the pituitary, forming the tuberoinfundibular pathway (green), where it functions to regulate prolactin release.

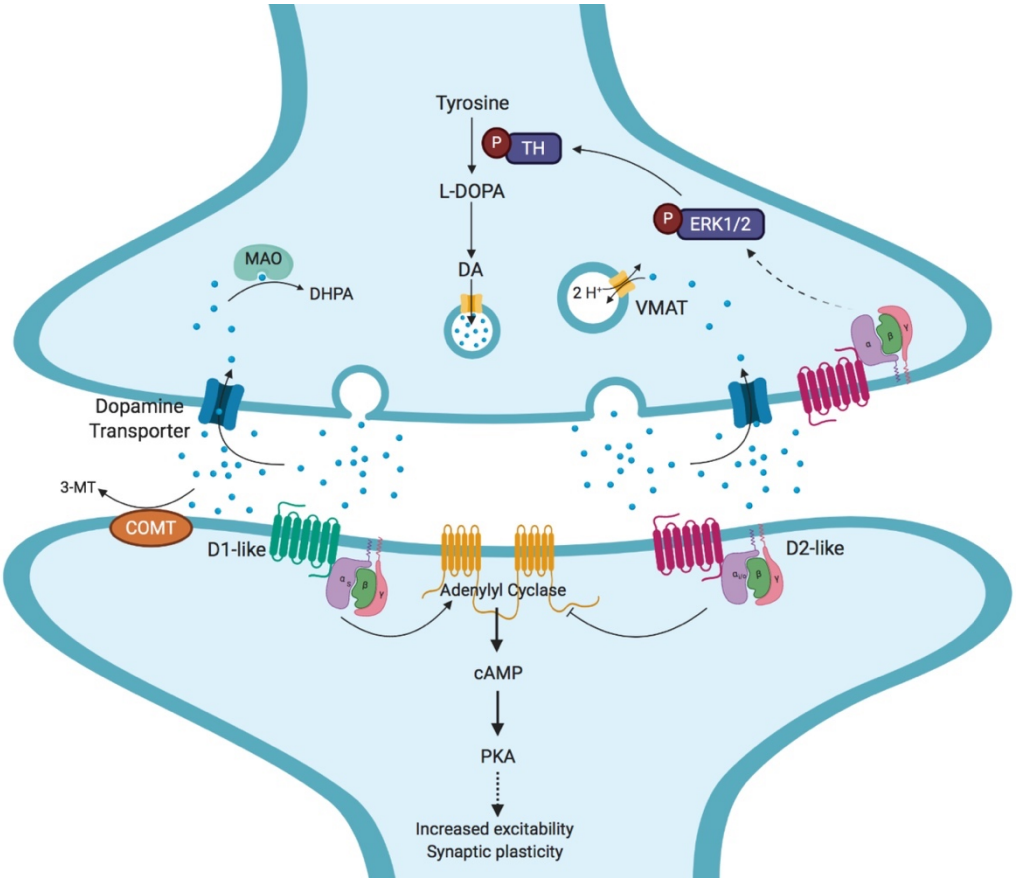

Figure 2. *Illustration of a dopaminergic synapse.*

DA (blue spheres) is packaged into vesicles in the presynaptic terminal via VMAT. Released DA stimulates post-synaptic D1-like and D2-like G-protein coupled receptors. Released DA is cleared from the synaptic cleft via the DAT or degraded via membrane-bound COMT. DA cleared from the synapse by the DAT is either repackaged into vesicles pre-synaptically or degraded by MAO in the presynaptic terminal.

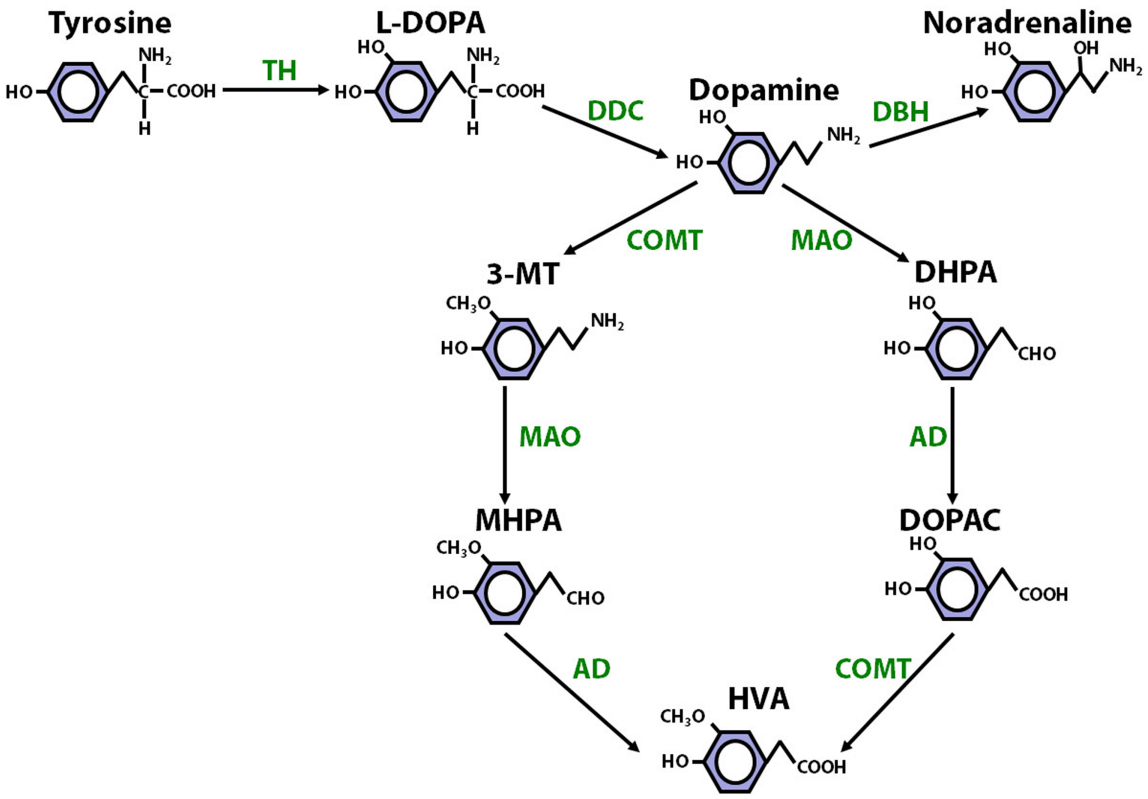

Figure 3. *The synthesis and degradation pathways of DA.*

Enzymes involved in the synthesis and catabolism of DA are shown in green. DA is synthesized from tyrosine via L-DOPA. The rate-limiting step in the synthesis of DA is the conversion of tyrosine to L-DOPA, which is mediated by tyrosine hydroxylase (TH). DA can be converted to NE, via the action of dopamine β-hydroxylase (DBH) or catabolised by the action of the enzymes catechol-O-methyltransferase (COMT), monoamine oxidase (MAO), and aldehyde dehydrogenase (AD). Other abbreviations: dopamine decarboxylase (DDC); 3-methoxytyramine (3-MT); 3,4 dihydrophenylacetaldehyde (DHPA); 3-methoxy-4-hydroxyphenylacetaldehyde (MHPA). Adapted from [42].

The role of DA in behavior

To understand the possible role of DA in neuropsychiatric disorders such as ASD, we must first consider the known roles of the DA signal in behavior. DA plays an essential role in both learning and in motivated behavior and can be conceptualized as providing an estimate for how worthwhile it is for an organism to devote limited resources to a particular motor or cognitive task[43]. DA plays an important role in motivated behavior, action selection, and reward processing[44,45]. DA signaling has been shown to drive behavioral activation and to promote effortful behavior. Rats in whom DA has been depleted or antagonized in the NAc redirect their behavior away from tasks that have high response requirements in favor of lower cost options[44]. Other groups have shown that reward rate is reflected by increased concentration of DA in the NAc and that DA levels in this brain region covary with motivational vigor[45]. That is, phasic changes in the concentration of DA affect an animal's willingness to do work for a reward[45].

In the same manner that DA can encode whether a physical movement warrants the allocation of energy, DA can also encode whether various cognitive processes (including attention and working memory) are similarly worthwhile[46]. Early work aimed at understanding DA's role in behavior demonstrated that DA depletion in the prefrontal cortex (PFC) of rhesus monkeys causes deficits in spatial working memory function[47]. A more recent study in humans demonstrates increased PFC DA during a verbal working memory task when compared to a less demanding attention task[48], supporting functional involvement of DA in working memory. DA signaling has also been linked with allocation of attention. When DA is depleted in the brains of rats, these rats display impairments in the integration of sensory information with motor performance in the home cage or neutral

testing environments. However, when the same animals are placed in a threatening or activating situation (such as in a cage with cats) where the salience of environmental stimuli is heightened, these rats demonstrate an immediate (although transient) restoration of sensorimotor performance[49]. This study demonstrates that, without appropriate DA signaling, an animal will not dedicate attentional resources to external cues that normally generate orienting movements.

Some of the earliest and most striking illustrations of the impact of DA dysfunction came from the study of Parkinson's Disease (PD). In PD, progressive degeneration of the dopaminergic neurons of the SN pars compacta (SNc) results in significant motor impairment characterized by rigidity, hypokinesia, resting tremor, and postural instability. These motor impairments may be the most well-recognized symptom of PD, but the disease is also accompanied by a number of non-motor symptoms[50]. Neuropsychiatric symptoms commonly associated with PD include depression[51], anxiety[52], repetitive behaviors[53], and deficits in attention[54]. These symptoms and disorders result from neurobiological factors associated with the underlying neurodegeneration (i.e. central loss of monoamines)[55] and are not due to psychosocial factors or disability[56]. Impulsive behaviors (such as pathological gambling, shopping, eating, and hobbying) and repetitive behaviors[57] can result from or can be worsened by DA replacement therapy[58], the primary treatment for PD. That is, artificially increasing levels of DA in the brain results in impulsive and repetitive behaviors.

Perhaps most interesting and directly related to the symptomatology of ASD is a behavior called punding that is observed in Parkinson's patients on high levels of DA replacement therapy and in chronic AMPH users[59]. Punding refers to a constellation of

stereotyped behaviors including intense fascination with repetitive manipulation of mechanical objects, handling and examining of common objects, excessive grooming, and engagement in extended monologues. Punding includes such behaviors as, for example, taking apart and putting together watches, flashlights, or radios, sorting and arranging common objects, repetitions of single words and phrases, etc.[59,60]. In both Parkinson's patients on DA replacement therapy and chronic AMPH users[61], there is an excess of available DA, which is thought to result in the observed obsessive and stereotyped behavior. Attempts by caretakers to interrupt punding leads to irritability in patients with Parkinson's[59], suggesting a persistent, irresistible, compulsive need for the patient to engage in these behaviors, despite potentially adverse personal, familial, or occupational consequences. Punding, and the insistence on performing the behaviors which punding defines, are reminiscent of the repetitive behaviors, restricted interests, and strict adherence to routines observed in individuals with ASD[62]. Work in animals also supports a role for DA in compulsive and repetitive behaviors. For example, D1 receptor agonism has been shown to drive increased repetitive grooming behavior in mice, as has D2 receptor knockout[63].

In addition to the role that DA has been shown to play in motor behavior, there is a great deal of evidence linking DA with a second key clinical domain in autism - social function. Work in rats has demonstrated that DA is phasically released in response to pro-social cues and that this release is associated with social-seeking behavior, indicating an important role for the dopaminergic system in driving social behavior[64]. Studies in prairie voles found that administering a DA receptor antagonist prior to mating abolished mating-induced partner preference formation, indicating that DA within the NAc can directly

influence social choice[65,66]. These studies also found that D1-like receptors were upregulated in the NAc of prairie voles who have pair bonded compared to those who have not. The authors further demonstrate that this D1-like receptor upregulation was important in maintaining pair bonding[65], again highlighting the role of the dopaminergic system in social choice. Such findings have been replicated in a number of vertebrates, including non-mammalian species. For example, when zebrafish are given a D1-receptor antagonist, a significant reduction in social preference is observed (without change in motor function or vision)[67]. In humans, activation of the VTA and the caudate nucleus (two DA-rich areas) occurred when subjects were shown images of their romantic partner[68]. Given that social dysfunction is pathognomonic of ASD and that DA is intricately involved in social regulation, it seems highly probable that dysfunction in the dopaminergic system could underlie at least some forms of ASD, either through altered social reward processing or altered social choice.

Autism Spectrum Disorder

Core symptomatology

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) defines ASD as a neurodevelopmental disorder characterized by two key diagnostic criteria. The first is core deficits in social communication and social interaction. This includes persistent impairment in social reciprocity, nonverbal communication, and in developing, maintaining, and understanding relationships. Examples of such social deficits include (but are not limited to): avoiding eye contact, flat or inappropriate facial expressions, failure to understand personal space boundaries, deficits in reciprocal to-

and-fro communication, and difficulty using and understanding gestures, tone of voice, or body language. The second diagnostic criterion is restricted, repetitive patterns of behavior, interests, or activities. This can include such behaviors as repetitive motor movements, inflexible adherence to routines, highly restricted interests that are abnormal in intensity, and hyper- or hyporeactivity to sensory input (see Table 1). The addition of hypo- or hyperreactivity to sensory input to the diagnostic criteria for ASD in the DSM-V is a tribute to the growing recognition of and evidence for sensory processing and perceptual differences in ASD[69–73].

The behavioral manifestations of ASD are highly variable and depend on the severity of the condition, the patient's developmental level, and the patient's chronological age (although ASD is a life-long disorder). The current diagnostic rubric for ASD encompasses a number of disorders under previous diagnostic guidelines including: infantile autism, childhood autism, Kanner's autism, high-functioning autism, atypical autism, pervasive developmental disorder not otherwise specified, childhood disintegrative disorder, and Asperger's disorder. These prior classifications highlight the true heterogeneity of the ASD population and the need to consider ASD by subtype in our scientific endeavors.

The National Institutes of Mental Health Research Domain Criteria (RDoC) initiative suggests such a way to classify individuals with ASD and other neuropsychiatric disorders. The DSM relies on assemblages of clinical symptoms to determine diagnosis and, in doing so, neglects to incorporate objective biological measurements into diagnosis. This limits the ability of scientists and clinicians to effectively subcategorize patients with ASD and other neuropsychiatric disorders. RDoC proposes to classify

mental disorders based on not only the clinically observable behavior (the basis of the DSM-5), but also on laboratory-based neurobiological measures, such as genetic makeup and psychophysical and cognitive evaluations. This framework, by incorporating biology, behavior, and context, is more directed toward identifying the mechanistic underpinnings of the various manifestations of autism, and thus could hold greater promise for personalized or precision treatment approaches. The RDoC framework is particularly important in conditions as heterogeneous as ASD, and raises an immediate set of questions around the disorder and which are centered on what measures or co-morbidities (i.e. co-occurring conditions) could be used to subcategorize ASD.

Physiologic and psychiatric comorbidities of ASD

To explore possible subcategories of the ASD diagnosis, it is important to assess the conditions and maladaptive features that can co-occur with ASD. In addition to the aforementioned core symptomatology that defines autism, there exist a number of psychiatric conditions and physiologic features and disorders that frequently co-occur with autism. The specific presentation and combination of these comorbidities varies greatly across individuals with autism. However, the patterns in which these features co-occur may ultimately inform our understanding of ASD and permit the identification of diagnostic subcategories within the ASD diagnosis. While a complete review of all disorders and behavioral features frequently co-occurring with ASD is beyond the scope of this review, we will consider some of the most common disorders and features and how they may provide the scaffolding for partitioning the ASD population based on shared neurobiological and behavioral features. Of great utility in this endeavor is a study performed in 2014 that used an electronic health record time-series analysis to identify

three comorbidity clusters in ASD[74]: one characterized by psychiatric disorders, one characterized by multisystem disorders (including gastrointestinal disorders), and one characterized by seizures[74]. These subgroups encompass the most common comorbidities of ASD and provide a useful context for our discussion.

Focusing first on psychiatric cormorbidities, in one study of 112 children with autism, 70.8% had at least one current psychiatric disorder in addition to autism and 41% had two or more[75]. The most common comorbid psychiatric disorders in these children were attention deficit hyperactivity disorder (ADHD; 28.2%, 95% confidence interval [CI] 13.2-45.1), oppositional defiant disorder (ODD; 28.1%, 95% CI 13.9-42.2), social anxiety disorder (29.2%, 95% CI 13.2-45.1), and generalized anxiety disorder (13.4%, 95% CI 0-27.4)[75]. A larger and more recent study of 658 children with ASD seeking treatment for disruptive behavior demonstrated high rates of ADHD (81.2%), ODD (45.5%), and any anxiety disorder (45.5%) in those individuals[76]. In this particular study, 66.1% of the sample had two or more concomitant psychiatric disorders[76]. The particularly high rate of ADHD in children with ASD is of great interest as this could point both to a shared neurobiological etiology for at least some individuals with these disorders as well as a potential diagnostic subcategory within ASD (e.g. ASD with ADHD). Indeed, ADHD alone can impair social functioning[77,78] and can lead to low levels of reciprocal friendships[79], as is also seen in ASD. It is not surprising therefore that, when ADHD co-occurs with ASD, this disorder may exacerbate the social dysfunction pathognomonic of ASD. While the precise etiology of ADHD is elusive, disruptions in the neurochemical environment created by the neuromodulators DA and NE are thought to play a central role in this condition[80,81]. Taken together with the high rates of ADHD in ASD, it is possible that

dysfunction in either of these neuromodulatory systems could contribute to the etiology of ASD, especially in those individuals with concurrent ASD and ADHD.

Functional gastrointestinal disorders (fGID) are reported in 30-70% of individuals with ASD[82]. In one study of 845 individuals with ASD, at least one fGID was present in 30.5% of these individuals[82]. The Rome Foundation defines fGIDs as disorders of gut-brain interaction (DGBI). This group of disorders is classified by gastrointestinal (GI) symptoms related to any combination of motility disturbances, visceral hypersensitivity, altered mucosal and immune function, gut microbiota, and/or central nervous system processing[83]. This combination of symptoms causes an illness experience in the patient that is not due to clearly identifiable anatomic disorder[82]. It has been shown that children with ASD are more likely to experience the symptoms of abdominal pain, constipation, and diarrhea than those without ASD[84]. Some have suggested that these GI symptoms may exacerbate the behavioral symptoms exhibited by children with ASD by presenting a source of emotional distress that then manifests as problematic behaviors[85]. Indeed, maladaptive behaviors correlate with GI issues in individuals with ASD[86]. In children with ASD, behavior scores for irritability, social withdrawal, stereotypy, and hyperactivity are significantly higher in children with frequent abdominal pain, gaseousness, diarrhea, and constipation[86]. There is also evidence suggesting that metabolites produced by microbes in the gut (including neurotransmitters such as DA and 5-HT[87]) can influence brain function and behavior[88]. Many studies have reported differences in the composition of the gut microbiota in patients with ASD[89–91] and it is possible that these differences in gut flora may contribute to both behavioral and GI symptoms in ASD[88].

Table 1. *DSM-5 Criteria for Autism Spectrum Disorder (299.0 [F84.])*

A. Persistent deficits in social communication and social interaction across multiple contexts Manifested by all of the following:	**B. Restricted, repetitive patterns of behavior, interests, or activities** Manifested by at least two of the following:
1. Deficits in social-emotional reciprocity, ranging, for example, from abnormal social approach and failure of normal back-and-forth conversation; to reduced sharing of interests, emotions, or affect; to failure to initiate or respond to social interactions.	1. Stereotyped or repetitive motor movements, use of objects, or speech (e.g., simple motor stereotypies, lining up toys or flipping objects, echolalia, idiosyncratic phrases).
2. Deficits in nonverbal communicative behaviors used for social interaction, ranging, for example, from poorly integrated verbal and nonverbal communication; to abnormalities in eye contact and body language or deficits in understanding and use of gestures; to a total lack of facial expressions and nonverbal communication.	2. Insistence on sameness, inflexible adherence to routines, or ritualized patterns of verbal or nonverbal behavior (e.g., extreme distress at small changes, difficulties with transitions, rigid thinking patterns, greeting rituals, need to take same route or eat same food every day).
3. Deficits in developing, maintaining, and understanding relationships, ranging, for example, from difficulties adjusting behavior to suit various social contexts; to difficulties in sharing imaginative play or in making friends; to absence of interest in peers.	3. Highly restricted, fixated interests that are abnormal in intensity or focus (e.g., strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interests). 4. Hyper- or hyporeactivity to sensory input or unusual interest in sensory aspects of the environment (e.g., apparent indifference to pain/temperature, adverse response to specific sounds or textures, excessive smelling or touching of objects, visual fascination with lights or movement).
***Specify* current severity:**	
Level 3*: "Requiring very substantial support"*	
Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others. For example, a person with few words of intelligible speech who rarely initiates interaction and, when he or she does, makes unusual approaches to meet needs only and responds to only very direct social approaches.	Inflexibility of behavior, extreme difficulty coping with change, or other restricted/repetitive behaviors markedly interfere with functioning in all spheres. Great distress/difficulty changing focus or action.

***Level 2**: "Requiring substantial support"*	
Marked deficits in verbal and nonverbal social communication skills; social impairments apparent even with supports in place; limited initiation of social interactions; and reduced or abnormal responses to social overtures from others. For example, a person who speaks simple sentences, whose interaction is limited to narrow special interests, and who has markedly odd nonverbal communication.	Inflexibility of behavior, difficulty coping with change, or other restricted/repetitive behaviors appear frequently enough to be obvious to the casual observer and interfere with functioning in a variety of contexts. Distress and/or difficulty changing focus or action.
***Level 1**: "Requiring support"*	
Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful responses to social overtures of others. May appear to have decreased interest in social interactions. For example, a person who is able to speak in full sentences and engages in communication but whose to-and-fro conversation with others fails, and whose attempts to make friends are odd and typically unsuccessful.	Inflexibility of behavior causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence.

C. Symptoms must be present in the early developmental period (but may not become fully manifest until social demands exceed limited capacities or may be masked by learned strategies in later life).

D. Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.

E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay. Intellectual disability and autism spectrum disorder frequently co-occur; to make comorbid diagnoses of autism spectrum disorder and intellectual disability, social communication should be below that expected for general developmental level.

Specify if:

With or without intellectual impairment

With or without language impairment

Associated with a known medical or genetic condition or environmental factor

Associated with another neurodevelopmental, mental, or behavioral disorder

With catatonia

As communication between the brain and the gut is bidirectional, the converse may also be true: that neurobiological differences in ASD may drive dysfunction of the enteric nervous system and/or drive changes in microbiota composition[92,93] that then contribute to the GI symptoms experienced by individuals with ASD. The enteric nervous system (ENS), which consists of a system of neurons that spans the length of the digestive system, serves to regulate digestive function[94]. As many neurotransmitters and signaling pathways are common between the ENS and the CNS (including DA, 5-HT, and acetylcholine), pathophysiologic changes affecting CNS function can similarly impact ENS function[95]. ENS deficits are reported to co-occur with a number of CNS disorders that involve disturbances in the DA system. One such disorder is Parkinson's Disease (PD), which as described above, is defined by degeneration of nigrostriatal dopaminergic neurons resulting in a movement disorder characterized by rigidity, hypokinesia, resting tremor, and postural instability. Dopaminergic neurons are also found in the ENS and are important for proper gut motility[96]. Studies in mice[97,98] and humans[99] have demonstrated that dopaminergic neurons in the ENS are similarly susceptible to degeneration in PD. Indeed, PD is often accompanied by disturbances in GI motility including dysphagia, impaired gastric emptying, and constipation[100], which are thought to be due to ENS DA neuron degeneration and dysfunction.

Epilepsy has been recognized as a common comorbidity of ASD for decades[101]. Epilepsy is defined as the occurrence of two or more unprovoked seizures, which are the physical manifestation of sudden, abnormal, excessive, hypersynchronous neuronal firing[102,103]. Studies estimate this neurologic disorder occurs in 8.6%-22% of individuals with ASD[104–106], a prevalence considerably higher than that reported in all children (0.32%-

4.4%)[107]. Importantly, children with ASD and epilepsy have higher rates of hyperactivity and are at an increased risk for more severe autism symptoms and maladaptive behaviors due to the increased likelihood of these children having low IQ[108]. Children with ASD and epilepsy (but without intellectual disability) have higher rates of irritability and hyperactivity symptoms[108]. Given the high rates of epilepsy in ASD, there likely exists a shared biological etiology for these two conditions when they co-occur. Indeed, several genetic syndromes have been identified in which ASD and epilepsy co-occur, including conditions caused by mutations in single genes (or monogenic disorders) and by genomic copy number variation[109]. Evidence points toward shared neurobiological features between these two conditions, including alterations in cortical architecture and changes in the excitatory-inhibitory neurotransmission balance[110] (which we will discuss separately). Both of these neurobiological changes, alone or in combination, could lead to instability of neural networks, resulting in altered neuronal excitability, and ultimately seizures and the behaviors associated with ASD[111–113].

These clinical comorbidities demonstrate two important concepts. First, that considering co-occurring conditions in individuals with ASD is potentially an effective means to subcategorize the ASD diagnosis. Second, that a shared genetic etiology between ASD and these comorbidities may point us toward an understanding of the neurobiological mechanisms within these subcategories. Therefore, perhaps as important to the identification of subtypes of ASD as considering common comorbidities is to consider the genetic susceptibilities and variants that may give rise to dysfunction in specific neurobiological circuits. Doing so will not only improve identification of individuals with ASD by determining genetic risk factors, but also will improve clinical efficacy in

identifying possible comorbidities of these genetic changes which may complicate or exacerbate the clinical presentation of ASD in these individuals. We will briefly review the genetic and environmental risk factors associated with ASD and how these may point us toward a better understanding of the various forms of ASD that may exist and their unique neurobiologies.

Genetic and environmental risk factors

The earliest evidence for the contribution of genetic factors to autism came from twin and family studies conducted in the 1970s[114]. Since these earliest studies, twin studies have shown pairwise concordance rates of 10-31% for dizygotic (DZ) and 60-92% for monozygotic (MZ) twins[7,114–116]. Sibling recurrence rates have been estimated between 7% to 25.9% and increase to 13.5% to 32.2% if 2 or more siblings are diagnosed with ASD[117,118]. A study of children born in Sweden found that risk of autism is increased 10-fold if a full sibling has a diagnosis[119]. These studies collectively demonstrate the high heritability of ASD and emphasize the importance of dissecting the genetic etiology of ASD.

Syndromic causes of ASD, such as fragile X syndrome (FXS) and Rett syndrome, were among the first identified genetic causes of ASD[120–122]. Syndromic forms of ASD are typically caused by chromosomal abnormalities or single gene mutations and differ from non-syndromic ASD in their association with co-occurring phenotypes or dysmorphic features[123]. While syndromic forms of autism are now considered separate clinical entities from non-syndromic ASD, their genetic bases can provide important insights that may help better elucidate the genetic underpinnings of non-syndromic ASD.

In 2007, *de novo* copy number variants (CNVs) were first associated with autism[124] and, in 2012, whole-exome sequencing revealed the contribution of *de novo* single-nucleotide variants (SNVs) to ASD risk[10]. CNVs, segments of DNA 1kb or larger present in the genome at a variable copy number in comparison to a reference genome[125], include deletions, duplications, triplications, insertions, and translocations[126]. Well-known CNVs include large-scale chromosomal abnormalities, such as trisomy 21 in patients with Down syndrome, but smaller-scale microdeletions and microduplications have also been linked with various diseases[127]. In comparison, SNVs are single base differences in the genome. CNVs and SNVs are considered *de novo* when these genetic changes are present for the first time in a family member as a result of spontaneous genetic variation during the formation of germ cells (sperm or egg) or after the union of sperm and egg cells[128,129]. An SNV is rare if it occurs with a minor allele frequency of less than 1% and a CNV is considered rare if it occurs in less than 1% of the population[130,131].

Both rare SNVs (especially *de novo* mutations[8,9,12]) and common SNVs are now known to contribute to risk for ASD[132]. Indeed, whole exome sequencing has shown that *de novo* insertions and deletions that disrupt the proteins that are encoded by the affected genes are significantly more common in affected individuals than in their unaffected siblings[8]. Chromosomal microarray studies have shown that *de novo* CNVs are found in 8-10% of sporadic ASD cases[14,124], however *de novo* SNVs in protein-coding regions of the DNA are more common than large *de novo* CNVs[133]. While common and *de novo* variants in both copy number and single nucleotides underlie a portion of the genetic etiology of ASD[10,132], the detection of SNVs in individuals with ASD in particular permits us to consider the contribution of discrete, single-gene changes on specific

neurobiological pathways. This ultimately will lead to improved understanding of the contribution of dysfunction in these specific pathways to the behaviors associated with ASD and its common comorbidities. It is therefore of utmost importance to continue identifying and studying these SNVs to further define subgroups of ASD based on the observed neurobiological and behavioral consequences of these mutations.

To date, thousands of genes have been associated with risk for ASD. The Simons Foundation Autism Research Initiative's Human Gene and CNV Modules list 1,089 genes implicated in ASD and 2,291 CNV loci reported in individuals with ASD, respectively[134]. The roles of these genes in brain development and function are as varied in scope as they are in number. Some are involved in brain development, including the production, growth, and organization of neurons, while others are involved in the formation and maintenance of synapses and ongoing cell-cell communication[135]. A more thorough understanding of the function of these genes and how changes in these genes impact their function is required before we can begin to understand how they may inform the creation of subtypes of ASD based upon common neurobiological differences.

To state that genetic risk is the only, or even the most important, factor contributing to the development of autism would be a misstatement. There is overwhelming evidence suggesting that genetic risk alone may not be sufficient to drive the development of ASD. This is apparent even in the seminal twin and sibling studies of ASD. These studies found that while the concordance rates of ASD in MZ twins is high, it is incomplete[7,115,116]. Furthermore, genetic risk factors for ASD can be found in individuals who do not meet the diagnostic criteria for ASD[136].

There is direct evidence demonstrating a link between various environmental factors and exposures and risk for ASD. For example, in utero exposure to the anticonvulsant valproic acid (VPA) is associated with a significantly increased risk of autism (with an absolute risk of 4.42% in children exposed to VPA in utero compared to 1.53% in children not exposed to VPA in utero)[137]. A systematic review of 111 publications examining estimated environmental toxicant exposures and toxicant biomarkers in ASD found that toxins and toxic exposures, such as pesticides, phthalates, polychlorinated biphenyls, solvents, toxic waste sites, air pollutants, and pesticides may promote neurotoxic mechanisms leading to the development of ASD[138]. However, this review also found that the majority of these studies have significant limitations including small sample size, inadequate matching of controls and cases, retrospective design, and recall and publication biases that limit their validity[138]. This does not exclude the possibility that toxins and toxic exposures may play a role in the pathogenesis of autism but does underline the necessity of future studies designed to prospectively determine the role of environmental exposures in ASD risk.

The possible role of environment in ASD risk only serves to further highlight the importance of modeling detected *de novo* mutations and rare inherited variants in the laboratory to determine their contribution to the observed clinical presentation of ASD. These models allow us to examine the molecular and neurophysiological contributors to ASD and its comorbidities in isolation from, or in controlled combination with, potentially interacting environmental influences. Indeed, a number of animal models of the identified *de novo* and rare inherited genetic variants associated with ASD have been created. These models allow the discovery of important insights regarding the differential impact

of these mutations on neurobiological pathways and how these neurobiological changes may promote the behaviors associated with autism. Before discussing these models, it is important to review some of the prevailing perspectives on the neurobiological changes that have been hypothesized to result in or contribute to ASD.

Neurobiological theories of autism

Given the immense variability in the clinical presentation of children with ASD and the numerous genetic risk factors associated with ASD described above, a single cause of ASD is unlikely to exist. Many theories attempt to unify the myriad genetic and environmental risk factors now associated with ASD by suggesting shared changes in synaptic function or structure, cortical architecture, and/or network function as a result of these risk factors. Of these many theories, among the most popular are the excitatory-inhibitory balance (E/I balance) disruption theory[111,139,140], the altered network connectivity theory[141–144], and the altered predictive coding theory[145,146]. In the following, we will consider each of these theories individually and also speak to the commonalities they share.

The E/I balance disruption theory proposes that ASD is due to alterations in the ratio of excitatory to inhibitory neurotransmission[111,112]. Much data exists to support alterations in both excitatory and inhibitory neurotransmission in the brains of individuals with ASD. A large portion of this research has focused on alterations in neuronal activity and network communication in response to the primary inhibitory neurotransmitter in the brain, GABA, and the primary excitatory neurotransmitter in the brain, glutamate. In a meta-analysis of multiple ASD mouse models, Gogolla and colleagues noted reductions in the number of parvalbumin-positive, GABAergic interneurons (a specific inhibitory

interneuron subpopulation) in the cerebral cortex[139]. In another review, Cellot et al. describe alterations in GABAergic signaling in eight of the most commonly used animal models of ASD[147]. Findings in human cortical tissue also support alterations in the E/I balance. Disruptions in cortical minicolumn architecture have been reported in the brains of individuals with ASD[113,148]. Since the formation and function of these cortical minicolumns is dependent on lateral inhibition by GABAergic interneurons during development[149,150], the observed changes may be due to altered inhibitory neurotransmission. One of the common comorbidities associated with ASD also supports changes in the E/I balance. As discussed above, there are high rates of seizures in individuals with ASD[104–106]. Seizures are the result of excessive, hypersynchronous neuronal activity, consequently reflecting an imbalance between excitatory and inhibitory neurotransmission. Therefore, in individuals with ASD and epilepsy, a shift in the E/I balance could govern the observed clinical phenotype.

We must note there are many pathologic processes that can disturb the E/I balance beyond primary disturbances in GABAergic interneuron populations, including disruption of extracellular ion homeostasis, changes in cellular metabolism, altered receptor function, or altered neurotransmitter uptake[103]. One example of such an indirect effect can be seen in the striatum. As described previously, a large population of GABAergic neurons, called medium spiny neurons (MSNs), reside in the striatum and receive input from the thalamus, various association areas of cortex, sensory cortex, as well as from dopaminergic neurons projecting from the SNc[17]. MSNs are typically active immediately prior to the initiation of a movement and may encode the decision to make a movement toward a specific target[18]. By perturbing dopaminergic input to these MSNs, one could

disrupt the E/I balance in the striatum. Given the important role of the striatum in action selection and reward-mediated behaviors[18,151], both of which are disrupted in ASD, change in striatal function represents a plausible etiology for ASD-associated behaviors[152]. We will return to the possible role of dopamine and dopaminergic signaling in ASD in a later section.

The altered network connectivity theory holds that distortions in neuronal communication within the cerebral cortex are the primary drivers of the behaviors observed in ASD[153]. Many studies supporting this theory rely on functional magnetic resonance imaging (fMRI), an imaging technique that measures changes in blood oxygenation associated with neuronal activity[154]. The interregional correlations between fluctuations of the fMRI signal can be used to determine the operational interactions (i.e. functional connectivity) of various brain regions, whether or not an apparent physical connection between these regions exists[155]. Somewhat paradoxically, results of several fMRI studies reveal both decreases[143,153,156] and increases[144,157] in functional connectivity within and between a number of brain regions in individuals with ASD as compared to typically developed individuals. Hahamy and colleagues posit that these seemingly contradictory results could be the product of distinct distortions of the typical functional connectivity pattern in individuals with ASD[141]. Their study of a large database of fMRI resting state scans demonstrated that individuals with ASD have higher inter-subject variability in their connectivity patterns, both within and between hemispheres, as compared to controls. Moreover, the authors found that greater distortions of the typical interhemispheric connectivity maps correlated with more severe ASD symptoms[141]. Importantly, these patterns of resting state functional connectivity are thought to reflect

both genetic and environmental factors, including the manner in which an individual with ASD interacts with the environment[158].

The predictive coding hypothesis rests on the assumption that our brain creates internal models of the external sensory environment that serve as the basis for our predictions about, and consequently perceptions of, the world. These models (or "priors") are generated based on incoming sensory information (so-called "bottom-up" information) and are generally updated when incoming sensory information does not match the predictions of these models (i.e. when there is prediction error). If there is a mismatch in the degree of importance (i.e. weighting) the brain places on either the internal model or on the incoming sensory information, this would lead to a fundamental shift in one's perception of the sensory environment. According to this theory, individuals with ASD both improperly form and fail to appropriately update their internal models of the sensory environment. Some have posited this as the basis for the observed hyper- and hypo-responsivity to sensory stimuli observed in individuals with ASD[159]. As the fidelity of the incoming sensory information and the sensory representations they inform is essential to the formation of a coherent percept of the world, any change could cascade into altered processing of social cues and communication in individuals with ASD[72,160]. Incoming ("bottom-up") sensory information is also subject to the influences of these internal models (so called "top-down" predictions). These "top-down" predictions are built on the previously received sensory information. Therefore, dysfunction at either level would likely drive dysfunction in the other[146,161]. Pellicano and Burr suggest that individuals with ASD have hypo-priors, or weakened internal models of the external environment, leading to an over-weighting of incoming sensory information[145]. These authors further suggest that an

increased reliance on incoming sensory information (i.e. reduced ability to predict the sensory environment) could support many of the behavioral features associated with ASD (including sensory hypersensitivities, insistence on sameness, and repetitive behaviors)[146].

Much overlap exists among these hypotheses and they may have some degree of mechanistic commonality. For example, since proper E/I balance is essential to synaptic function in the brain, which in turn forms the basis of network communication, the observed changes in functional connectivity could be the result of E/I imbalances. The converse is also true: connectivity changes, by altering the patterns of ongoing neuronal activity, could drive E/I dyshomeostasis. Similarly, E/I balance disruptions are not entirely separable from the neurobiological mechanisms underlying the proposed changes in predictive coding. E/I balance disruptions could distort the representation of primary sensory information in the brain via changes in lateral inhibition and local communication or could alter communication between brain regions (i.e. network connectivity) and, ultimately, drive a failure of the brain to construct and update accurate internal models of the world.

These theories, when taken in combination with the large number of genetic variants associated with ASD, the pleiotropic nature of these variants, and the many comorbidities associated with ASD, point toward the distinct possibility that many disparate neurobiological mechanisms ultimately give rise to the complex and variable behavioral symptomatology associated with ASD. This heterogeneity provides a framework from which to study the genetic variants associated with ASD, with the ultimate goal of understanding their impact on brain function and, in turn, behavior. Such studies

will undoubtedly provide greater clinical insight as to the various molecular subtypes of ASD that may exist and, in doing so, allow for more precise outcome predictions and the development of more targeted interventions.

Among the many neurobiological mechanisms that could drive the aforementioned neurobiological changes (i.e. E/I imbalance, altered network communication, and sensory prediction error or altered predictive coding) is dysfunction of the dopaminergic system. DA is known to regulate both the release of excitatory and inhibitory neurotransmitters as well as neuronal responses to these neurotransmitters[162–165]. Inhibitory neurotransmitters such as GABA can similarly modulate dopaminergic function[166,167]. Therefore, a change in dopaminergic function could result in an E/I imbalance or an E/I imbalance could result in dopaminergic system dysfunction. Additionally, as DA is a regulator of ongoing neuronal activity, it is highly likely that DA dysfunction could lead to abnormal patterns of neuronal activation. These abnormal patterns of activation could in turn cascade into altered neuronal connectivity and therefore altered connectivity patterns of the cerebral cortex. In regards to the predictive coding theory of ASD, there is evidence to suggest a fundamental role for DA in the appropriate weighting of prior expectations and new sensory information[168]. Cassidy et al. found that people who weight their priors more strongly (i.e. overestimate the precision of their predictions) during perceptual inference have higher levels of striatal DA. Specifically, this study found that individuals with more striatal DA are more likely to report target auditory stimuli embedded in a stream. Taken together, these lines of reasoning suggest several possible mechanisms by which DA dysfunction could cascade into the behavioral symptoms associated with ASD.

Evidence for DA dysfunction in ASD

The role disruption to the dopaminergic system plays in ASD has been of interest since at least the 1970s[169]. Many studies between 1975 and 1990 focused on attempting to identify biomarkers of ASD by using indices of DA function derived from plasma, platelets, urine, and cerebrospinal fluid. These indices included measures of the levels of DA, of its downstream metabolite HVA (the end product of DA metabolism), and of the enzymes responsible for the metabolism of DA (i.e. dopamine-β-hydroxylase [DBH] and monoamine oxidase [MAO]). These studies, summarized in Table 2, failed to show replicable differences between individuals with ASD and controls in the concentration of DA, the concentration of products of DA metabolism, or in the activity of the enzymes involved in DA metabolism[170–176]. While disappointing in their inability to identify a consistent biomarker of ASD, these studies do not, however, exclude the possibility of central dopaminergic dysfunction playing an important role in ASD. Indeed, as is often noted in these studies, there exists much inter-individual variability in these measures, which could support the idea of multiple subtypes of ASD, each with a unique neurophysiological disturbance at its core. Hence, DA dysfunction may represent one of many different subtypes of ASD.

Contrary to these often-conflicting results in peripheral measures of dopaminergic system function in ASD, brain imaging studies have revealed more consistent results (see Table 3). One of the earliest imaging studies of DA-system dysfunction in ASD, a positron emission tomographic (PET) scanning study in 1997, found a 39% reduction in the accumulation of fluorine-18-labelled fluorodopa (FDOPA, a radiolabeled analog of DOPA used to visualize dopaminergic nerve terminals) in the anterior medial prefrontal cortex in

individuals with autism compared to the control group[177]. Since then, MRI studies have repeatedly demonstrated abnormalities in dopaminergic structures and their connectivity[178–189]. Multiple groups have found evidence for altered growth trajectory of the caudate nucleus in individuals with ASD (one of the major targets of the dopaminergic system)[178,179,181,184,186,190]. Sears and colleagues demonstrated significant enlargement in caudate volume in patients with ASD in two independent sample groups[178]. This study was limited by its inclusion of subjects exposed to neuroleptics. A follow up study in medication-naïve individuals with ASD (again in two independent sample groups) confirmed the finding of caudate nucleus enlargement in individuals with ASD[179]. McAlonan and colleagues found significant localized grey matter reduction in fronto-striatal networks, suggesting abnormal connectivity of dopaminergic structures with cortical structures in subjects with ASD[180]. Subsequent studies focused on dissecting changes in functional brain networks repeatedly demonstrated increased connectivity of striatal regions with cortex in individuals with ASD relative to control groups[185,187–189]. In several studies, authors uncovered a significant positive association between functional connectivity in fronto-striatal networks and severity of repetitive behaviors in individuals with ASD[184,188,190], linking altered dopaminergic system function with the behaviors associated with ASD. While these studies provide compelling evidence that differences in dopaminergic structures and their connectivity patterns exist in individuals with ASD, they do not provide definitive causal evidence. These changes could occur as a result of dysfunction in other neurotransmitter signaling pathways or structural protein differences that cascade into the observed differences in DA-related structure and connectivity.

Table 2. *DA-Related Biochemical Studies in Individuals with ASD*

Year	Authors	*n*	Measures	Sample Type	Findings	Limitations & Considerations
1987	Gillberg and Svennerholm	ASD = 25 Other psychotic disorders = 12 Control = 20	HVA	CSF	Mean CSF HVA elevated in ASD group ($p < 0.001$) Mean CSF HVA elevated in other psychotic disorders group ($p < 0.01$)	ASD group included 4 with FXS
1987	Launay et al.	ASD = 22 Control = 22	DA, NE, Epinephrine MAO in plasma only DOPAC and MHPG in urine only	Plasma	Mean NE elevated in ASD ($p < 0.02$) Mean epinephrine elevated in ASD ($p < 0.01$) No difference in mean DA	Wide age range of subjects
				Platelets	Mean DA lower in ASD group ($p < 0.05$) Mean epinephrine lower in ASD group ($p < 0.05$) No difference in mean MAO activity or norepinephrine	
				Urine	*ns*	
1975	Boullin et al.	ASD = 9 Control = 9	MAO activity	Platelet	*ns*	
1989	Minderaa et al.	ASD = 36-40 Control = 20-28	PRL, HVA, DA	Plasma	No difference in mean HVA or PRL	
				Urine	No difference in mean HVA or DA	
1977	Cohen et al.	ASD = 10 Nonautistic psychotic = 10 Central processing disturbance = 7 Aphasic = 7 Control = 9	HVA, 5-HIAA, Probenecid	CSF	Lower 5-HIAA in ASD group ($p = 0.04$)	Control group consisted of children with other medical conditions including vertebral disk disease, nonspecific headache, and conversion disorder

						Probenecid administered to artificially block egress of metabolites of dopamine and serotonin
1986	Garnier et al.	ASD = 19 Control = 15	DBH, HVA	Plasma	Higher DBH in more impaired ASD group ($s < 0.01$)	No difference was noted when ASD group as a whole was compared to control group
				Urine	Higher HVA in ASD group ($s < 0.02$)	
1977	Lake et al.	ASD = 11 Control = 12	NE, DBH activity, MAO activity	Plasma	Higher NE in ASD group ($p < 0.001$) Lower DBH activity in ASD group ($p < 0.05$)	

Table 3. DA-Related Imaging Studies in ASD

Year	Authors	*n*	Technique	Region	Findings	Limitations & Considerations
1997	Ernst et al.	ASD = 14 Control = 10	PET - FDOPA	Anterior medial cortex	39% reduction in FDOPA in ASD group ($p = 0.016$)	
1999	Sears et al.	Sample 1: ASD = 35 Control = 36 Sample 2: ASD = 13 Control = 25	MRI (volumetric)	Caudate nucleus	Increased volume of the caudate nuclei in ASD group in both samples (Sample 1: $p = 0.01$; Sample 2: $p = 0.003$)	Wide age range of subjects; no information on neuroleptic use in subjects
2004	McAlonan et al.	ASD = 17 Control = 17	MRI (connectivity defined as interregional grey matter volume correlation)	Fronto-striatal networks	Significant localized grey matter reduction in ASD group ($p < 0.01$)	
2007	Langen et al.	Sample 1: ASD = 21 Control = 21 Sample 2: ASD = 21 Control = 21	MRI (volumetric)	Caudate nucleus	Significant enlargement in the ASD group (Sample 1: $p < 0.05$; Sample 2: $p < 0.001$)	
2009	Langen et al.	ASD = 99 Control = 89	MRI (volumetric & gray matter density)	Caudate nucleus	Increased volume with age in ASD subjects ($p < 0.042$) & decreased volume with age in control subjects ($p = 0.002$)	
2011	Di Martino et al.	ASD = 20 Control = 20	MRI (functional connectivity)	Cortico-striatal networks	Prominent ectopic striatal functional connectivity in ASD group (increased functional connectivity between striatum and associative and limbic cortex) ($p < 0.05$)	
2012	Langen et al.	ASD = 21 Control = 22	DTI	Fronto-striatal networks	Lower fractional anisotropy of white matter tracts connecting putamen to	

					frontal cortex ($p < 0.019$) in the ASD group	
2013	Padmanabhan et al.	ASD = 42 Control = 48	fMRI (resting-state)	Cortico-striatal networks	Increased connectivity of striatal regions with parietal cortex and decreased connectivity with prefrontal cortex in the ASD group	
2013	Delmonte et al.	ASD = 28 Control = 27	MRI (functional & structural)	Fronto-striatal networks	Increased functional connectivity between striatum and multiple regions of the frontal cortex in the ASD group Increased functional connectivity between ACC and caudate was associated with deactivation to social rewards in the caudate ($p = 0.006$) Greater connectivity between the MFG and caudate was associated with higher restricted interests and repetitive behaviors ($p = 0.008$)	
2013	Di Martino et al.	ASD = 56 ADHD = 45 Control = 50	fMRI	Whole brain	ASD + ADHD group shared ADHD-specific abnormality of increased local connectivity in the right striatum ($p = 0.017$)	
2014	Langen et al.	ASD = 49 Control = 37	MRI	Striatum	Increased growth rate of striatal structures in ASD group specific to the caudate nucleus ($p = 0.005$), which correlated with repetitive behavior ($p = 0.009$)	
2016	Schuetze et al.	ASD = 373 Control = 384	MRI (surface-based)	Basal ganglia	Greater surface area in bilateral dorsal medial globus pallidus in individuals with more severe restricted, repetitive symptoms on ADOS ($q < 0.01$) Steeper increase in concavity of the caudal putamen and pallidum with age in the ASD group	
2017	Abbott et al.	ASD = 50 Control = 52	MRI (intrinsic functional connectivity)	Cortico-striatal networks	Cortico-striatal overconnectivity of limbic and frontoparietal seeds in ASD group	ASD group not controlled for medications or comorbidities

2018	Akkermans et al.	ASD = 24 OCD = 25 Control = 29	fMRI (resting-state)	Fronto-striatal networks	Significant positive association of repetitive behavior with functional connectivity between the left cuneate nucleus and the right premotor cortex ($p < 0.05$)	

There are many reasons why studies aimed at determining possible peripheral biomarkers of dopaminergic abnormalities in individuals with ASD might produce such variable findings, while the results of the many imaging studies related to the DA system in ASD are more consistent. First, as discussed above, most studies related to peripheral biomarkers of DA dysfunction in ASD focus on measurements of the concentration of DA and its metabolites (e.g. HVA) in peripheral body fluids. This is problematic as (1) DA cannot cross the blood-brain barrier and (2) there is no evidence to suggest that plasma or cerebral spinal fluid (CSF) HVA concentration measurements are sensitive enough to estimate DA activity in the brain[191]. In one study, the concentration of HVA was measured in four brain areas (dorsal frontal cortex, orbital frontal cortex, caudate, and putamen), in CSF, and in plasma. Correlations were tested between CSF and plasma and between these fluids and brain regions. Only the concentration of HVA in dorsal frontal cortex and in CSF were significantly correlated, limiting the value of plasma HVA measurements for determining DA activity in the brain[191]. Moreover, DA is also produced and release by various peripheral tissues including the pancreas[192], the adrenal medulla[193], the kidney[194], and peripheral leukocytes[195], complicating our interpretations. Finally, primary DA dysfunction is not required for observable changes in dopaminergic brain structures to occur. Genetic variants that impact synaptic scaffolding could lead to abnormal patterning of cortex and subcortical brain structures[196], such as the striatum and basal ganglia, without impacting the gross availability of DA and production of its metabolites.

Interventions and therapeutics for ASD targeting the DA system

Treatment for ASD is largely focused on developmental and behavioral therapies designed to improve social function, improve communication, promote academic functioning, and decrease maladaptive behaviors[197]. Currently, there are no psychopharmacological agents that treat the core symptoms of ASD. Psychopharmacotherapy is initiated only after potential contributors to problematic behaviors (e.g. psychosocial stressors, co-occurring psychiatric disorders, medical issues, and difficulties using functional communication[197]) are either addressed with behavioral and educational interventions or ruled out[198]. Psychotropic medications are then be used to treat specific symptoms such as hyperactivity, inattention, impulsivity, aggression, and anxiety.

Two of these symptoms, inattention and hyperactivity, are behaviors which have been linked with dysregulation or dysfunction of the DA system. These behaviors may result as a response to the sensory changes associated with ASD (e.g. hyperarousal resulting from changes in sensory processing[197]) or may be due to co-occuring psychiatric disorders, such as ADHD. Given the known role of the DA system in ADHD and the emerging evidence for DA dysfunction in ASD, individuals with ASD and co-occurring ADHD maybe represent a distinct subgroup that may have DA dysfunction or dysregulation as a core component contributing to their clinical presentation.

Strong evidence suggests that drugs targeting the DA system may reduce hyperactivity and improve inattention in some children with ASD[199]. In one randomized, controlled, crossover trial of methylphenidate, which acts via blockade of the DA and NE

transporters[200], 49% of enrolled subjects with ASD showed an improvement in parent- and teacher-rated hyperactivity, while 18% of enrolled subjects discontinued the medication due to adverse effects (most commonly irritability)[201]. In this study, methylphenidate did not improve Aberrant Behavior Checklist (ABC) subscale ratings for social withdrawal, stereotypy, or inappropriate speech[201]. This study highlights both the possible responsivity of ASD-related behavioral symptoms to modulation of DA systems as well as the inherent inter-individual variability that exists in ASD. In the future, it will be important to define, either genetically or through a comprehensive behavioral profile, individuals who might benefit most from therapies targeting the DA system and those who might be classified as non-responders to these targeted therapeutics.

The atypical antipsychotic drug risperidone (which functions via D_2 and serotonin 5-HT_{2A} receptor antagonism[202]) may also hold therapeutic value in the treatment of hyperactivity and inattention in some children with autism[199]. In a randomized, double-blind, placebo-controlled experiment, treatment with risperidone resulted in significant reduction in ABC subscale ratings for hyperactivity, lethargy/social withdrawal, and irritability. There is also strong evidence for the benefit of risperidone in treating other features of autism (including restricted, repetitive, and stereotyped behaviors, interests, and activities)[199]. At this time, evidence suggests limited efficacy of risperidone therapy for improving social communication in individuals with ASD[203]. Still, the positive value of risperidone for the treatment of the non-social symptoms of ASD highlights the possibility that dopaminergic dysfunction may lay at the underlie at a least a portion of the behavioral change observed in a subset of individuals with ASD. Identifying these individuals through genetic testing, cognitive testing, and psychophysical evaluation is essential for the

practice of personalized, precision medicine to improve outcomes and quality of life for affected individuals.

CHAPTER II

MODELING DOPAMINE DYSFUNCTION IN AUTISM SPECTRUM DISORDER: FROM INVERTEBRATES TO VERTEBRATES

Introduction

While human studies certainly suggest the involvement of the dopaminergic system in ASD in at least a subset of individuals, the question remains how the various genetic risk factors associated with ASD might lead to dysfunction of dopaminergic systems and, ultimately, the associated clinical phenotype. Animal models provide a particularly useful system for exploring the impact of *de novo* mutations and rare inherited variants on the neurobiological mechanisms that may contribute to the constellation of symptoms associated with ASD. We will consider here evidence from invertebrate and vertebrate models demonstrating how changes in DA system function, either due to directed mutation to the DA system or via genetic variants associated with ASD, provide important insights into the links between DA signaling and phenotypic characteristics of ASD.

Drosophila melanogaster (Fruit Fly)

The common fruit fly, *Drosophila melanogaster*, has long been used as a model system in neuroscience[204]. The utility of the fly model is reinforced by the numerous structural, functional, and genetic parallels between the brains of vertebrates and *Drosophila*. At the genetic level, 77% of human disease genes have homologs in

Drosophila[205] that can be easily manipulated to study the association between genotype and phenotype. Structurally, in the same way the vertebrate brain is organized into a tripartite pattern (forebrain, midbrain, and hindbrain), the *Drosophila* brain also follows a tripartite organization[206]. At the functional level, *Drosophila* brains contain the same key neurotransmitters (i.e. glutamate, GABA, DA, homologs of epinephrine and NE, and 5-HT) as the vertebrate brain and the requisite synthetic enzymes, transporters, and receptors for these neurotransmitter systems[207]. Given these numerous parallels, it is perhaps not surprising that there now exist many studies of ASD-associated genes and their associated neurobiology in *Drosophila*[208]. Several of these studies, summarized in Table 4, reveal clear interplay between the genes associated with ASD, the DA system, and the behaviors associated with both ASD and DA.

At the broadest level, genetic and physiologic work in *Drosophila* has sought to determine the role of DA and the constituent components of the DA system in behavior. This body of evidence shows that perturbations to the DA system can result in a number of abnormal social and motor behaviors. For example, loss-of-function mutations to the VMAT (the transporter responsible for packaging monoamines into vesicles for synaptic release) have been shown to drive increased social spacing in flies[209], while overexpression of VMAT drives abnormal courtship behaviors, decreases successful mating, drives stereotypic grooming, and increases locomotion[210]. Alterations to the synthetic pathway of DA can result in similarly abnormal social and motor behaviors in *Drosophila*. Loss of tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of DA, results in abnormal social spacing in female flies and reduced locomotion, while disinhibition of TH results in increased social spacing in male flies[209]. Knockout of *TH* in

neurons specifically results in reduced activity and locomotor deficits[211]. Stimulation of D1 and D2 receptors in flies has been shown to drive repetitive grooming behavior[212]. These studies provide direct links between dysfunction of the DA system and behaviors associated with ASD (i.e. social deficits and repetitive behaviors) as well as behaviors associated with other neuropsychiatric disorders, including ADHD.

A great deal of work has linked genetic mutations associated with syndromic forms of ASD with DA dysfunction in *Drosophila*. Fragile X Syndrome (FXS) is the most common inherited cause of intellectual disability and the most frequent monogenic cause of ASD. To be clear, FXS is considered a separate clinical entity from ASD, however one in three individuals with FXS also have ASD. As such, studying this syndrome provides a valuable window into the neurobiology of ASD. This syndrome results from a CGG repeat expansion in the *FMR1* gene[213], leading to transcriptional silencing of *FMR1* and resultant reduction or absence of fragile X mental retardation protein (FMRP)[214,215]. At least two groups have demonstrated that mutation to the *Drosophila* homolog of *FMR1*, *dfmr1*, has significant impacts on the DA system in flies[216,217] and drives behavioral abnormalities typically associated with ASD including social deficits and repetitive behaviors[217]. Zhang and colleagues demonstrated that *dfmr1* null mutants have significant increases in the synthesis of DA and 5-HT in the brain and in the number of dense core vesicles that package these neurotransmitters for secretion[216]. Subsequent work by Tauber *et al.* confirmed and extended these findings by showing that *dfmr1* mutant flies exhibit elevated levels of VMAT mRNA, which is necessary for the packaging of DA into presynaptic vesicles[217]. This group also identified behavioral abnormalities consistent with FXS and ASD in *dfmr1* mutant flies including reduced courtship (a series of

stereotypical behaviors carried out by male flies that is used to assay social behavior that permits the exchange of somatosensory, chemosensory, auditory, and visual information between male and female flies[218]), impaired climbing, impaired flight as adults, and repetitive grooming behavior[217]. Importantly, depletion of monoamines with reserpine (a drug that reduces uptake and intracellular stores of monoamines via irreversible blockade of VMAT) in these flies suppressed the observed repetitive grooming behavior, directly linking DA dysregulation due to *dfmr1* mutation with the observed behavioral changes[217]. Beyond ASD-related behavioral symptoms, *dfmr1* mutants also demonstrate FXS-related behavioral changes including deficits in commonly used learning/memory assays. Specifically, *dfmr1* mutants show deficits in assays of olfactory association (i.e. learning to associate an odor with an aversive or appetitive stimulus)[219,220]. While these findings are not specific to DA or to ASD, the presence of learning/memory deficits in *dfmr1* mutants supports the validity of these mutants for modeling FXS and studying the neurobiological mechanisms underlying this condition. Together, these studies in *Drosophila* provide suggestive evidence that FXS, a syndromic form of ASD, may include a component of DA dysfunction.

The *UBE3A* gene encodes a protein that acts both as a ubiquitin ligase and as a co-activator of transcription[221,222]. While maternally inherited deletion of this gene is known to cause Angelman Syndrome[223], duplication or triplication of chromosomal regions encompassing *UBE3A* causes a highly penetrant form of ASD[224]. In *Drosophila*, overexpression in sensory neurons of dUBE3A (the *Drosophila* homologue of UBE3A) results in decreased dendritic branching, suggesting that the appropriate level of dUBE3A is essential for the proper neuronal development[225]. While this finding is not specific to

dopaminergic neurons, overexpression of dUBE3A has also been shown to elevate the rate-limiting cofactor in monoamine synthesis (tetrahydrobiopterin [THB]) and to significantly increase DA levels in *Drosophila*[226]. This increase in DA drives hyperactivity in flies[226], a behavior commonly observed in both ADHD. Conversely, loss of dUBE3A has been shown to decrease THB and significantly reduce DA pools, resulting in hypoactivity in *Drosophila*[226]. These findings provide additional evidence of a possible role for an ASD-associated genetic mutation in DA homeostasis and behaviors associated with ASD.

Members of the neuroligin family (*NLGN*) have been implicated as candidate genes for ASD[227,228]. Neuroligins are post-synaptic cell surface proteins that bind with neurexins to guide the formation of functional synapses[229–232] and are essential for normal synaptic function[233]. Five neuroligin genes have been identified in humans, with at least three of these also being identified in rodents[230,234]. Neuroligins-1, -2, and -3 are principally expressed in the CNS, where neuroligin-1 is enriched post-synaptically at exclusively at excitatory synapses[229] and neuroligin-2 is expressed post-synaptically at inhibitory synapses[235]. Neuroligin-4 is expressed primarily in the cerebral cortex and is localized to excitatory synapses[236]. Early work connected NLGN3 and NLGN4 with ASD[228,237], but more recent work also suggests a role for NLGN2[238]. Thus, *Drosophila* deficient in neuroligin-2 exhibit a number of abnormal behaviors including reduced social interaction (as measured by increased inter-individual spacing when placed in groups and reduced motivation to engage in courtship behaviors) and impaired transitions between behaviors, including transitions between walking and turning and from courtship singing to subsequent behavior[239].

Increasing empirical evidence associates DAT variants with ASD and with the behavioral features associated with ASD[127,240–242]. A number of these ASD-associated DAT variants have been modeled in *Drosophila*. Results of these studies show that these variants disrupt DA signaling and drive behavioral abnormalities consistent with ASD[127,240]. The human DAT (hDAT) T356M mutation (a threonine to methionine substitution at position 356) results in an "open to the outside" conformation of the transporter (thereby driving anomalous DA efflux through the transporter) and reduces DAT-mediated DA uptake in cells. *Drosophila* expressing the T356M form of the hDAT display increased locomotion[240]. Similarly, the hDAT ΔN336 mutation promotes a DAT intracellular gate conformation that is "half-open and inward facing", also resulting in reduced DA uptake. *Drosophila* expressing the hDAT ΔN336 exhibit impaired social behavior during escape, hyperactivity, and prolonged freezing and reduced fleeing in response to predatory cues[127]. These two mutations, identified in individuals with ASD, directly link altered DA function with ASD-associated behaviors in *Drosophila*.

Collectively, these studies in *Drosophila* demonstrate that ASD-associated genetic changes can lead to behavioral abnormalities that recapitulate many aspects of the ASD phenotype, and that these mutations can drive abnormalities in the dopaminergic system. *Drosophila* reproduce rapidly, reach adulthood in 10-15 days, and can be easily genetically manipulated[243], making them ideal candidates for first-pass identification of behavioral and physiological changes that may result from genetic variants. The advent of new techniques and technologies for studying complex behavior (i.e. social behaviors and repetitive behaviors) in *Drosophila* further serves to increase the attractiveness of

this organism for use in screening studies of new variants associated with ASD as they are discovered.

Danio rerio (Zebrafish)

Danio rerio (zebrafish) are attractive as a model organism to study ASD for a number of reasons. These vertebrate animals are inexpensive, highly prolific, develop quickly, have high genetic homology with humans, are easy to manipulate genetically, and display robust behavioral phenotypes (including social and reward-related behaviors)[244,245]. Zebrafish are also appealing from a neuroscientific perspective as zebrafish possess all the neurotransmitters, receptors, transporters, and enzymes required for glutamate, GABA, acetylcholine, and biogenic amine (DA, NE, 5-HT, and histamine)-mediated neurotransmission[246–249]. Although zebrafish lack the midbrain dopaminergic system seen in mammals (e.g. the substantia nigra and ventral tegmental area)[250], they do have a significant telencephalic population of DA neurons that may be the zebrafish homolog of the substantia nigra[249]. Despite this difference in the organization of the dopaminergic system, zebrafish do respond behaviorally to pharmacological manipulations of the DA system in a manner consistent with mammalian models[251]. For these reasons, zebrafish have much potential for high-throughput genetic screening (similar to the proposed screening studies put forth above) in the study of DA dysfunction in ASD and other neuropsychiatric disorders.

Behaviors that are frequently altered in ASD (social behavior and motor behaviors) have been associated with DA in zebrafish. Mahabir and colleagues demonstrated that, in two different strains of zebrafish, maturation of shoaling (the aggregation of swimming

fish for social purposes) correlates with increases in the level of DA and DOPAC in the brain with age[252]. In another study, zebrafish exposed to the D_1 receptor antagonist SCH23390 showed significant reductions in social preference, again providing evidence for the role of the dopaminergic system in regulating social behaviors[67]. Similarly, motor behaviors have been linked to the zebrafish dopaminergic system. Targeted ablation of dopaminergic neurons in zebrafish results in decreased locomotor activity[253,254]. Exposing developing zebrafish to exogenous DA during key periods during development suppresses episodes of swimming, while ablating dopaminergic neurons during the same period in development increases episodes of swimming[255]. Exposing zebrafish larvae to either quinpirole (a D_2 receptor agonist) or SCH23390 (a D_1 receptor antagonist) reduces larval movement, while exposing larvae to SKF-38392 (a D_1 receptor agonist) increase larval movement[254]. These results demonstrate a key role for DA in the development of social behaviors and motor function in zebrafish. With this information in hand, the question then becomes whether mutations associated with ASD can drive ASD-like behaviors in zebrafish and to what degree these changes in behavior stem from dopaminergic dysfunction. Multiple transgenic zebrafish models have been created as a tool to study the role of ASD-associated mutations on behavior and brain morphology. We will consider some of the more important of these models here, with an emphasis on the evidence for dopaminergic dysfunction resulting from these mutations.

Mutation to *SHANK3* has been identified as a causative factor of ASD[256]. SHANK3 is a postsynaptic protein whose major functions include modulating the scaffolding of glutamatergic postsynaptic densities and promoting normal development of MSN morphology in the striatum[257]. As described earlier, MSNs are the major target of

dopaminergic projections arising from the substantia nigra and the ventral tegmental area[17]. In zebrafish, knockdown of *shank3* (the zebrafish orthologue of *SHANK3*) results in disruptions in motor behaviors (i.e. unproductive swim attempts), seizure-like behavior (a common comorbidity of ASD, as described above), and delayed mid- and hindbrain development (regions of the zebrafish brain responsible for motor control)[258]. Zebrafish with *shank3b* loss-of-function mutations show impaired shoaling behavior (as measured by increased inter-fish distance resulting in larger and looser schools) and reduced frequency and duration of social contacts with conspecifics[259]. These animals also exhibit repetitive swimming patterns, such as repetitive or sterotyped figure "8" swimming, circling, cornering, and walling (swimming repetitively up and down one side of the test chamber)[259]. Given the known role of dopaminergic neurons in the development of both motor and social behaviors in zebrafish[252–255], it is likely that DA dysfunction plays a role in the phenotype observed in these *shank3* mutants.

As described earlier, exposure to the anticonvulsant VPA in utero is a known risk-factor for ASD[137]. Zebrafish exposed to VPA during development exhibit loss of social preference, hyperactivity, and anxiety-like behavior[260]. Importantly, Baronio and colleagues demonstrated that exposing zebrafish embryos to VPA results in reduced mRNA expression of the tyrosine-hydroxylase encoding gene *th1* (the rate limiting enzyme in the production of dopamine) and reduced mRNA expression of the dopamine-β-hydroxylase encoding gene *dbh* (the enzyme that converts DA into NE). The authors also report reductions in the number of TH1-immunoreactive cells. However, this does not mean VPA acts only to alter expression of mRNAs associated with DA synthesis. VPA inhibits histone deacetylase (which is associated with epigenetic regulation of gene

expression). Studies suggest VPA alters the expression of over 1300 genes, including subunits of the GABA receptor[261]. While VPA does not solely target the DA system, there is reasonable evidence that this known risk factor for ASD (in utero VPA exposure) can drive ASD-associated behaviors in zebrafish that are accompanied by alterations to the dopaminergic system in these animals.

Given the high comorbidity of ASD and ADHD, it is likely that the two disorders share neurobioloigical substrates. As such, considering dysfunction of the DA system in the context of ADHD is also useful to our understanding of how DA dysfunction may play a role in ASD. Dopaminergic dysfunction has been specifically studied in zebrafish models of ADHD-risk alleles. One such ADHD-susceptibility gene is *LPHN3*, which encodes latrophilin 3. Latrophilins function as both adhesion molecules (essential for the formation of synapses) and as G-protein-coupled receptors (whose activation sets in motion an intracellular signaling cascade with multiple targets that act in concert to regulate neuronal activity)[262]. At the behavioral level, loss of function of the *LPHN3* ortholog (*lphn3.1*) in zebrafish results in hyperactivity and increased bursts of swimming, suggesting motor impulsivity[263]. This is accompanied by impaired DA system development, including misplacement of DA neurons in the ventral diencephalon[263].

Much work remains to establish a definitive link between dopaminergic dysfunction and alterations in behaviors associated with ASD-associated mutations in zebrafish. However, the current evidence suggests that ASD-associated mutations do drive dysfunction in social and motor behaviors in zebrafish. Given that these behaviors are behaviors known to be under the influence of the dopaminergic system, future work should aim to determine whether changes in dopamine signaling are sufficient to drive

changes in these behaviors consistent with the pattern observed in ASD (i.e. social deficits and restricted and repetitive interactions or activities).

Mus musculus (Mouse)

Perhaps the strongest evidence supporting a role for DA dysfunction in ASD comes from work performed in mouse genetic models. These models shed light on the impact of genetic mutations associated with ASD on underlying neurobiological pathways and the complex behaviors supported by these pathways. As in *Drosophila melanogaster* and *Danio rerio*, work in mice has historically focused on dissecting the impact of mutations associated with the most common, syndromic forms of ASD. However, a growing number of studies now model rare variants associated with ASD and their role in the pathophysiology of ASD, providing new insight into possible mechanistic underpinnings.

Among the earliest mouse genetic models of ASD-associated conditions were the *Mecp2*-null mouse and the *Mecp2*$^{308/y}$ mouse. These models replicate the protein truncating mutation causative of Rett Syndrome, a syndrome which (while considered a separate clinical entity from ASD by the DSM-5) shares many clinical characteristics with ASD (including withdrawal from social engagement and repetitive behaviors). Both the *Mecp2*-null mouse and the *Mecp2*$^{308/y}$ mouse have a number of behavioral changes similar to the behaviors observed in Rett Syndrome (of which many are applicable for ASD). These behavioral changes include loss of social preference (i.e. reduced exploration of novel social targets)[264] and a number of motor deficits. These motor deficits include impaired motor coordination as measured by a reduced latency to fall on the

accelerating rotarod task and impaired motor skill learning evidenced by limited improvement on the accelerating rotarod task over consecutive days of training[265–267]. In addition to the behavioral abnormalities observed in *Mecp2*-null mice are a number of abnormalities in dopaminergic synapses and dopaminergic brain structures[266–268]. Such abnormalities include marked reductions in the population size of tyrosine hydroxylase-expressing neurons in the substantia nigra pars compacta[267] and reduced DA content in the striatum[267,268,268]. Importantly, Su et al. demonstrated that selective deletion of MeCP2 in the striatum was sufficient to disrupt DA content and locomotor activity, directly linking disruption of a gene known to be causative of ASD-associated behaviors and DA dysfunction.

As discussed previously, mutation to *SHANK3* (a postsynaptic scaffolding protein involved in the normal development of medium spiny neurons in the striatum) has been identified as a causative factor of ASD[256]. *Shank3B*-null mice and mice with disruptions in major isoforms of *Shank3* (i.e. *Shank3*$^{e4\text{-}9}$) exhibit behaviors associated with ASD, including reduced social interaction and repetitive behaviors (i.e. repetitive grooming)[269,270]. *Shank3B*-null mice also display alterations in the morphology of striatal MSNs and volumetric enlargement of the caudate[269]. Knock-down of *Shank3* specifically in ventral tegmental neurons results in a similar behavioral phenotype (abnormal social behaviors and repetitive behaviors) as well as altered neuronal activity patterns in DA neurons[271], suggesting that reduction of *Shank3* in dopaminergic neurons is sufficient to drive the ASD-associated behavioral changes exhibited by these mice.

As discussed previously, a number of neuroligin family genes (*NLGN*) have been implicated as candidate genes for ASD[227,228]. The *NLGN* genes encode post-synaptic cell

surface proteins critical for guiding the formation of synapses[229–232]. In mice, knock-down of neuroligin-2 significantly impacts the formation of dopaminergic synapses in the striatum, resulting in a reduction in the density of dopaminergic synapses on medium spiny neurons (MSNs) and an increase in the number of GABAergic synapses on MSN dendrites, indicating a shift in the excitatory-inhibitory ratio[272]. Overexpression of neuroligin-2 similarly results in the reduction of the excitatory-inhibitory ratio and drives a number of behavioral abnormalities (including stereotyped jumping behavior, anxiety, and impaired social interactions)[273]. While the authors of this study attribute these behavioral changes to potentiation of inhibitory responses in the frontal cortex[273], it is likely that the observed excitatory-inhibitory imbalance extends to and results in the dysfunction of other regions of the brain, including striatal structures, which are both a major target of and source of input to the cortex. This concept is supported by work demonstrating that knockout of and mutation in neuroligin-3, another member of the neuroligin family, results in selective reduction of synaptic inhibition on D1-dopamine receptor-expressing MSNs in the ventral striatum that is accompanied by enhanced formation of repetitive motor routines[274]. Loss of neuroligin-4, which is most highly expressed in the olfactory bulb, striatum, cortex, and hippocampus, results in social deficits in mice (reduced social preference and reduced ultrasonic vocalizations) as well as a reduction in total brain volume. These mice also exhibit impaired olfaction, a function that is highly dependent on dopaminergic signaling[275,276].

The DAT T356M mutation, as previously discussed, was identified in an individual with ASD and results in dysfunction of the DAT (i.e. constitutive reverse transport of DA)[240]. While the specifics of this model will be discussed in the following chapters, in

brief we found that mice homozygous for this mutation (DAT T356M$^{+/+}$ mice) displayed significant impairments in the uptake of released DA and reduced total tissue content of DA[277]. DAT T356M$^{+/+}$ mice also exhibited a number of behavioral changes similar to the behavioral characteristics of ASD. These behavioral changes included reduced social preference (i.e. equal time spent exploring an inanimate target as spent with a social target), repetitive rearing behavior, and profound increases in spontaneous locomotor activity[277]. Antagonism of the DAT reduced the observed hyperlocomotion in DAT T356M$^{+/+}$ animals, suggesting that DAT-mediated leak of DA may underlie hyperactivity in these animals[277]. This research (summarized in Table 4) provides new, direct evidence for a role of DA dysfunction (specifically anomalous DA efflux) in the behavioral changes typically associated with ASD and ADHD.

Table 4. *Animal models of ASD, their known behavior changes, and evidence for dopaminergic dysfunction*

Year	Authors	Species	Mutation or Manipulation	Social Behavior	Motor & Repetitive Behavior	Other Behavior	Evidence for DA or Neuronal Dysfunction
***Danio rerio* (Zebrafish)**							
2013	Mahabir et al.	Zebrafish	WT (2 strains: AB & TU)	Maturation of shoaling behavior (index of social behavior) differs between the two strains and correlates with DA & DOPAC levels			DA & DOPAC levels increase differentially with age in the two strains & correlates with behavioral differences between the strains
2012	Scerbina et al.	Zebrafish	WT (AB Strain) treated with D1 receptor antagonist	Significant reduction of social preference			
2008	Thirumalai and Cline	Zebrafish	WT				Endogenous DA release suppresses swim circuits in developing zebrafish
2017	Kacprzak et al.	Zebrafish	DAT-KO			KO: anxiety-like phenotype in fish WT + COC: anxiety-like phenotype D1R inhibition: rescue of anxiety-like phenotype (not seen with D2/3R inhibition)	WT + COC: reduced DAT mRNA abundance
2015	Kozol et al.	Zebrafish	*syngap1* knockdown		Disruptions in motor behaviors (unproductive swim attempts)	Seizure-like behaviors	Delayed mid- and hindbrain development (responsible for locomotor behaviors)

			shank3 knockdown				
2018	Liu et al.	Zebrafish	*shank3b* loss-of-function mutant	Reduced social interaction (increased inter-fish distance during shoaling) and reduced social preference	Reduced locomotor activity	Repetitive behaviors (circling and figure “8” swimming patterns)	
2011	Souza et al.	Zebrafish	DA		90% decrease in larval movement ($p = 0.007$)		
			Quinpirole (D_2R agonist)		94% decrease in larval movement ($p = 0.011$)		
			SCH-23390 (D_1R antagonist)		94% reduction in larval movement ($p = 0.011$)		
			SKF-38392 (D_1R agonist)		120% increase in larval movement ($p = 0.005$)		
			MPTP		60% decreased in larval motor activity ($p = 0.002$)		25% decrease in TH expression in brains of 5 dpf larvae
2015	Jay et al.	Zebrafish	Targeted ablation of supraspinal DAergic neurons		Decreased locomotion		
2012	Lange et al.	Zebrafish	lphn3.1 (risk factor for ADHD)		Hyperactivity and swimming bursts (impulsivity) rescued by methylphenidate and atomoxetine		Reduction and misplacement of DA neurons in the ventral diencephalon
2015	Zimmerman et al.	Zebrafish	VPA exposure	Social interaction deficit (loss of social preference)	Hyperactivity	Anxiety	

2017	Baronio et al.	Zebrafish	VPA exposure	Loss of social preference			Reduced th1 Reduced dbh Reduced number of TH1-immunoreactive cells
***Drosophila melanogaster* (Fruit fly)**							
1997	Yellman et al.	Decapitated Drosophila	D1-like agonists		Stimulate hind-leg grooming		
			D2-like agonist (quinpirole)		Stimulate hind-leg grooming and locomotion		
2006	Chang et al.	Drosophila	VMAT-A overexpression	Prolonged courtship behavior & decrease in successful mating	Stereotypic grooming behavior Increased locomotion		
2011	Riemensperger et al.	Drosophila	*TH* knockout in neurons		Reduced activity & locomotor deficits that increase with age4/3/20 11:28:00 AM	Hypophagia	
2017	Fernandez et al.	Drosophila	*VMAT* loss-of-function mutants	Increase in social spacing (similar to non-social flies)			
			Tyrosine hydroxylase knockout	Females exhibit increased social spacing	Reduced locomotion		
			RNAi against inhibitor of tyrosine hydroxylase (*catsup*)	Males exhibit increased social spacing			
2011	Tauber et al.	Drosophila	*dfmr1* mutant	Reduced courtship (social interaction)	Impaired climbing and impaired flight as adults	Excessive grooming	VMAT blockade suppresses excessive grooming *Dfmr1* mutant flies exhibit elevated levels of VMAT mRNA

							Reserpine (depletes monoamines) suppresses excessive grooming
2005	Zhang et al.	Drosophila	*dfmr1* null mutant				Significant increase in dopamine in brain Elevation of the dense core vesicles that package monoamine neurotransmitters for secretion
2015	Cartier et al.	Drosophila	Syntaxin 1 mutant (STX1 R26Q)		No effect of AMPH on locomotion		Reduced AMPH-induced DA efflux Enhanced DAT-mediated uptake of DA
			DAT mutant (hDAT R51W)		Significantly reduced effect of amphetamine on locomotion		Reduced AMPH-induced DA efflux
2013	Hamilton et al.	Drosophila	hDAT T356M		Significantly increased locomotion		Reduced DAT-mediated DA uptake Anomalous dopamine efflux
2009	Lu et al.	Drosophila	*dUBE3A* overexpression in sensory neurons				Decreased dendritic branching
2011	Ferdousy et al.	Drosophila	*dUBE3A* overexpression		Hyperactivity		Elevated THB (rate-limiting cofactor in monoamine synthesis) and significantly increased DA
			Dube3a knockout		Hypoactivity		Loss of *Dube3a* results in decreased tetrahydrobiopterin (rate-limiting cofactor in monoamine synthesis)

							and significant reduction of DA pools
2013	Hahn et al.	Drosophila	*dnl2* (orthologue of neuroligin) mutant	Impaired social interaction Alters acoustic communication signals Less female-directed courtship			
***Mus musculus* (Mouse)**							
2014	Mergy et al.	Mouse	DAT A559V		Reduction in novelty-induced rearing Increased darting speed Blunted behavioral response to AMPH		Anomalous DA efflux (significant elevation of basal extracellular DA) Loss of AMPH-induced DA efflux
2011	Panayotis et al.	Mouse	*Mecp2*-null		Reduced grip strength Poorer performance on rotarod		Morphological and function alterations in substantia nigra pars compacta (decreased Th protein levels; fewer Th-positive neurons; reduced Th phosphorylation; reduced [DA] in caudate-putamen)
2015	Su et al.	Mouse	*Mecp2*-null				MeCP2 mantains local dopamine content in a non-cell autonomous manner in the rostral striatum
2015	Kao et al.	Mouse	*Mecp2*-null		Hypoactivity Impaired motor coordination Impaired motor skill learning		Significant reduction in striatal dopamine content Down-regulation of TH

							Up-regulation of dopamine D2 receptors
2001	Guy et al.	Mouse	*Mecp2*-null		Stiff, uncoordinated gait Reduced spontaneous movement Hindlimb clasping Irregular breathing		
2002	Mineur et al.	Mouse	*Fmr1* knockout			Diminished learning on radial maze Increased locomotor activity	
2008	Wang et al.	Mouse	*Fmr1* knockout			Increased locomotor activity	Impaired D1 receptor signaling D1 receptor hyperphosphorylation D1 receptor agonist partially rescues hyperactivity
2013	Rogers et al.	Mouse	*Fmr1* knockout				Attentuated cerebellar-evoked medial prefrontal cortex dopamine release Inactivation of the VTA decreased dopamine release by 50% in WT and 20-30% in knockouts Inactivation of the ventrolateral thalamus decreased dopamine release by 15% in WT and 40% in knockout animals

							[altered cerebellar modulation of mPFC dopamine release related to reorganization of neuronal pathways mediating this release]
2011	Peça et al.	Mouse	*Shank3B* null	Reduced interaction with social partners		Repetitive grooming Reduced rearing Anxiety-like behavior (reduced time in open arms of elevated zeromaze)	Altered morphology of striatal medium spiny neurons (increased dendritic length and surface area) Volumetric enlargement of the caudate
2011	Wang et al.	Mouse	*Shank3*$^{e4\text{-}9}$	Abnormal social behaviors (reduced social interaction) and communication patterns (abnormal USVs)	Repetitive behaviors (increased head pokes) Poorer performance on rotarod	Learning and memory deficits	
2016	Bariselli et al.	Mouse	*Shank3* knockdown in VTA neurons	Loss of social preference			Altered glutamatergic transmission in DA neurons of the VTA Bursting rate of DA neurons is significantly lower
2016	Uchigashima et al.	Mouse	Neuroligin-2 (NL2) knockdown			Reduced density of dopamine synapses on MSN dendrites	

						and increased GABAergic synapses	
2008	Jamain et al.	Mouse	*Nlgn4* knockout	Reduced social interaction Reduced ultrasonic vocalizations		Impaired olfaction	Reduced total brain volume Highest levels of NL-4 is in the olfactory bulb, striatum, cortex, and hippocampus
2014	Rothwell et al.	Mouse	*Nlgn3* knockout and R451C mutation		Poorer performance on rotarod Increased activity in open field test (hyperactivity) Enhanced acquisition of repetitive motor routines		Selective reduction of synaptic inhibition on NAc D1-MSNs
2012	Reith et al.	Mouse	*Tsc2* knockout in Purkinje cells	Loss of social preference	Repetitive marble burying Poorer performance on rotarod at 5 months of age		Loss of Purkinje cells
2012	Tsai et al.	Mouse	*Tsc1* knockout in Purkinje cells	Loss of social preference Increased ultrasonic vocalizations	Increased grooming		Purkinje cell abnormalities including: Increased area of the soma, increased dendritic spine density, and abnormal axonal collaterals Reduced excitability of purkinje cells

2019	DiCarlo et al.	Mouse	DAT T356M	Loss of social preference Loss of social dominance	Repetitive rearing Increased spontaneous locomotor activity		Reduced [DA] in the striatum Reduced pTH and pERK Slowed clearance of DA from the extracellular space

Discussion

This chapters highlights a number of invertebrate and vertebrate models with behavioral changes that recapitulate those seen in humans with ASD (i.e. social behaviors, repetitive behaviors, anxiety, and hyperactivity) that may also have underlying dopaminergic dysfunction or changes in structures known to be involved in the DA system. These models illustrate several important concepts. First, that modeling of complex behaviors known to be altered in ASD (i.e. social behaviors, repetitive behaviors, anxiety, and hyperactivity) is possible in invertebrates such as *Drosophila* and zebra fish and in vertebrates (i.e. mice). Second, that the behavioral changes observed as a result of a given ASD-associated mutation are often conserved across species. Finally, that DA dysfunction may play a role in these behavioral changes. This is particularly important as the scientific and clinical communities continue to uncover a growing number of genetic variants associated with ASD. As these variants are detected the question becomes: how do these variants (alone or in concert) act to alter brain function and contribute to the ASD phenotype?

As we look forward to the future of autism research, we must set our sights on an improved mechanistic understanding of ASD so we may improve diagnostics and interventions to optimize individual outcomes. Not all individuals with ASD are equally affected and not all individuals exhibit the same constellation of symptoms. We must endeavor to better understand the factors contributing to the presentation of ASD, including genetic and environmental influences, if we hope to effect meaningful change upon the status quo. The animal models reviewed here, summarized in Table 4, represent

the beginning of such efforts. Through these models, we can begin to gain a picture of the neurobiological commonalities, and differences, that may exist among the various genetic risk factors we now associate with ASD. The possibility exists that doing so will ultimately reveal a unifying thread between these biological changes, but more likely we will uncover the intricacies of each that will lead to better understanding of and appreciation for the unique and varied clinical presentations of ASD.

We propose a standardized, high-throughput screening process for genetic variants identified in individuals with ASD in the interest of furthering these goals. *Drosophila* and zebrafish are inexpensive, reproduce rapidly, reach adulthood quickly, and can be easily genetically manipulated, making these model species ideal candidates for first-pass identification of behavioral and physiological changes that may result from genetic variants. The advent of new techniques and technologies for studying complex behavior (i.e. social behaviors and repetitive behaviors) in *Drosophila* and zebrafish further serves to increase the attractiveness of these organisms for use in screening studies of new variants associated with ASD as they are discovered. Such screening studies could involve targeted genetic manipulation followed by a standardized, pre-specified battery of behavioral and neurobiological assays designed to characterize the impact of the particular genetic manipulation. Behavioral assays could include social testing (e.g. social spacing and courtship assays in *Drosophila*, which are paralleled by indices of shoaling and social preference in zebrafish), observation for repetitive grooming or locomotor behaviors using high speed cameras, hyperactivity testing (e.g. observation of locomotion), and fear/anxiety testing (e.g. measurement of fleeing in response to predatory cues and measures of anxiety-like behaviors such as thigmotaxis). Targeted

drug screens on mutant *Drosophila* and zebrafish can be used both to guide studies related to the mechanistic underpinnings of the variants as well as determine which, if any, of the behavioral changes observed are responsive to potential therapeutic agents. For example, drug screens with various dopamine-receptor agonists might inform whether hypo- or hyper-sensitivities to these drugs exist, which may in turn highlight the potential neurobiological pathways involved.

Based on these beahvioral findings and the hypothesized function of the gene in question, one could then rely on a tailored set of neurobiological assays to assess neuronal function. For example, if a novel variant in the gene encoding the DAT were discovered and animals expressing this variant displayed reduced sensitivity to cocaine, this would be suggestive of for altered DAT function. To determine the nature of this dysfunction, one could then use amperometric techniques and DA-uptake assays to determine the impact of the variant on transporter function (i.e. DA release and uptake profiles of the variant transporter). Similar strategies could be applied for the other neurotransmitter systems. These first-pass experiments would then serve as the rationale for pursuing genetic manipulations, further behavioral testing, and further neurobiological testing in mammalian (i.e. rodent) models. While this proposal is not necessarily novel[278], there are also few laboratories with the technical ability and requisite knowledge to perform all of the potential studies outlined. This highlights the critical importance of the establishment of collaborative networks designed specifically to facilitate this goal.

Here we have delineated how a collection of such experiments, from *Drosophila* to the mouse, has built support for the concept of a DA-dependent form of ASD, which may represent a unique subtype of ASD. We have reviewed literature suggesting DA

dysfunction, either resulting from or contributing to perturbances in network connectivity, excitatory and inhibitory signaling, and allocation of attention, energy, and working memory may indeed form a plausible explanation for the behavioral and physiological symptoms observed in ASD and in the conditions that co-occur frequently with ASD (including epilepsy, GI disorders, and ADHD). We have reviewed evidence suggesting that DA dysfunction may be a direct result of variants associated with ASD or may be impacted secondarily by variants in neurobiological systems that interact with the DA system. We have found evidence across multiple institutions, laboratories, and model organisms for DA disturbances as a result of many genetic variants associated with ASD. Many of the specific behavioral and neurobiological changes reviewed replicated between the different species considered, again supporting the use of more rapidly generated animal models as a screening method before proceeding to rodent models.

While DA system dysfunction may not be the primary driver of disease as it relates to some variants, the reviewed experiments have provided important insight into the how these variants may cascade into DA disturbances, which in turn may manifest as the behavior associated with ASD or exacerbate these behaviors. For some individuals in whom DA dysregulation is the primary insult, we have reviewed evidence suggesting that therapeutics targeting the DA system may alleviate at least some of the observed behavioral changes. These findings not only lend credence to the idea of the existence of a DA-dependent subtype of ASD, but also provides a path forward for identifying and understanding the mechanistic underpinnings of other forms of ASD, beyond the strict behavioral guidelines outlined in the DSM-V.

Unifying hypothesis

Given the important roles of DA in behavior, the evidence suggesting DA dysfunction may play a role in ASD, and the identification of variants in DA-associated genes in individuals with ASD, the study of such variants and their impacts on neurobiology and behavior become of utmost importance. The hypothesis underlying this work is that dysfunction in the dopaminergic system, and specifically dysfunction due to an ASD-associated DAT variant (the DAT T356M variant), will promote ASD-associated behaviors including social deficits, repetitive behaviors, hyperactivity, and changes in sensory sensitivity. Moreover, manipulations to the DA system (i.e. DAT antagonists) will improve behavioral changes observed in mice homozygous for the DAT T356M variant.

Specific aims

The aims of this dissertation are as follows:

1. To determine the impact of the DAT T356M variant on striatal dopamine neurotransmission (Chapter III).
2. To determine the impact of the DAT T356M variant on the behaviors associated with ASD and ADHD and the reversibility of these behaviors (Chapter III).
3. To determine the impact of the DAT T356M variant on sensory behaviors and sensorimotor gating (Chapter IV).

CHAPTER III

AUTISM-LINKED DOPAMINE TRANSPORTER MUTATION ALTERS STRIATAL DOPAMINE NEUROTRANSMISSION AND DOPAMINE-DEPENDENT BEHAVIORS

Introduction

The dopamine (DA) system is a critical regulator of motor activity, motivation, attention, and reward processing[151,279–281]. Dopaminergic projections originating from the substantia nigra and ventral tegmental area terminate in the striatum, where they function to regulate ongoing activity, gate motor function, and influence thalamocortical signaling [27,282]. Given the integral role of this neurotransmitter system in critical brain functions, it is not surprising that dysregulation of the dopaminergic system has been implicated in a number of neuropsychiatric disorders, including Parkinson's disease [283], substance use disorder[284], bipolar disorder (BPD)[285], ADHD[286], and recently Autism Spectrum Disorder (ASD)[184,241,242,287]. ASD is a neuropsychiatric disorder characterized by impairments in social communication and interaction and by patterns of restricted and repetitive behaviors, interests, or activities[288]. As previous work has demonstrated that features of ASD may be due to or exacerbated by abnormal DA signaling[182,289], the study of dopaminergic dysfunction as it relates to this disorder is vitally important.

The dopamine transporter (DAT), a presynaptic membrane protein found in dopaminergic terminals, regulates the temporal and spatial availability of DA[290] by rapidly clearing released DA from the synapse. The tight regulation of synaptic DA by DAT fine-tunes the phasic nature of DA signaling and thus the coding of both salience[291] and a

quantitative reward prediction error signal[151,292], which are critical for synaptic plasticity, reward processing, and behavioral learning[293,294]. It is not surprising, therefore, that genetic variants in the DAT gene have been linked to a number of neuropsychiatric disorders, including ADHD[295,296] and ASD[241,242] as well as neurological conditions such as Parkinson's Disease[297] and parkinsonism-dystonia[298]. Changes in the development of the striatum, a brain region enriched with DA projections and DAT expression, are associated with repetitive behaviors in autism. Disruptions in social reward processing in the striatum[299] have also been found in individuals with ASD, and impairments in prediction and predictive coding have been suggested as causative factors in the clinical manifestation of ASD[300]. Taken together, this evidence suggests that DAT dysfunction leading to altered dopaminergic signaling could support many aspects of the altered behaviors associated with neuropsychiatric disorders such as ASD and ADHD.

Until recently, studies of DAT dysfunction in neuropsychiatric disorders and its downstream consequences for behaviors have limited to DAT knockout (KO)[290], DAT heterozygous KO[301], DAT knock-down mice[302], as well as DAT knock-in mice expressing a mutant incapable of binding synaptic scaffold proteins[303]. DAT KO mice exhibit reduced DA levels, increased extracellular DA, hyperactivity, and loss of sensitivity to cocaine and amphetamine[290]. In comparison, DAT knock-down mice exhibit hyperactivity in novel environments, which is inhibited by amphetamine[302], while DAT heterozygous KO animals do not exhibit spontaneous hyperlocomotion[301]. While the present genetic mouse models have been instrumental in understanding the biological and behavioral importance of DAT function, these models are less representative of known human genetic mutations in DAT and thus have more limited applicability to human disease.

More recent work has identified single nucleotide polymorphisms of DAT in individuals with ASD and associated comorbidities[241,242,295]. One such polymorphism is the substitution DAT Ala559Val. This mutation was identified in two individuals with ADHD[304], two individuals with ASD[242], and one with BPD[305]. In vitro, this mutation was shown to promote DAT-mediated leak of cytoplasmic DA[306]. While homozygous Ala559Val mice do exhibit increased darting speed upon imminent handling, they do not replicate most of the hallmark clinical features associated with the conditions in which this mutation was identified[307]. Specifically, they do not exhibit spontaneous locomotor hyperactivity or stereotypies[307] as might be expected in ADHD and ASD, respectively.

Here we investigate the first de novo ASD-associated mutation identified in DAT, a threonine to methionine substitution at site 356 (DAT T356M)[9] using a novel genetically modified knock-in mouse model. This mutation, identified in an individual with ASD (for patient genetic information and clinical evaluation, see supplemental material of Hamilton et al. 2013[240]), is positioned in the seventh transmembrane domain of DAT near the ion binding site[308]. In vitro work has demonstrated that this mutation drives reduced DA reuptake, as well as anomalous DA efflux[240], and significantly reduces the affinity of the transporter for cocaine and methylphenidate (suggesting an important role for T356 in inhibitor binding)[308]. Models based on the bacterial homologous transporter, LeuT, predict that the T356M mutation promotes an outward-facing transporter conformation when substrate is bound. In *Drosophila melanogaster*, the T356M variant has been shown to generate hyperlocomotion[240], but the impact of this mutation on DA homeostasis and DA-dependent behaviors in the mammalian brain has not been investigated. Here, we demonstrate that DAT T356M homozygous (DAT T356M$^{+/+}$) mice display profound

impairments in DA neurotransmission and clearance. These impairments translate to severe alterations of specific behaviors, which closely resemble those seen in the ASD clinical phenotype. While this work provides a better understanding of DAT biology in vivo, it also defines the impact of DAT dysregulation on specific behaviors and provides insights into the potential molecular mechanisms underlying the etiology of ASD and its comorbidities.

Methods & materials

All behavioral, biochemical, and electrophysiological experiments were performed under a protocol approved by the Vanderbilt University Animal Care and Use Committee. Male and female mice maintained on a C57BL/6 background were used for all studies. All animals were derived from matings of DAT T356$^{+/-}$ parents.

Antibodies

Details of all primary and secondary antibodies used in this study are summarized in Table 5.

Generation of the DAT T356M$^{+/+}$ mouse

Mice were generated by genOway S.A. (Lyon, France). The point mutation was inserted into the exon 8 of the mouse *Slc6a3* gene and was expressed under the control of the endogenous *Slc6a3* promoter. It should be noted that the mouse orthologue of the T356M variant is T355M. The T356M terminology will be used in the manuscript to refer to this mutation to maintain consistency with the nomenclature used in previous studies referring to this variant. A neomycin positive selection cassette flanked by *loxP* sites was

inserted into intron 7 in a region devoid of experimentally validated regulatory regions and of predicted transcription factor binding sites conserved between mouse and human (Figure 4). Isolated homology fragments were used to generate the targeting vector. Polymerase chain reaction (PCR) validation was used to confirm the recombination over the 3' homology arm. Southern blot was used to validate recombination over the 3' and 5' homology arms.

After linearization to enhance homologous recombination, ES cells were electroporated with the targeting vector and subjected to positive selection. The presence of the correct recombination event was validated by PCR and Southern blot. Recombined ES cell clones, derived from black-coated C57BL/6 mice, were injected into blastocysts, derived from an albino C57BL/6 mouse strain, which were then implanted in pseudo-pregnant females to produce chimeric males carrying the combined locus. Chimerism was assessed by coat color marker comparison. Male mice with >50% chimerism were bred with C57BL/6 mice ubiquitously expressing the Cre-recombinase to excise the *lox*P flanked neomycin selection cassette and to generate heterozygous mice carrying the neo-excised point mutant knock-in allele (Figure 5). Progeny were genotyped by PCR and the recombinase-mediated excision event was validated by Southern blot. Heterozygous animals identified by PCR screening and Southern blot were investigated by sequencing analysis to confirm the integration of the mutation.

Chronoamperometry

DA release and reuptake were measured using chronoamperometry in striatal slices. Striatal hemislices (300 µm) from 6-10 week old WT and DAT T356M$^{+/+}$ mice were

prepared with a vibratome (Leica VT1000S) in an ice cold oxygenated (95% O_2/5% CO_2) artificial cerebral spinal fluid (aCSF) solution consisting of (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 10 dextrose, 26 $NaHCO_3$, 0.25 ascorbic acid. Slices were then recovered in an oxygenated NMDG-HEPES bath (consisting of, in mM,: 100 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 10 $MgSO_4$•$7H_2O$, 0.5 $CaCl_2$•$2H_2O$, 5 L-ascorbic acid, 3 sodium pyruvate, 2 thiourea, 12 N-acetyl-L-cysteine adjust to pH 7.3-7.4) for 12 minutes at 32-34ºC. Slices were then moved to an oxygenated aCSF bath (as above) at 28ºC for a minimum of 1 hour prior to recording. Carbon fiber electrodes were advanced angularly into the desired recording site in the striatum so that the tip of the electrode was positioned at a depth of 75-100 µm beneath the tissue surface. DA release was stimulated by a 100 ms 100-200 µA potential pulse delivered by a bipolar electrode while the carbon fiber electrode measured DA release and clearance dynamics. Data were collected with an Axopatch 200B amplifier. Decay time was calculated as time for the current to decay from 80% of peak current to 20% of peak current (t_{80}-t_{20}). For these experiments, n = 17 slices for each genotype from 6 animals of each genotype.

Voltammetry

Fast-scan controlled adsorption voltammetry (FSCAV) was used to measure basal dopamine levels in the striatum. Mice between the ages of 8-16 weeks old were anesthetized with an intraperitoneal (*i.p.*) injection of urethane (25% (w/v) in sterile saline) at a volume of 7 µL per 1 g body weight. A heating pad from Braintree Scientific was used to maintain mouse body temperature (37°C) throughout the duration of the experiment. Stereotaxic surgery was performed with coordinates determined with reference to bregma, according to Franklin and Paxinos (2008). A pseudo Ag/AgCl reference was

placed in the contralateral hemisphere. A stainless-steel stimulation electrode (diameter 0.2 mm, MS303/2-A/SPC; Plastics One, Roanoke, VA) was lowered into the MFB. A 50 µm nafion-coated carbon fiber microelectrode (CFM), made as previously described [309], served as the working electrode and was lowered in the striatum. FSCAV was performed using instrumentation and software (WCCV 3.05) developed by Knowmad Technologies LLC (Tucson, AZ). Dopamine presence was confirmed by first collecting FSCV files. A 30 second file was collected and a stimulation applied at 5 seconds (60 Hz biphasic, 360 µA, 120 pulse stimulation, 2 ms per phase) through a linear constant current stimulus isolator (NL800A Neurolog; Digitimer Ltd.) to evoke dopamine release. The release was measured at the CFM in the striatum by applying a DA triangular waveform (-0.4 V to 1.4 V to -0.4 V scan rate 400 V/s). Once the presence of DA has been confirmed, FSCAV was applied for ambient DA concentration measurements. A DA FSCAV waveform was applied as previously described [309]. Signals collected were processed with WCCV 3.05 software using LabVIEW 2009, which included signal deconvolution, filtering, and smoothing. The cyclic voltammogram (CV) at the 3rd scan (following the controlled adsorption period) was extracted to integrate the dopamine oxidation peak approximately between 0.45 V and 0.9 V. Calibration curves were acquired prior to experiments and plotted as charge, in pC, versus dopamine concentration. A separate calibration set (n=5 electrodes) was obtained using both pre and post calibrations to determine the factor by which the sensitivity of the electrode changes post experiment. Each pre-calibration was then adjusted by this factor and used to calculate in vivo values which were specific to each electrode. Following data collection, a large voltage was applied to the electrode (~10 V for 45 s) to lesion the tissue. Mice were subsequently euthanized *via* cervical

dislocation followed by decapitation, and the brain was removed from the skull and stored in 4% paraformaldehyde in phosphate buffered saline (PBS) solution. Prior to sectioning, the brain was transferred into a 30% sucrose solution until it was saturated with the medium. The brain was then flash-frozen, sectioned into 20 µm slices, mounted onto frosted glass slides, and the lesion identified to confirm electrode placement.

Western blot

Mice were decapitated under isoflurane anesthesia at 7-10 weeks of age. The brain was rapidly dissected and placed in ice-cold sucrose solution consisting of (in mM): 210 sucrose, 20 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1 $MgCl_2$, 26 $NaHCO_3$, 10 dextrose. The striatum was then dissected on ice and snap frozen. Dissected tissue was homogenized in 200 µL of homogenization buffer (RIPA buffer [Thermo Fisher Scientific], cOmplete protease inhibitor cocktail [Millipore Sigma], phosphatase inhibitor cocktail 3 [Millipore Sigma], 1 mM sodium orthovanadate) on ice. Protein concentrations of all samples were determined by a bicinchoninic acid (BCA) protein assay using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23225). Samples were resolved using NuPAGE Bis-Tris pre-cast gels (Thermo Fisher Scientific, 10% 10-well, NP0301BOX). The Precision Plus Protein Kaleidoscope Prestained Ladder was run alongside samples for protein size reference. Proteins were transferred from gels onto nitrocellulose membranes using the iBlot system (Thermo Fisher Scientific, IB23001). Western blot analysis was performed using antibodies listed in Table 5 and visualized using Western Lightning Plus ECL. Adobe Photoshop software was used to crop full blots. Western blots were quantified using NIH ImageJ Gel analysis tool.

Table 5. *Antibodies used for western blot experiments*

Antibody	Species	Manufacturer	Dilution/Remarks
Anti-DAT (MAB369)	Rat	Millipore Sigma	1:1000
Anti-Rat-HRP	Goat	Thermo Fisher Scientific	1:5000
Anti-TH	Rabbit	Cell Signaling Technology	1:1000
Anti-pTH (Ser 31)	Rabbit	Cell Signaling Technology	1:1000
Anti-pERK1/2	Rabbit	Cell Signaling Technology	1:1000
Anti-ERK1/2	Rabbit	Cell Signaling Technology	1:1000
Anti-Actin	Rabbit	Santa Cruz Biotechnology	1:5000
Anti-Rabbit-HRP	Donkey	Thermo Fisher Scientific	1:5000

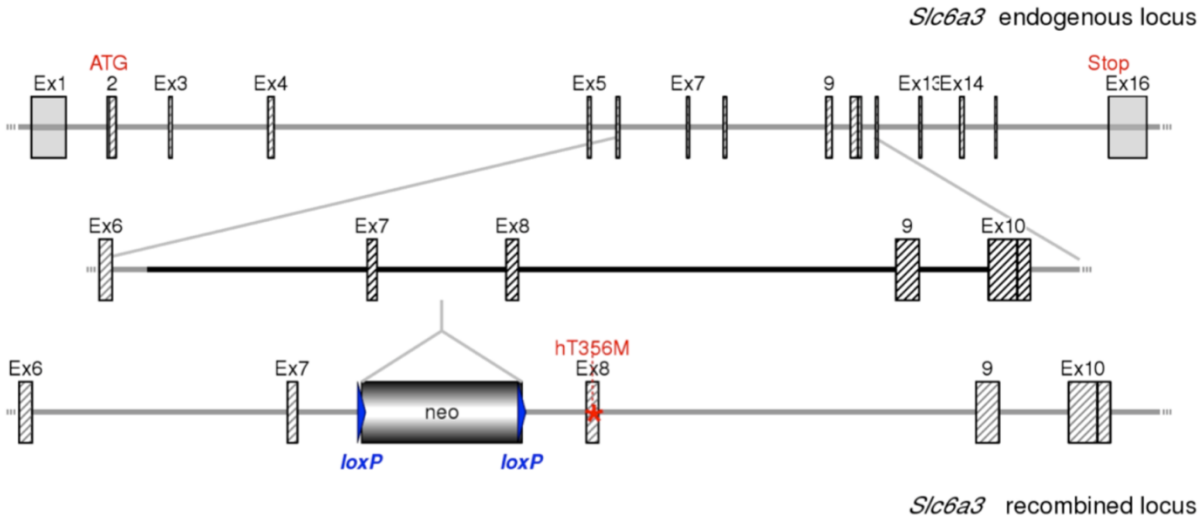

Figure 4. *Schematic representation of the selected targeting strategy.*

This schematic demonstrates the targeting strategy for disrupting the murine *Slc6a3* gene function by insertion of the mouse orthologue of human T356M point mutation. The T355M point mutation is inserted into the exon 8 of the mouse *Slc6a3* gene. Mutated *Slc6a3* gene is expressed under the control of the endogenous *Slc6a3* promoter. Hatched rectangles represent *Slc6a3* coding sequences, grey rectangles indicate non-coding exon portions, solid lines represent chromosome sequences. The neomycin-positive selection cassette is indicated. *loxP* sites are represented by blue triangles. The initiation (ATG) and Stop (Stop) codons are indicated.

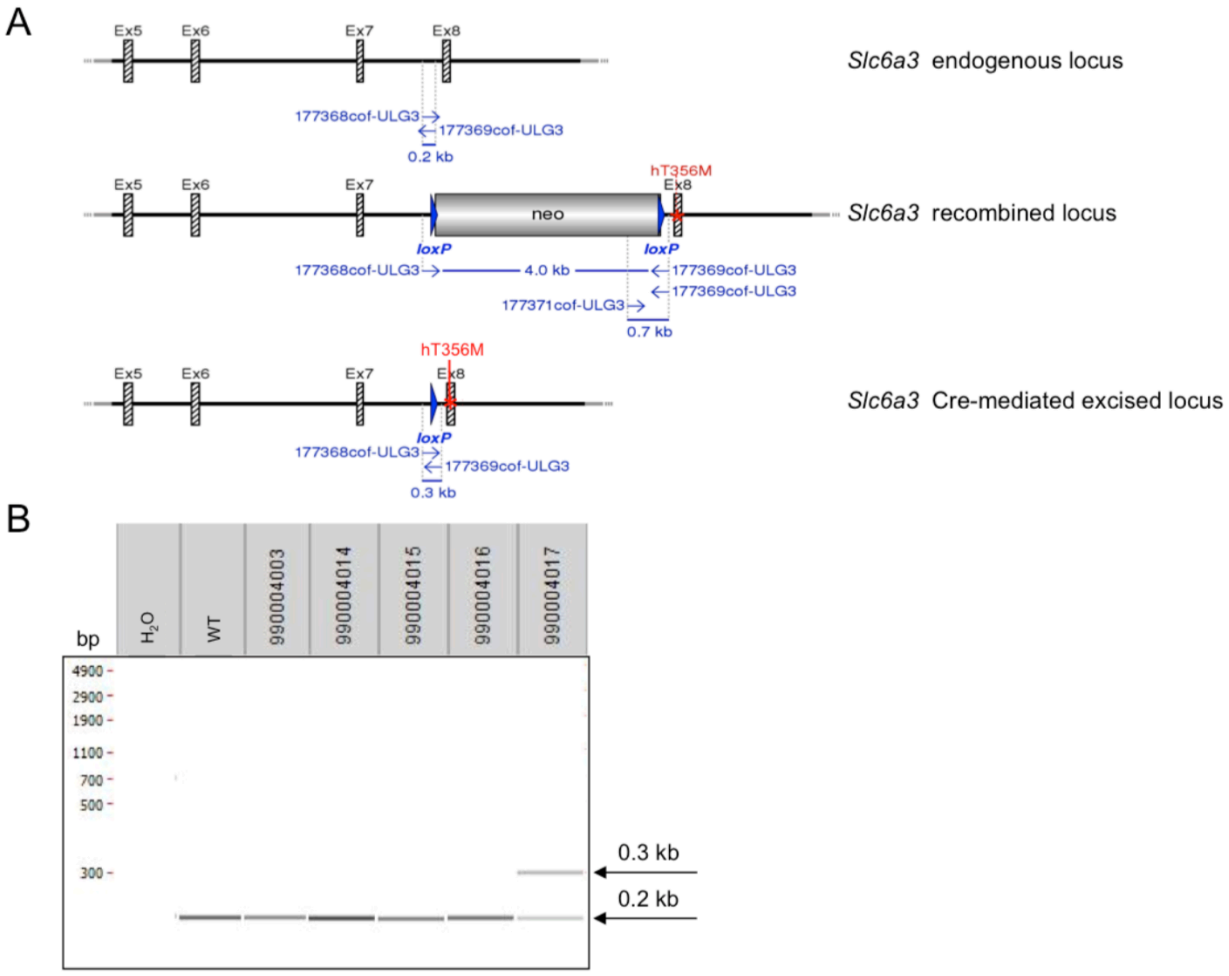

Figure 5. *Schematic representation of the generation of the heterozygous neo-excised point mutant knock-in mouse line.*

(A) Schematic representation of the *Slc6a3* wild-type (top), recombined (middle), and neo-excised (bottom) point mutant alleles with binding sites of the PCR screening primers (in blue). **(B)** Representative PCR of animal of interest. C57BL/6 wild-type genomic DNA (WT) was used as a positive control. PCR without DNA (H_2O) served as a negative control. The heterozygous appears on the far right.

High-performance liquid chromatography (HPLC)

Measures of the concentration of biogenic amines were obtained by the Neurochemistry Core Facility at Vanderbilt University. The striatum was collected from animals aged 7-10 weeks old in the same manner as described for western blots and stored at -80ºC. Frozen brain tissue was homogenized using a tissue dismembrator (Misonix XL-2000, Qsonica) in 100-750 µl of 0.1M TCA, which contains 10^{-2} M sodium acetate, 10^{-4} M EDTA, and 10.5 % methanol (pH 3.8). Ten microliters of homogenate were used for protein assay. Protein concentration was determined using the BSA Protein Assay Kit (Thermo Scientific). Then samples were spun in a microcentrifuge at 10,000g for 20 minutes. The supernatant was removed for biogenic monoamines analysis. Biogenic amine concentrations were determined utilizing an Antec Decade II (oxidation: 0.65) electrochemical detector operated at 33°C. Twenty microliter samples of the supernatant were injected using a Water 2707 autosampler onto a Phenomenex Kintex C18 HPLC column (100 x 4.60 mm, 2.6 µm). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). Solvent was delivered at 0.6 ml/min using a Waters 515 HPLC pump. Using this HPLC solvent the following biogenic amines eluted in the following order: Norepinephrine, Epinephrine, DOPAC, Dopamine, 5-HIAA, HVA, 5-HT, and 3-MT. Data acquisition was managed by Empower software (Waters Corporation, Milford, MA USA). Isoproterenol (5 ng/mL) was included in the homogenization buffer for use as an internal standard to quantify the biogenic amines of interest.

Weight measurements

All mice not undergoing experimental manipulation (including behavioral testing) were weighed weekly beginning 7 days after birth (P7). Animals undergoing behavioral testing were excluded from weight measurements.

Behavioral paradigms

All behavioral experiments were performed in the Vanderbilt University Neurobehavioral Core Facility. All behavioral tests were performed at the same time of day (13:00-17:00) using mice 8-10 weeks of age. Mice were group housed (3-5 mice/cage) on a 12:12-h light-dark cycle with food and water available ad libitum. Mice were housed with corncob bedding. Mice were transferred to testing rooms followed by a 30-minue acclimation period before the start of each test. Unless otherwise stated, each apparatus used was cleaned with a 10% ethanol solution between each animal or trial to provide a standardized testing environment.

Behavior observations

Mice were placed individually into clean cages (containing bedding only) and recorded using a video camera for 15 minutes. Videos were then scored by observers blinded to genotype for the instances of the following behaviors: digging, grooming, rearing, tail flick, jumping, and climbing.

Elevated zero maze

Anxiety behaviors were assessed using an elevated zero maze (62.5 cm outer diameter, 5 cm path width, 15 cm wall height, Stoelting). Animals were placed in center

of the open portion of the maze at the start of each trial. Each trial was recorded by a video camera mounted to the ceiling and analysis was performed using ANY-maze software (Stoelting). Data analyzed included the percent of time in closed sections, number of entries into open and closed sections, and distance traveled in the maze.

Marble burying

Cages (189 mm x 297 mm x 128 mm) were prepared by filling clean, empty cages with 4.5 cm of Diamond Soft Bedding (Envigo Teklad 7089). Mice were placed individually into filled cages for a 5-minute acclimation period. Mice were then removed from the cages and 15 blue marbles (15 mm diameter) cleaned with a 10% ethanol solution were gently placed in the cages. Marbles were evenly spaced in a 3 x 5 configuration in each cage. A photograph was taken of each cage prior to returning mice to the cage and again 30 minutes later when mice were removed from the cage. Photographs of cages before and after testing were compared and scored by three independent, blinded observers. A marble was considered buried if more than 2/3 of the marble was covered with bedding at the end of 20 minutes.

Inverted screen

Mice were individually placed on a 7.5 cm x 7.5 cm wire mesh screen which was mounted horizontally on a metal rod and elevated 40 cm above the apparatus floor. The rod was then slowly rotated 180º, ensuring all animals achieved a four-paw grasp on the wire mesh before the apparatus was fully inverted. Time to fall was measured for each animal on three trials. The maximum time allowed was 180 seconds.

Horizontal pole climb

The horizontal climb tests the ability of a mouse to maintain balance on a horizontal rod (cloth tape wrapped, 1.5 cm diameter) suspended between two platforms. The pole is suspended 40 cm above the apparatus floor. The mouse was placed on the rod and its movements were observed for 1 minute. Time to reach either platform or time to fall was measured for three trials for each mouse.

Open field locomotion

Spontaneous locomotor activity in an open field was measured using 27 x 27 x 20.5 chambers (Med Associates) placed within sound-attenuating boxes (64 x 45 x 42 cm) that were light- and air-controlled. Locomotion was detected by infrared beam disruption by the body of the mouse. 16 evenly-spaced photocells located 1 cm above the chamber floor measured horizontal movement while 16 photocells located 4 cm above the chamber floor captured vertical movements (jumping and rearing). Animals were placed in the activity chambers and their activity was recorded for 60 minutes. Total distance traveled was analyzed using the Student's t-test. Distance traveled across time was analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. Data from this test was also used to measure anxiety-like behaviors. Thigmotaxis, the tendency of the mice to remain close to the walls of the open field, can be used as an index of anxiety. Percent of time mice spent in the periphery of the open field (~52% of area) vs. the center was determined.

Rotarod

Rotarod was used to assess the balance and motor coordination of the mice on a rotating rod, as well as the propensity for acquisition of repetitive motor routines [310]. The rotarod apparatus consists of a rubber-covered cylindrical rod (3 cm in diameter) suspended 30 cm above the apparatus floor and separated into 5 equal compartments by plastic dividers with a radius of 15 cm. The starting rotation rate was 4 rpm and increased steadily to 40 rpm over a period of 4 minutes. Mice were placed on the rod and the timer was started. The time to fall or to complete two passive rotations was measured for each animal with a maximum time of 5 minutes. Animals completed three trials each day for three days. The time to fall or rotate for each day was calculated as an average over the time to fall or rotate for the three trials completed in a given day.

Tube test

The tube test was used to assess social dominance (a test for normal social behavior). One DAT T356M$^{+/+}$ knock in mouse and one WT mouse were placed head first at opposite ends of a clear plastic tube (2.5 cm inner diameter, 13 cm length) and released simultaneously. Each trial ended when one mouse completely retreated from the tube or after 3 minutes. The mouse remaining in the tube was designated the winner and the retreating mouse was designated the loser. If neither animal retreated from the tube after 3 minutes the trial was designated a draw. Each pair of animals participated in two trials, starting on alternating ends of the tube on each trial. A χ^2 one-sample analysis was used to determine whether the scores of the mutant mice were significantly different from an outcome expected by chance (50:50 win-lose).

Three chamber test

The three chamber test was used to assess social preference (a test for normal social behavior in mice). The apparatus consists of a 60 x 42 x 22 cm box divided into three equal-sized compartments. Removable doors initially confined the test mouse to the center chamber, where the mouse habituated for 5 minutes. After 5 minutes, the doors were removed and the mouse was allowed to freely explore the apparatus. A stimulus mouse was then introduced in an inverted wire cup in one side chamber, while a clean, empty, inverted pencil cup was introduced in the opposite chamber. The stimulus mouse was always an age and sex-matched WT mouse, either known to the subject mouse (familiar social stimulus) or unknown to the subject mouse (novel social stimulus). The subject mouse was then allowed to explore all three chambers freely for 10 minutes. A research assistant blinded to genotype then coded videos for time spent in each chamber and time spent interacting with the stimulus mouse or the empty inverted wire cup.

ACT-01 reversal of hyperactivity

Mice were allowed to habituate to the test room for 15 minutes prior to treatment. Mice naïve to the open field apparatus were treated with either 50 mg/kg of ACT-01 or with vehicle via IP injection 30 minutes prior to testing. The same protocol for measuring spontaneous locomotor activity was then followed as is described under the “Open Field Locomotion” section of this methods section. ACT-01 was provided by Hao Chen (DRI Biosciences Corporation, Frederick, MD).

GBR12909 reversal of hyperactivity

Mice were allowed to habituate to the test room for 15 minutes prior to treatment. Mice naïve to the open field apparatus were treated with either 20 mg/kg of GBR12909 or with vehicle via IP injection 30 minutes prior to testing. The same protocol for measuring spontaneous locomotor activity was then followed as is described under the "Open Field Locomotion" section of this methods section.

Statistics

All statistical analyses were performed using GraphPad Prism software (version 8.0.2). Statistical methods are indicated in the figure legends and the results section. Data are presented as mean ± SEM. Differences are considered statistically significant at $P < 0.05$. Unpaired 2-tailed Student's t-test was used for 2-group comparisons, unless stated otherwise. Either 1- or 2-way ANOVA with posthoc testing was used for multiple comparisons. Sidak's multiple comparisons test was used for posthoc testing. This test was used after 1- and 2-way ANOVA when comparing selected pairs of means. This method assumes each comparison is independent of the others.

Study approval

All behavioral, biochemical, and chronoamperometry experiments were performed under a protocol approved by the Vanderbilt University Animal Care and Use Committee. For FSCAV, a protocol approved by the Institutional Animal Care and Use Committees of the University of South Carolina was followed.

Results

DAT T356M+/+ mice display impaired DA clearance while maintaining normal DAT expression

DAT T356M$^{+/-}$ and DAT T356M$^{+/+}$ mice were generated as described in the methods section. No significant differences were observed between WT and the DAT T356M$^{+/-}$ among all behavioral phenotypes analyzed in this study (see below). Therefore, electrophysiological and biochemical experiments were performed on WT and DAT T356M$^{+/+}$ animals. We first sought to determine whether the T356M mutation, by promoting anomalous DA efflux, affects striatal DA clearance. We performed ex vivo chronoamperometry to measure electrically-stimulated DA release and clearance in striatal slices (Figure 6A). We quantified peak DA release (Figure 6B) and the decay time of the amperometric current (Figure 6C) at baseline and in the presence of 10 μM cocaine (COC) for both WT and DAT T356M$^{+/+}$ mice. DAT is a known target for the psychoactive drug COC, which has been shown to impair DA reuptake from the synapse via competitive inhibition of the DAT[311,312]. To determine whether this mutation disrupted transporter sensitivity to cocaine (and to ensure our signal was dopaminergic), we measured release and dynamics of DA in the presence of 10 μM cocaine in the striatum of WT and DAT T356M$^{+/+}$ mice.

A two-way ANOVA was performed to examine the effect of genotype and treatment type on peak DA release. There was a signficiant interaction between the effects of genotype and treatment on peak DA release ($F(1,32) = 11.00$, $p = 0.0023$). Simple main effects analysis was performed to determine which groups differed significantly. At baseline, Peak DA release did not differ between WT and DAT T356M$^{+/+}$ mice (-COC;

WT = 1.921 ± 0.17 μM; DAT T356M$^{+/+}$ = 1.526 ± 0.18 μM; n = 17; p = 0.1908 by Sidak's multiple comparisons test). These data indicate intact capacity of presynaptic dopaminergic terminals to properly release stored DA in DAT mutant animals. Cocaine did significantly decrease the peak of the amperometric current in the DAT T356M$^{+/+}$ mice with respect to WT animals (Figure 6B. +COC; WT = 1.766 ± 0.19 μM; DAT T356M$^{+/+}$ = 0.916 ± 0.13 μM; n = 17; p = 0.23 and <0.001, respectively, by Sidak's multiple comparisons test). This decrease in the presence of cocaine might be due to an increase in buffering capacity for DA at the synapse mediated by DAT. One possibility for this observation is that DAT T356M has a decreased affinity for cocaine[308] therefore increasing the availability of DAT to bind DA upon release.

A two-way ANOVA was performed to examine the effect of genotype and treatment type on the rate of DA clearance (i.e. the decay time of the amperometric current (t_{80}-t_{20})). There was a signficiant interaction between the effects of genotype and treatment on the decay time ($F(1,32) = 7.482$, $p = 0.0101$). Simple main effects analysis was performed to determine which groups differed significantly. At baseline, the decay time of the amperometric current (t_{80}-t_{20}) was significantly greater in DAT T356M$^{+/+}$ mice compared to WT mice (Figure 6C. -COC; WT = 121.5 ± 12.39 ms; DAT T356M$^{+/+}$ = 244.9 ± 19.83; n = 17; p = 0.001 by Sidak's multiple comparisons test). This increase reflects significantly reduced DAT-mediated DA clearance in DAT T356M$^{+/+}$ mice. Notably, cocaine significantly increased the decay time of the DA signal in both WT and DAT T356M$^{+/+}$ mice (Figure 6C. +COC; WT = 334.1 ± 25.82 ms; DAT T356M$^{+/+}$ = 384.9 ± 32.21 ms; n = 17; p = <0.001 and 0.006, respectively, by Sidak's multiple comparisons test). These data indicate that a) the mutant transporter does retain sensitivity to cocaine; b) the

amperometric signal is fine-tuned by DAT activity; and c) the mutant transporter is capable of DA reuptake, although to a lesser extent.

We then sought to determine whether the T356M mutation alters basal extracellular DA concentration in the striatum. We performed in vivo FSCAV to measure basal dopamine levels [309]. Basal values from a 20-minute collection period, capable of demonstrating stability, were used to calculate an average basal concentration for each animal. The basal concentration of extracellular DA was significantly higher in the striatum of DAT T356M$^{+/+}$ animals compared to WT animals (WT = 268.35 ± 27.52 nM; DAT T356M$^{+/+}$ = 359.54± 28.67 nM; n = 5 WT, n = 7 DAT T356M$^{+/+}$; p = 0.0453 by Welch's *t*-test). This increase in basal levels of DA provides evidence that the T356M mutation promotes elevated basal extracellular DA in the striatum, possibly by DAT-mediated anomalous DA efflux.

The observed decreased DA clearance in DAT T356M$^{+/+}$ mice could stem from either impairment of the DAT (i.e. reduced function or anomalous DA efflux) or from decreased expression. To determine whether DAT expression was decreased in DAT T356M$^{+/+}$ mice, we used immunoblotting to quantify DAT expression relative to WT animals. We found no difference in DAT expression in the striatum between DAT T356M$^{+/+}$ mice and WT animals (Figure 6D. n = 5; p = 0.877 by Student's two-tailed *t*-test). These data suggest that the DAT T356M mutation impairs DA clearance by directly reducing transporter function and not by altering DAT availability. However, it should be noted that this data does not exclude the possibility of altered DAT expression at the plasma membrane.

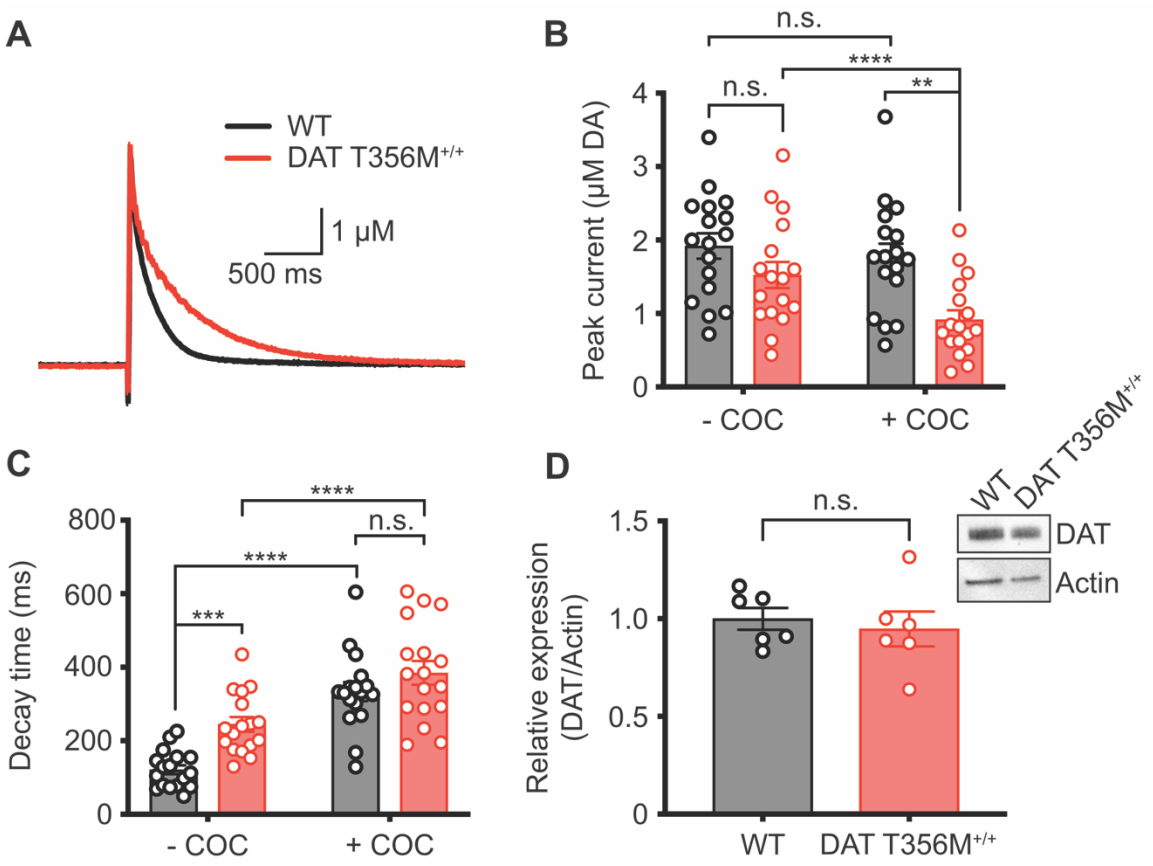

Figure 6. *The DAT T356M mutation impairs striatal DA reuptake while maintaining normal DAT expression.*

(A) Stimulated dopaminergic current recorded (using carbon fiber amperometry) from acute striatal slices of WT (black) and DAT T356M$^{+/+}$ (red) mice. **(B)** Peak dopaminergic current recorded from acute striatal slices of WT (black) and DAT T356M$^{+/+}$ (red) mice. There was no significant difference in the peak dopamine released between WT and DAT T356M$^{+/+}$ mice at baseline (-COC; WT = 1.921 ± 0.17 µM; DAT T356M$^{+/+}$ = 1.526 ± 0.18 µM; n = 17; p = 0.1908 by two-way ANOVA). With addition of cocaine, peak DA released decreased significantly in DAT T356M$^{+/+}$ striatum, but not WT striatum (+COC; WT = 1.766 ± 0.19 µM; DAT T356M$^{+/+}$ = 0.916 ± 0.13 µM; n = 17; p = 0.23 and <0.001, respectively, by two-way ANOVA). **(C)** Decay time ($t_{80} - t_{20}$) of the dopaminergic signal

recorded in acute striatal slices of WT (black) and DAT T356M$^{+/+}$ (red) mice. The decay time was significantly longer at baseline in DAT T356M$^{+/+}$ striatum when compared to WT striatum (-COC; WT = 121.5 ± 12.39 ms; DAT T356M$^{+/+}$ = 244.9 ± 19.83; n = 17; p = 0.001 by two-way ANOVA). Addition of cocaine increased decay time of the dopaminergic signal in both WT and DAT T356M$^{+/+}$ mice (+COC; WT = 334.1 ± 25.82 ms; DAT T356M$^{+/+}$ = 384.9 ± 32.21 ms; n = 17; p = <0.001 and 0.006, respectively, by two-way ANOVA followed by Sidak's multiple comparisons test), as expected, and confirming the identity of the current as dopaminergic. ***P = 0.001; ****P< 0.0001 **(D)** Immunoblotting for DAT showed no difference in DAT expression in the striatum between WT and DAT T356M$^{+/+}$ mice (n = 6; p = 0.877 by two-tailed *t*-test).

DAT T356M+/+ mice have increased striatal DA metabolism and decreased tyrosine hydroxylase phosphorylation at Ser31

Based on our finding that the DAT T356M mutation reduces rate of DA clearance, we sought to determine whether this resulted in increased DA metabolites and altered DA synthesis in the striatum. We measured the striatal tissue content of biogenic amines and their metabolites in WT and DAT T356M$^{+/+}$ mice. A two-way ANOVA was performed to examine the effect of genotype and amine type on the concentration of the measured amines. There was a signficiant interaction between the effects of genotype and amine type on amine concentration ($F(5,54) = 25.99$, $p < 0.0001$). Simple main effects analysis was performed to determine which groups differed significantly. The concentration of DA was greatly reduced in DAT T356M$^{+/+}$ mice compared to WT mice (

Figure 7A. WT = 153.5 ± 15.38 ng/mg; DAT T356M$^{+/+}$ = 65.55 ± 6.71 ng/mg; n = 6 WT, 5 DAT T356M$^{+/+}$; $p < 0.0001$ by Sidak's multiple comparisons test). Similary, a two-way ANOVA was performed to examine the effect of genotype and the relative concentration of DA and its metabolites in the striatum on this ratio. There was a signficiant interaction between the effects of genotype and the relative concentration of DA and its metabolites in the striatum on amine concentration ($F(5,54) = 25.99$, $p < 0.0001$). Simple main effects analysis was performed to determine which groups differed significantly. The concentration of DA relative to its metabolites was significantly reduced in the striatum of DAT T356M$^{+/+}$ mice compared to WT mice (

Figure 7B. n = 6 WT, 5 DAT T356M$^{+/+}$; see Table 6), supporting increased metabolism of DA in the striatum of DAT T356M$^{+/+}$ mice. In contrast, there was no

difference in serotonin (5-HT) content or in the ratio of 5-HT to its metabolite 5-hydroxyindoleacetic acid (5-HIAA). To determine the impact of altered DA metabolism in the DAT T356M$^{+/+}$ mouse on DA synthesis, we measured the expression of the rate limiting enzyme, tyrosine hydroxylase (TH), and its phosphorylated (i.e. active) form. The activity of TH is regulated, in part, by phosphorylation of the residue Ser31[313]. Phosphorylation at this site results in increased activity of TH and, as a consequence, increased DA synthesis[313]. Ser31 is phosphorylated by ERK1/2[314], which is coupled to the DA D_2 autoreceptor (D_2R)[315]. Therefore, we measured changes in phosphorylation of TH in Ser31 (pTH Ser 31), and found that pTH Ser 31 was reduced in DAT T356M$^{+/+}$ mice relative to WT mice (

Figure 7C. n = 12 from 4 animals for each genotype; p = 0.0172 by Student's two-tailed *t*-test), with no change in total TH expression. Decreased TH phosphorylation could be a result of continuous stimulation of the D_2R due to a reduced DA clearance in the DAT T356M$^{+/+}$ mouse, which has been shown to decrease D_2R function[316,317]. It is also consistent with previous literature demonstrating reduced TH Ser31 phosphorylation as well as increased extracellular DA levels in animals with loss of the DAT[317] . Decreased D_2R function would in turn result in reduced ERK1/2 activation and, therefore, reduced TH phosphorylation at Ser31. Reduced phosphorylation of TH at Ser31 would result in reduced DA synthesis. This is consistent with our HPLC data, which demonstrate reduced total tissue content of DA.

To determine whether ERK1/2 activation was indeed reduced in DAT T356M$^{+/+}$ mice, we measured pERK1/2 expression (its activated form). We found reduced pERK1 (but not pERK2) striatal expression relative to total ERK (

Figure 7D & 2E. n = 6; p = 0.03 by Student's two-tailed *t*-test), indicating downregulation of ERK1 activity in the striatum. This is consistent with our finding of reduced phosphorylation of TH at Ser 31.

Reduced body weight in DAT T356M+/+ male mice

Male DAT T356M$^{+/+}$ mice gained weight significantly more slowly than WT mice in the period immediately following weaning through the fifth week of life (Figure 8A. P21 – W 5; n = 37 WT, 39 DAT T356M$^{+/+}$; * p = 0.02, ** p < 0.009 by two-way ANOVA followed by Sidak's multiple comparisons test). In adulthood (at 10 weeks of age), the body weight of male DAT T356M$^{+/+}$ mice averaged 3.09 g (± 0.92 g) lower than that of adult male WT mice. Conversely, female DAT T356M$^{+/+}$ body weights never differed significantly from WT mice (Figure 8B. n = 36 WT, 28 DAT T356M$^{+/+}$). As observed in DAT homozygous KO animals, slower weight gain in early life may be associated with impaired food intake [290] (however caloric intake was not directly measured) or with increased activity (see behavioral results section).

DAT T356M+/+ mice are hyperactive, exhibit repetitive behaviors, and social deficits

As dysregulated DA homeostasis and signaling have been associated with movement disorders and anxiety, we first sought to determine whether T356M produced deficits in strength and motor coordination and promoted anxiety-like behaviors. These behavioral traits were assessed prior to other testing as these behaviors (if present) would complicate interpretation of other behavioral findings (see below). DAT T356M$^{+/+}$ animals exhibited no motor strength or coordination deficits (as measured by inverted screen, pole

climb, and rotarod). For all behavioral tests in the present work, both male and female animals were used. As there was no effect of sex in any of the behavioral tasks, male and female animals were combined for all analyses. DAT T356M$^{+/+}$ animals had no difference in: time to fall on inverted screen (Figure 9A. WT = 120. ± 21.79 s, DAT T356M$^{+/+}$ = 109.6 ± 19.29 s; n = 10 WT, 11 DAT T356M$^{+/+}$; p = 0.72 by Student's two-tailed *t*-test), time to reach the platform on pole climb (Figure 9B. WT = 10.11 ± 2.360 s, DAT T356M$^{+/+}$ = 8.545 ± 1.836 s, n = 9 WT, 11 DAT T356M$^{+/+}$; p = 0.6 by Student's two-tailed *t*-test). A two-way ANOVA was performed to examine the effect of genotype and day on time to fall or rotate on rotarod. There was a significant main effect of day ($F(2,38) = 14.97.00$, $p < 0.0001$) and of genotype ($F(1,19) = 5.556$, $p = 0.0293$) on time to fall or rotate. Post-hoc analysis revelaed that the DAT T356M$^{+/+}$ mice outperformed the WT on day 3 only (Figure 9C. WT Days 1, 2, 3 (respectively) = 129.43 ± 15.89 s, 159.06 ± 21.52 s, 167.03 ± 22.1 s; DAT T356M$^{+/+}$ Days 1, 2, 3 (respectively) = 153.73 ± 10.16 s, 207.36 ± 15.28 s, 236.45 ± 15.22 s; n = 10 WT, 11 DAT T356M$^{+/+}$; p = 0.68 (day 1), 0.14 (day 2), 0.02 (day 3) by Sidak's multiple comparisons test). Additionally, we observed no anxiety phenotype as measured by the elevated zero maze test (Figure 9D. WT = 56.94 ± 3.12%, DAT T356M$^{+/+}$ = 62.55 ± 1.75%; n = 10 WT, 11 DAT T356M$^{+/+}$; p = 0.13 by Student's two-tailed *t*-test).

We then sought to determine whether the T356M mutation promoted behaviors that have been associated with ASD or with the ADHD clinical phenotype, a common comorbidity of ASD. We tested animals for hyperactivity, social preference and dominance, and repetitive behaviors. We found that spontaneous locomotor activity was significantly elevated in DAT T356M$^{+/+}$ mice (Figure 10A. WT = 3648 ± 312.4 cm; DAT T356M$^{+/+}$ = 20571 ± 1062 cm; n = 15 WT, 16 DAT T356M$^{+/+}$; p < 0.0001 by Student's two-

tailed *t*-test). These animals traveled, on average, over five times the distance of their WT littermates in the same period of time (60 min). A two-way ANOVA was performed to examine the effect of genotype and time in the chamber on distance traveled. A two-way ANOVA was performed to examine the effect of genotype and treatment type on the rate of DA clearance (i.e. the decay time of the amperometric current (t_{80}-t_{20})). There was a signficiant interaction between the effects of genotype and treatment on the decay time ($F(1,32) = 7.482$, $p = 0.0101$). Simple main effects analysis was performed to determine which groups differed significantly.There was a significant main effect of time ($F(2.865,42.98) = 17.42$, $p < 0.0001$) and of genotype ($F(1,15) = 59.64$, $p < 0.0001$) on distance traveled. The observed hyperactivity in DAT T356M$^{+/+}$ mice persisted for the duration of the test (Figure 10B**.** for all bins, $p < 0.0001$ by two-way ANOVA followed by Sidak's multiple comparison test). We also used this assay to measure thigmotaxis in the first five minutes of exposure to the open field (a measure of anxiety). We found no difference in the percent of time spent on the edges of the activity chambers during this period of time (Figure 11. WT = 50.06% ± 5.355%; DAT T356M+/+ = 57.54 ± 4.089%; n = 10 WT, 11 DAT T356M+/+; $p = 0.28$ by Student's two-tailed *t*-test).

To assess whether DAT T356M$^{+/+}$ mice exhibited repetitive behaviors, we video monitored the animals for 15 minutes and quantified episodes of specific behaviors (self-grooming, climbing on wire cage lid, rearing, tail flick, jumping, and digging) (Figure 10C. See Table 7). A two-way ANOVA was performed to examine the effect of genotype and behavior type on incidence of behaviors. There was a signficiant interaction between the effects of genotype and behavior on incidence of behaviors ($F(5,174) = 21.91$, $p < 0.0001$). Simple main effects analysis was performed to determine which behaviors

differed significantly. We observed that DAT T356M$^{+/+}$ mice exhibited repetitive rearing behavior, performing this specific behavior nearly twice as often as their WT littermates (WT = 71 ± 5; DAT T356M$^{+/+}$ = 123 ± 9; n = 15 WT, 16 DAT T356M$^{+/+}$; $p < 0.0001$ by Sidak's multiple comparisons test). There was no difference in the frequency of self-grooming or digging behaviors, although a trend toward reduced digging was observed in DAT T356M$^{+/+}$ mice (Figure 10C. WT = 28 ± 4; DAT T356M$^{+/+}$ = 16 ± 3; n = 15 WT, 16 DAT T356M$^{+/+}$; $p = 0.0773$ by Sidak's multiple comparisons test). There was also no difference in episodes of tail flick or jumping, both of which can be used as indicators of stress and anxiety.

In our sample, we observed a significant positive association between distance traveled and bouts of rearing (Figure 12, Pearson's $r = 0.6538$, $p < 0.001$). This association did not vary according to genotype (genotype*predictor product term > .05 in the regression model testing the possibility of a moderated effect). Based on this finding, we sought to determine whether generalized hyperactivity mediated, or explained at least in part, the effect of genotype on rearing. This possibility can be statistically tested using modern mediation analyses that test the indirect effect of genotype on rearing through hyperactivity[318]. Two pathways comprise this indirect effect. The first pathway, referred to as the "a path," represents the relation between genotype and hyperactivity. The second pathway, referred to as the "b path," represents the relation between hyperactivity and rearing, controlling for genotype. An indirect effect is statistically significant when the confidence interval for the product of the unstandardized coefficients for these two paths does not include zero. Bias-corrected confidence intervals for the effects of interest were generated using 1000 bootstrap samples with the confidence level set at 95%. Results

indicated that the hypothesized mediation relation (a x b) was significant, 95% CI [0.87085, 49.9104]. The significant indirect effect confirms that hyperactivity mediates the relation between genotype and rearing behavior. This mediation is considered complete because the direct effect of genotype on rearing, the "c′ path," becomes non-significant when controlling for hyperactivity. This is consistent with findings in individuals with ASD, which show that ADHD symptoms are positively associated with stereotypic behaviors[319] and suggests that intervening on hyperactivity in individuals with ASD may translate into improvements in repetitive behaviors. This is important as, currently, there is little to no evidence indicating that existing interventions improve or decreased repetitive behaviors in children with ASD.

As the striatum has been implicated in action selection (the task of resolving conflicts between competing behavioral alternatives), we used marble burying to determine the impact of this mutation on the capacity for action selection and propensity for repetitive behaviors in DAT T356M$^{+/+}$. We found that DAT T356M$^{+/+}$ mice buried significantly fewer marbles than WT mice (See Figure 13; WT = 39.02 ± 7.95% of marbles buried, DAT T356M$^{+/+}$ = 8.26 ± 2.97% of marbles buried; n = 15 WT, 16 DAT T356M$^{+/+}$; p = 0.0009 by Student's two-tailed *t*-test), which can be interpreted to indicate a loss of motivated behavior or altered action selection. We next tested DAT T356M$^{+/+}$ mice for deficits in social interactions using the three chamber test of sociability [320]. A two-way ANOVA was performed to examine the effect of genotype and target on time in each chamber. There was a signficiant interaction between the effects of genotype and target type on time spent in each chamber ($F(1,58) = 7.336$, $p = 0.0089$). Simple main effects analysis was performed to determine for which group a signficant difference in time spent

with the social vs. the empty chamber existed. We found that DAT T356M$^{+/+}$ mice exhibited a loss of preference for social novelty (spending equal time in the empty chamber and the chamber containing a social partner), a behavior associated with the ASD clinical phenotype (Figure 10D; WT = 115.41 ± 11.06 s with social target, 66.07 ± 8.12 s with empty chamber; n = 15; p = 0.001 by Sidak's multiple comparisons test; DAT T356M$^{+/+}$ = 72.51 ± 5.91 s with social target, 66.125 ± 5.97 s with empty chamber; n = 16; p = 0.81 by Sidak's multiple comparisons test). Finally, we used the tube test to further investigate the effect of the DAT T356M mutation on normal social behaviors. Our results demonstrate a loss of social dominance (indicative of a social deficit) in DAT T356M$^{+/+}$ mice against both familiar and novel social stimulus mice (Figure 10E; n = 24 bouts from 12 pairs of mice; $p < 0.05$ by χ^2). Together, this pattern of behavioral alterations suggests the T356M mutation, via impairments of the transporter function, results in altered DA homeostasis that contributes to an ASD-associated phenotype, including aberrant social behaviors and repetitive behaviors. This mutation also drives hyperactivity, a behavior associated with ADHD, which is a common comorbidity of ASD[75,129] and which has been linked to DAT mutation[241,295]. In contrast to the observed differences between homozygous and WT animals, there were no significant differences between WT mice and DAT T356M$^{+/-}$ in any of the behavioral paradigms tested.

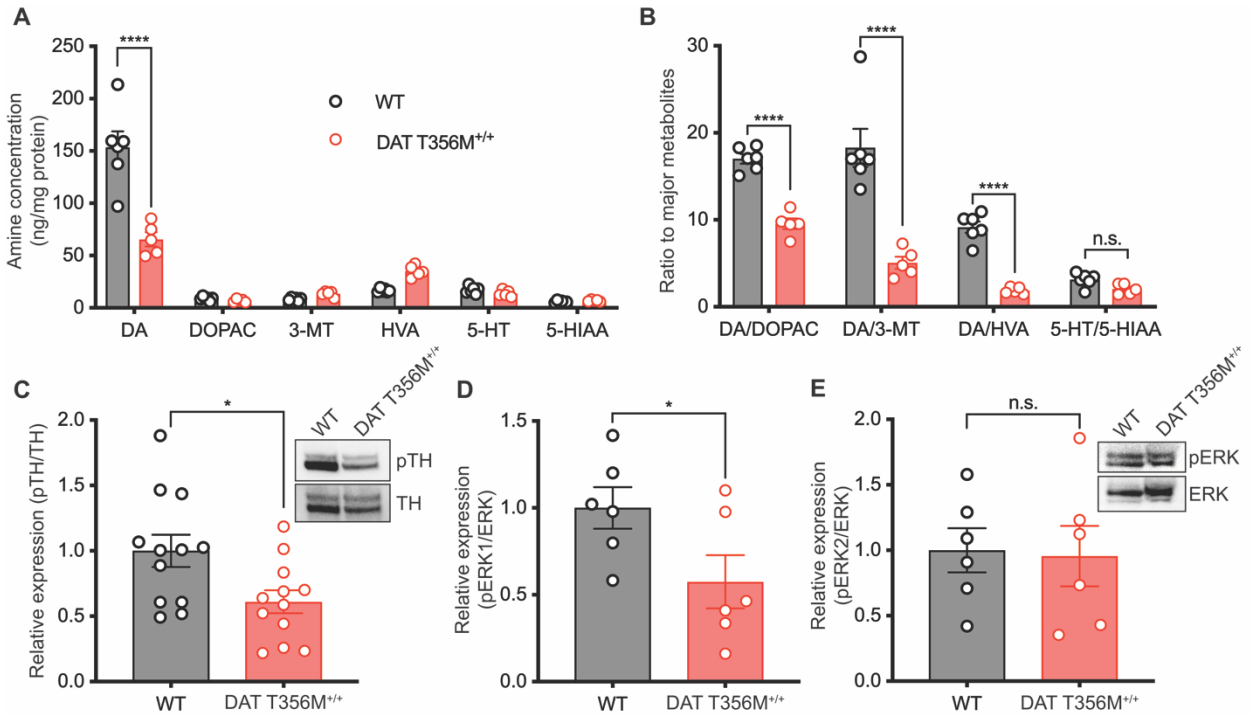

Figure 7. *The DAT T356M mutation drives increased striatal DA metabolism and reduced striatal DA synthesis.*

(A) The tissue concentration of DA (measured by HPLC) is significantly reduced in the DAT T356M$^{+/+}$ striatum compared with WT striatum (WT = 153.5 ± 15.38 ng/mg; DAT T356M$^{+/+}$ = 65.55 ± 6.71 ng/mg; *n*= 6 WT, 5 DAT T356M$^{+/+}$; *P* < 0.0001, 2-way ANOVA followed by Šidák's multiple comparisons test). There was no difference in the concentration of other biogenic amines (serotonin). ****P < 0.0001. (B) The ratio of the tissue content of DA in striatum to its metabolites is significantly lower in DAT T356M$^{+/+}$ mice compared with WT mice, providing evidence for increased metabolism of DA in the striatum (likely due to reduced reuptake of released DA). ****P < 0.0001. (C) Immunoblotting revealed significantly decreased pTH expression in the striatum of DAT T356M$^{+/+}$ mice when compared with WT mice (n = 12 from 4 animals; P = 0.0172,

Student's 2-tailed *t* test). **P* = 0.0172. (D and E) Immunoblotting revealed significantly decreased p-ERK1 expression in the striatum of DAT T356M$^{+/+}$ mice when compared with WT mice (*n* = 6; *P* = 0.03, Student's 2-tailed *t* test). **P* = 0.0279 (D).

Table 6. *Ratio of DA to its downstream metabolites in striatum.*

Ratio	**WT** (mean ± SEM)	**DAT T356M$^{+/+}$** (mean ± SEM)	***p***
DA/DOPAC	17.01 ± 0.55	9.547 ± 0.63	<0.0001
DA/3-MT	18.28 ± 2.175	5.047 ± 0.70	<0.0001
DA/HVA	9.149 ± 0.65	1.87 ± 0.17	<0.0001
5-HT/5-HIAA	3.115 ± 0.32	2.042 ± 0.26	0.9026

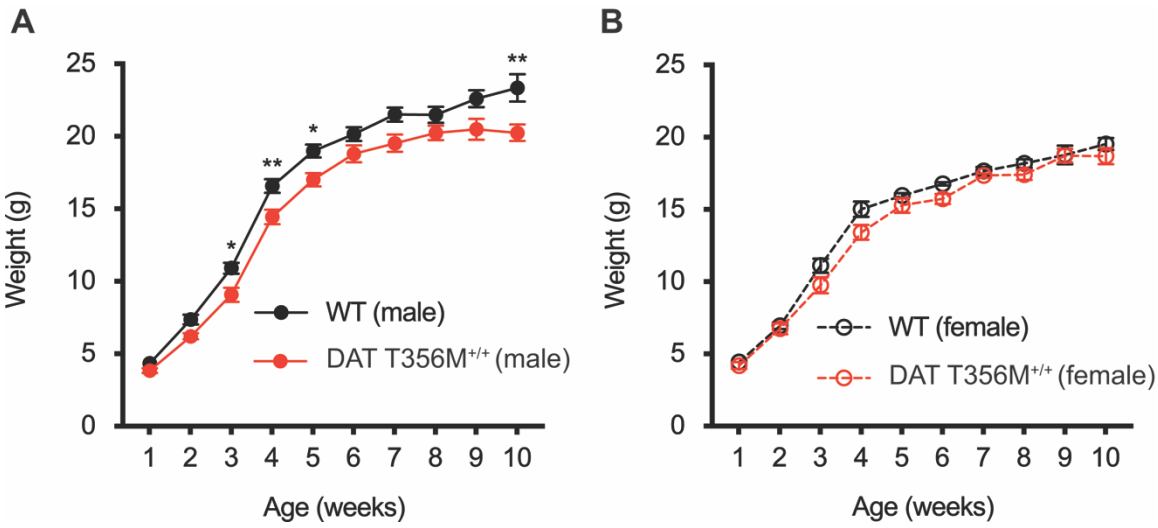

Figure 8. *DAT T356M+/+ male, but not female, mice exhibit slower weight gain in early life and have lower body weights in adulthood than DAT WT male mice.*

(A) Male DAT T356M$^{+/+}$ mice gain weight significantly more slowly in the period following weaning (P21 – week 5 of life; n = 37 WT, 39 DAT T356M$^{+/+}$; *P = 0.02, **P < 0.009, 2-way ANOVA followed by Šidák's multiple comparisons test). In adulthood (at 10 weeks of age), the body weight of male DAT T356M$^{+/+}$ mice averaged 3.09 g ± 0.92 g lower than that of adult male WT mice. (B) Female DAT T356M$^{+/+}$mice never differed statistically in body weight compared with WT female mice.

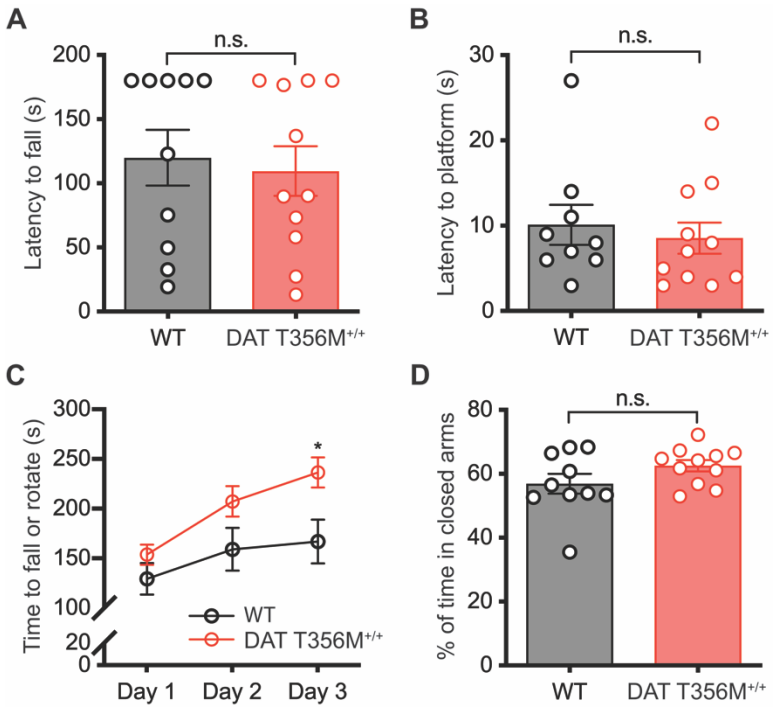

Figure 9. *DAT T356M+/+ mice do not demonstrate deficits in strength, coordination, motor learning, or anxiety.*

(A) There was no difference in latency to fall on the inverted screen test (a proxy for strength) between WT and DAT T356M$^{+/+}$ mice (WT = 120.0 ± 21.79 s, DAT T356M$^{+/+}$ = 109.6 ± 19.29 s; *n* = 10 WT, *n* = 11 DAT T356M$^{+/+}$; *P* = 0.72, Student's 2-tailed *t* test). (B) There was no difference in latency to reach the platform on the pole climb test (a proxy for coordination) between WT and DAT T356M$^{+/+}$ mice (WT = 10.11 ± 2.360 s, DAT T356M$^{+/+}$ = 8.545 ± 1.836 s; *n* = 9 WT, 11 DAT T356M$^{+/+}$; *P* = 0.6, Student's 2-tailed *t* test). (C) On days 1 and 2 of the rotarod test of coordination and motor learning, there was no statistically significant difference in performance between WT and DAT T356M$^{+/+}$ mice. However, on the third day of testing, DAT T356M$^{+/+}$ took a significantly longer time to fall or rotate than WT mice, indicating improved motor learning and

indicating a propensity for the formation of repetitive motor routines in DAT T356M$^{+/+}$mice (WT days 1, 2, 3 = 129.43 ± 15.89 s, 159.06 ± 21.52 s, 167.03 ± 22.1 s, respectively; DAT T356M$^{+/+}$ days 1, 2, 3 = 153.73 ± 10.16 s, 207.36 ± 15.28 s, 236.45 ± 15.22 s, respectively; n = 10 WT, n = 11 DAT T356M$^{+/+}$; P = 0.68 [day 1], P = 0.14 [day 2]), P = 0.02 [day 3], 2-way ANOVA followed by Šidák's multiple comparisons test). (D) There was no difference in the percentage of time spent in the closed arms of the elevated zero maze between WT and DAT T356M$^{+/+}$ mice, indicating no anxiety-like phenotype in the DAT T356M$^{+/+}$ mice (WT = 56.94% ± 3.12%, DAT T356M$^{+/+}$ = 62.55% ± 1.75%; n = 10 WT, n = 11 DAT T356M$^{+/+}$; P = 0.13, Student's 2-tailed t test). *P = 0.0157.

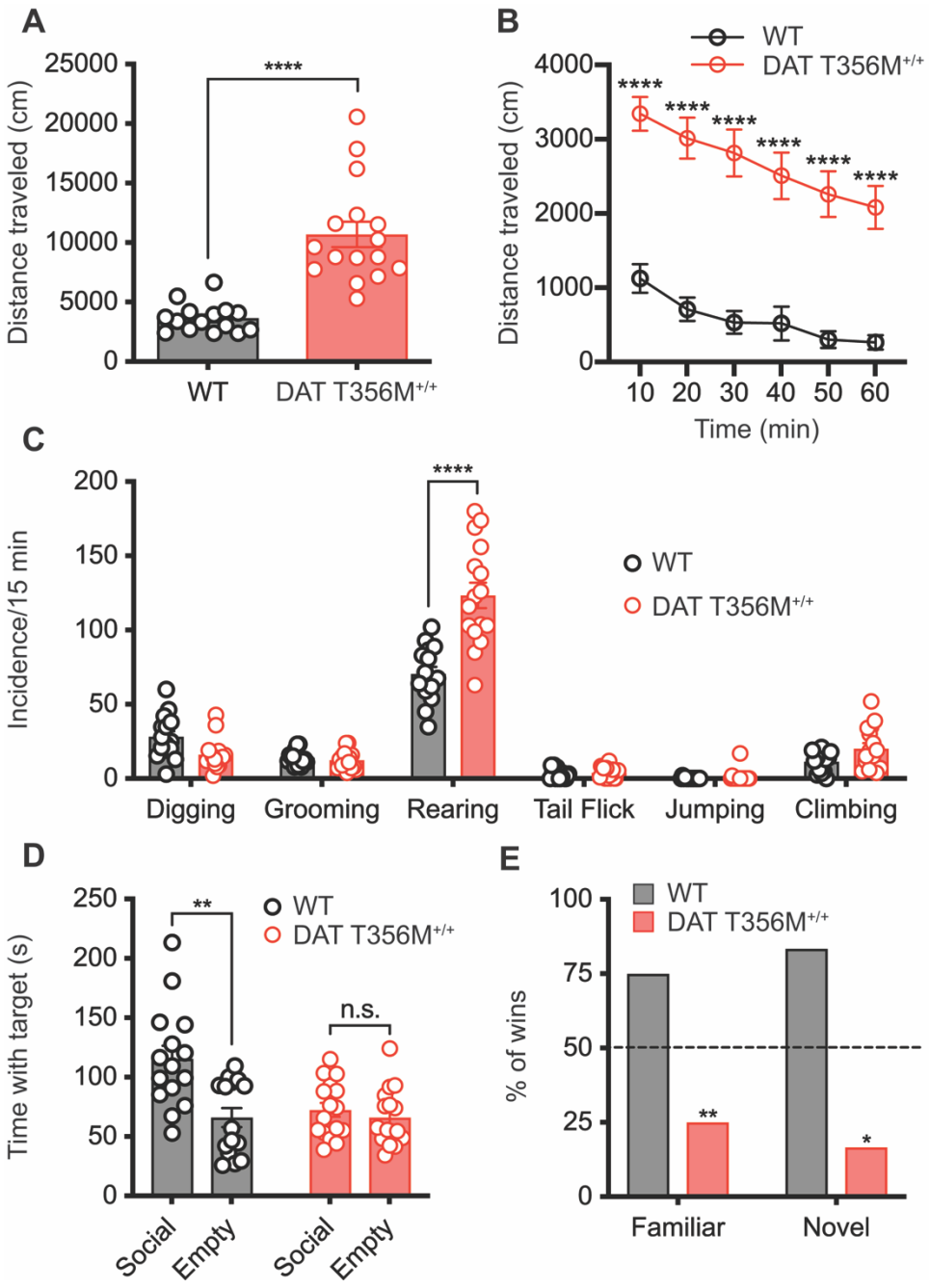

Figure 10. *DAT T356M+/+ mice exhibit spontaneous, persistent hyperlocomotion, repetitive rearing behavior, and altered social behaviors.*

(A) DAT T356M$^{+/+}$ mice traveled significantly further than WT mice during a 60-minute test period (WT = 3648 ± 312.4 cm; DAT T356M$^{+/+}$ = 20571 ± 1062 cm; *n* = 15 WT, 16 DAT T356M$^{+/+}$; ****P < 0.0001, Student's 2-tailed *t* test). (B) DAT T356M$^{+/+}$ mice exhibited

hyperlocomotor activity across all 10-minute intervals of the 60-minute test (for all intervals, ****$P < 0.0001$ by 2-way ANOVA followed by Šidák's multiple comparison test). (C) DAT T356M$^{+/+}$ mice exhibited repetitive rearing behavior (WT = 71 ± 5; DAT T356M$^{+/+}$ = 123 ± 9; n= 15 WT, n = 16 DAT T356M$^{+/+}$; ****$P < 0.0001$, 2-way ANOVA followed by Šidák's multiple comparisons test). (D) WT animals displayed a statistically significant preference for the social target, while DAT T356M$^{+/+}$ mice exhibited no preference for either target (WT = 115.41 ± 11.06 s with social target, 66.07 ± 8.12 s with empty chamber; n = 15; P = 0.001, 2-way ANOVA followed by Šidák's multiple comparisons test; DAT T356M$^{+/+}$ = 72.51 ± 5.91 s with social target, 66.125 ± 5.97 s with empty chamber; n = 16; P = 0.81, 2-way ANOVA followed by Šidák's multiple comparisons test). **$P = 0.0012$. (E) DAT T356M$^{+/+}$ mice won significantly fewer bouts against both familiar and novel mice than would be expected by chance (dashed line indicates chance-level performance; n = 24 bouts from 12 pairs of mice; *$P < 0.05$, **$P <$ 0.01 by χ^2).

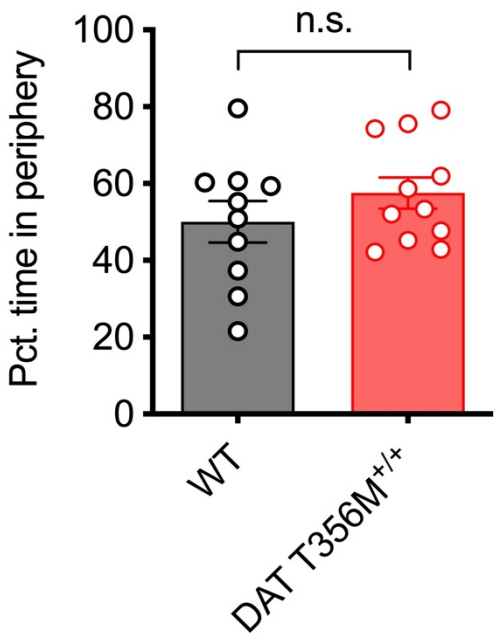

Figure 11. *DAT T356M$^{+/+}$ mice do not exhibit increased thigmotaxis.*

Percent of time spent in the periphery of the open field chambers during the first 5 minutes of testing (the neophobic period) does not differ between WT and DAT T356M$^{+/+}$ mice (WT = 50.06% ± 5.355%; DAT T356M+/+ = 57.54 ± 4.089%; n = 10 WT, 11 DAT T356M+/+; p = 0.28 by Student's two-tailed *t*-test).

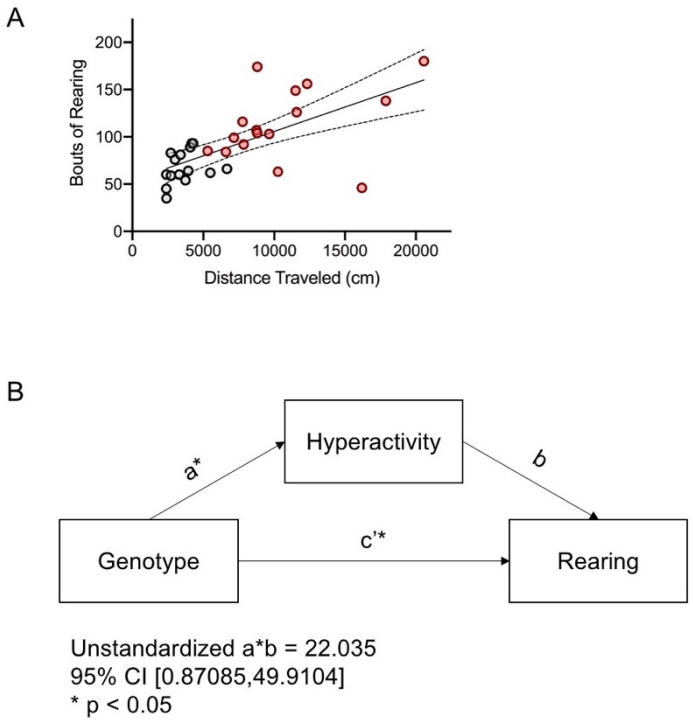

Figure 12. *Hyperactivity mediates repetitive rearing in DAT T356M$^{+/+}$ mice.*

(A) Bouts of rearing are positively associated with distance traveled in WT and DAT T356M$^{+/+}$ mice (Pearson's r = 0.6538, $p < 0.001$). (B) Modern mediation analysis reveals an indirect effect of genotype on rearing behavior through hyperactivity. The "a" path represents the relation between genotype and hyperactivity, the "b" path represents the relation between hyperactivity and rearing, controlling for genotype. Results indicated that the hypothesized mediation relation (a x b) was significant, 95% CI [0.87085, 49.9104]. The significant indirect effect confirms that hyperactivity mediates the relation between genotype and rearing behavior. This mediation is considered complete because the direct effect of genotype on rearing, the "c'" path, becomes non-significant when controlling for hyperactivity.

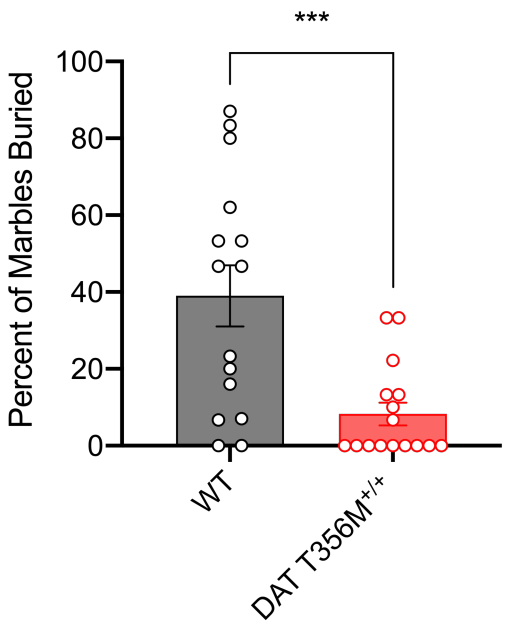

Figure 13. *DAT T356M$^{+/+}$ mice do not exhibit repetitive marble burying.*

DAT T356M$^{+/+}$ mice buried significantly fewer marbles than WT mice in the marble burying assaying (WT = 39.02 ± 7.95% of marbles buried, DAT T356M$^{+/+}$ = 8.26 ± 2.97% of marbles buried; n = 15 WT, 16 DAT T356M$^{+/+}$; *** P = 0.0009 by Student's two-tailed *t*-test).

Table 7. *Incidence of behaviors observed in WT and DAT T356M$^{+/+}$ mice.*

Behavior	**WT** (mean ± SEM)	**DAT T356M$^{+/+}$** (mean ± SEM)	**p**
Digging	28 ± 4	16 ± 3	0.0773
Grooming	14 ± 1	13 ± 1	0.9997
Rearing	71 ± 5	123 ± 9	<0.0001
Tail Flick	3 ± 1	4 ± 1	0.9999
Jumping	0 ± 1	1 ± 1	>0.9999
Climbing	11 ± 2	20 ± 4	0.3523

DAT antagonists decrease hyperlocomotion in DAT T356M+/+ mice

Our group has demonstrated that the DAT T356M mutation drives anomalous DA efflux that is prevented by exposure to DAT blockers [242]. We therefore hypothesized that blockade of the DAT by a specific DAT antagonist (ACT-01; see Table 8) would prevent DA efflux through the mutant transporter, thereby reducing the hyperlocomotion observed in DAT T356M$^{+/+}$ mice. ACT-01 was selected as this compound specifically blocks the DAT without off-target interactions with the norepinephrine or serotonin transporters (see Table 8). Specificity of ACT-01 for the DAT was determined by RTI-55 displacement as well as inhibition of [^{3}H]DA uptake, [^{3}H]5-HT uptake, and [^{3}H]NE uptake in HEK293 cells expressing, respectively, the hDAT, the human serotonin transporter (hSERT), and the human norepinephrine transporter (hNET) (see Table 8 for Kis, Hill coefficients, and IC_{50}s). This allowed us to directly assess of the impact of DAT blockade on behavior. A two-way ANOVA was performed to examine the effect of treatment type and time on distance traveled. There was a signficiant main effect of treatment type on distance traveled ($F(1,25) = 16.97$, $p = 0.0004$). Posthoc testing was performed to determine at which time points the treatment groups differed signfiicanlty. ACT-01 treatment significantly reduced hyperlocomotion as early as 20 minutes into the observation period (Figure 14A. n = 6-9; $p = 0.0032$ at 20 minutes; $p < 0.032$ at 30 minutes by Sidak's multiple comparisons test). Total locomotor activity was also reduced by acute treatment with ACT-01 (Figure 14B. DAT T356M$^{+/+}$ Vehicle = 6847 cm ± 901.2 cm; DAT T356M$^{+/+}$ ACT-01 = 2361 cm ± 595.2 cm; n = 6-9; $p = 0.0045$ by Student's *t*-test). Due to the novel nature of this compound, we also used a previously validated DAT antagonist, GBR12909, to further support our findings. A two-way ANOVA was performed to examine the effect of

treatment type and time on distance traveled. There was a signficiant main effect of treatment type ($F(1,24) = 24.39$, $p < 0.0001$) and time ($F(3,24) = 8.536$, $p = 0.0005$) on distance traveled. Posthoc testing was performed to determine at which time points the treatment groups differed signfiicanlty.Treatment with GBR12909 significantly reduced spontaneous locomotor activity in DAT T356M$^{+/+}$ mice in the first 10 minutes of observation (Figure 14C. $n = 4$; $p = 0.045$ by by Sidak's multiple comparisons test). Total locomotor activity was also reduced by acute treatment with GBR12909 (Figure 14D. DAT T356M$^{+/+}$ Vehicle = 5188 cm ± 411.7 cm; DAT T356M$^{+/+}$ GBR12909 = 2578 cm ± 716.5 cm; $n = 4$; $p = 0.0196$ by Student's two-tailed t-test). These data support our hypothesis that anomalous DA efflux, mediated by the mutant DAT, leads to hyperlocomotion, which is corrected by blockade of the DAT.

Table 8. *Effect of ACT-01 on HEK-hDAT, HEK-hSERT and HEK-hNET cells*

HEK-hDAT cells	ACT-01	Cocaine
[^{125}I]RTI-55 Binding Ki (nM)	710 ± 170	595 ± 59
Hill coefficient	-0.87 ± 0.07	-1.06 ± 0.11
[^{3}H]Dopamine Uptake IC_{50} (nM)	276 ± 36	370 ± 110
HEK-hSERT cells	ACT-01	Cocaine
[^{125}I]RTI-55 Binding Ki (nM)	>9100#	468 ± 49
Hill coefficient		-0.93 ± 0.04
[^{3}H]Serotonin Uptake IC_{50} (nM)	>10 µM	286 ± 19
HEK-hNET cells	ACT-01	Cocaine
[^{125}I]RTI-55 Binding Ki (nM) Hill coefficient	>9,800#	2,240 ± 320 -1.12 ± 0.15
[^{3}H]NE Uptake IC_{50} (nM)	>6,000	203 ± 14

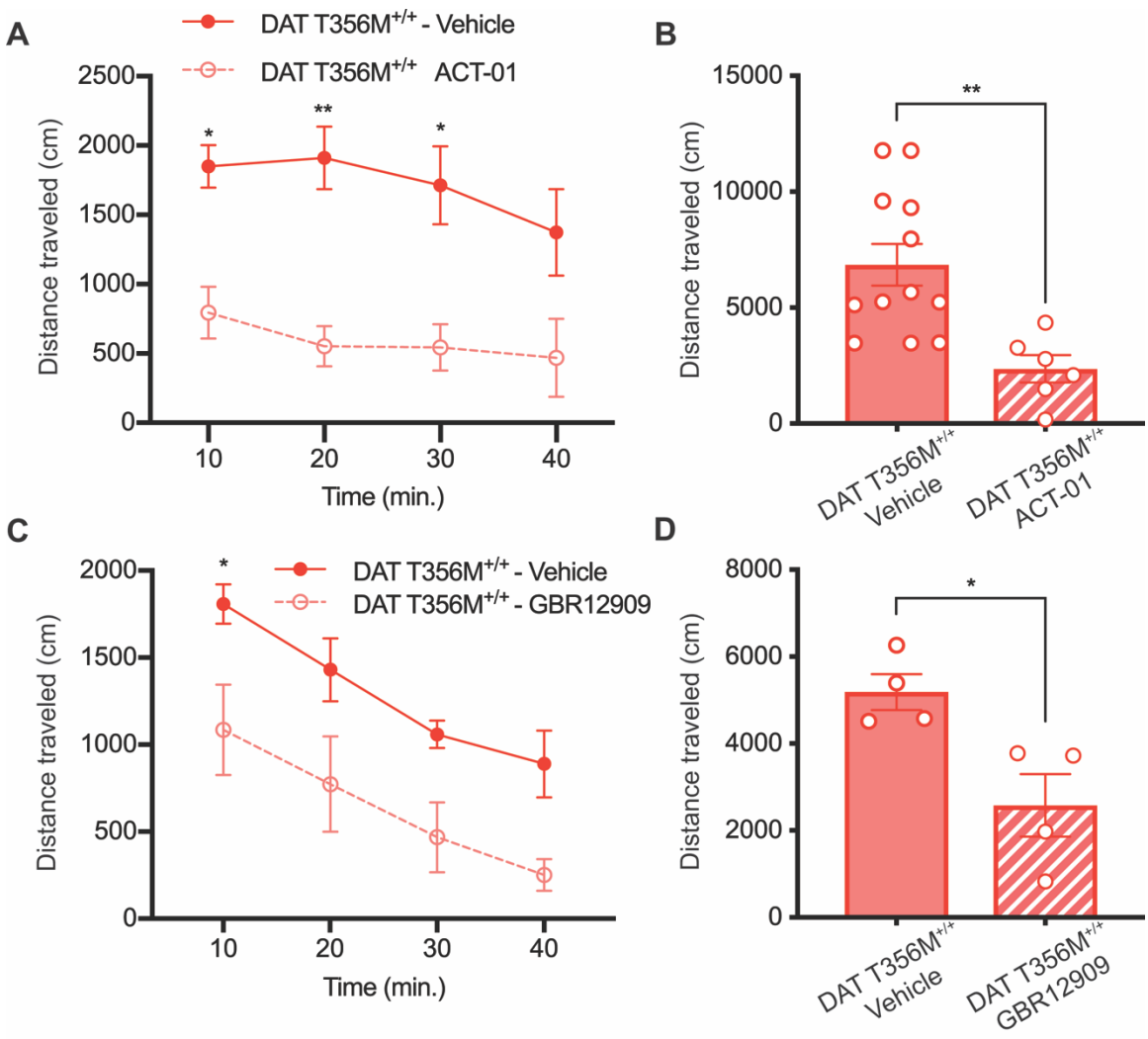

Figure 14. *Treatment with DAT inhibitors reduces spontaneous locomotor activity in DAT T356M+/+ mice.*

(A) ACT-01 treatment significantly reduced spontaneous locomotor activity in DAT T356M$^{+/+}$ mice as early as 20 minutes into the observation period compared with vehicle-treated animals (n = 6–9; **P= 0.0032; *P < 0.032, 2-way ANOVA followed by Šidák's multiple comparisons test). (B) Total locomotor activity in DAT T356M$^{+/+}$ mice was reduced by acute treatment with ACT-01 (DAT T356M$^{+/+}$ vehicle = 6847 cm ± 901.2 cm; DAT T356M$^{+/+}$ ACT-01 = 2361 cm ± 595.2 cm; n = 6–9; **P = 0.0045, Student's t test).

(C) GBR12909 treatment significantly reduced spontaneous locomotor activity in DAT T356M$^{+/+}$ mice as early as 10 minutes into the observation period compared with vehicle-treated animals (n = 4; *P = 0.045, 2-way ANOVA followed by Šidák's multiple comparisons test). (D) Total locomotor activity in DAT T356M$^{+/+}$ mice was rescued by acute treatment with GBR12909 (DAT T356M$^{+/+}$ vehicle = 5188 cm ± 411.7 cm; DAT T356M$^{+/+}$ GBR12909 = 2578 cm ± 716.5 cm; n = 4; *P = 0.0196, Student's 2-tailed t test).

Discussion

Our studies demonstrate that the DAT T356M mutation drives reduced transporter function, synaptic hyperdopaminergia, and reduced DA synthesis in the striatum. Impaired transporter function (specifically, reduced rate of DA reuptake from the extracellular space) aligns with previous work in transfected cells demonstrating reduced capacity of DAT T356M for DA reuptake and the presence of anomalous DA efflux through these defective transporters[240,308]. Although the expression of the T356M DAT is comparable to that of WT DAT, the possibility exists that altered DAT trafficking promoted by the T356M mutation drives impaired DA clearance. We believe this to be unlikely given previous work demonstrating intact surface expression of the mutant transporter in cultured cell lines. Furthermore, the amperometric data presented here demonstrates reduced (but not eliminated) transporter function that is sensitive to cocaine, suggesting the presence of the transporter on the membrane. Our work strongly supports a mechanism whereby the reduced rate of DAT-mediated reuptake of released DA from the extracellular space results in D_2R desensitization, reduced DA synthesis as a result of elevated synaptic DA levels, and ultimately reduced total tissue content of DA. These results align with previous work in DAT KO animals, which demonstrated that desensitization of the D_2R leads to reduced phosphorylation (activation) of tyrosine hydroxylase, the rate limiting enzyme in the DA synthesis pathway [317,321].

It is now understood that dopaminergic signaling in the striatum governs action selection (the task of resolving conflicts between competing behavioral alternatives)[322–324]. Under physiological conditions, the DA signal is tightly regulated to support a quantitative prediction error signal (a physiological correlate of reward encoded by

dopaminergic neurons as the difference between the current reward and the predicted reward based on previous experience)[292]. This prediction error signal is critical to reward processing and learning of motivated behaviors[292,293,325,326]. Evidence presented here strongly supports a mechanism whereby transporter dysfunction drives dysregulation of the dopaminergic signal. It follows, therefore, that change in the nature of the DA signal (here, alteration in the phasic and quantitative nature of the signal due to transporter dysfunction) would lead to alterations in motivated behaviors (such as social behaviors). Here we show that DAT T356M$^{+/+}$ animals have deficits in social behaviors as demonstrated by loss of social preference in the three chamber task and loss of social dominance in the tube test. This supports our hypothesis that the DAT T356M drives aberrant, ASD-associated behaviors, which may be due, at least in part, to altered social motivation due to the underlying alteration in dopaminergic signaling. Furthermore, the work presented here demonstrates deficits in marble burying in DAT T356M$^{+/+}$ mice. Marble burying represents an innate behavior of laboratory mice that is motivated by investigation [327]. A deficit in marble burying can therefore be interpreted to represent a change in motivated behavior in mice. Our finding of reduced marble burying DAT T356M$^{+/+}$ mice therefore supports our hypothesis that DAT dysfunction leading to synaptic hyperdopaminergia drives altered action selection and motivated behavior. Moreover, impulsivity and repetitive behaviors can be interpreted as deficits in inhibitory action control [328] and have been previously related to altered dopaminergic signaling [182,329–331]. The repetitive rearing behavior observed in DAT T356M$^{+/+}$ mice further supports our hypothesis that dopaminergic dysregulation due to this mutation drives altered action selection and control. Finally, we demonstrate increased rotarod learning in DAT

T356M$^{+/+}$ mice. The superior performance of the DAT T356M$^{+/+}$ animals on the rotarod test on the third day of testing provides evidence suggesting that this autism-associated mutation drives an increased propensity for the formation of repetitive behaviors [274] (a core diagnostic feature of ASD), presumably by altering DA homeostasis in the striatum [274]. This enhanced learning on the rotarod task has been observed previously in multiple mouse lines with ASD-associated mutations and has been linked to altered DA neuron signaling.

In addition to the role of DA in action selection and reward learning, it has been shown that DA regulates spontaneous locomotor activity [332–335] and that hyperactivity can be driven in a DA-dependent fashion[336]. It follows therefore that enhanced synaptic DA levels in the striatum due to the DAT T356M mutation could drive hyperactivity. Indeed, our work demonstrates significant hyperactivity in DAT T356M$^{+/+}$ mice. We further demonstrate the DAT-dependence of this hyperactivity via blockade of the DAT, which reduced spontaneous locomotor activity in DAT T356M$^{+/+}$ mice. This blockade was obtained both with a specific and novel DAT blocker (ACT-01) as well as with a traditional DAT blocker (GBR12909). Taken together, these results suggest anomalous efflux of DA and reduced DA reuptake by the mutant transporter (both of which contribute to synaptic hyperdopaminergia) as driving factors in the observed hyperactivity. As increased locomotion was observed in flies expressing this variant in dopaminergic neurons and due to the high comorbidity of ASD and ADHD, we chose to explore the reversibility of the observed hyperlocomotion. Future work should aim to determine whether blockade of the DAT may eliminate or alleviate the more complex behavioral changes observed in DAT T356M $^{+/+}$ animals.

It is interesting to note that the DAT M356 variant was identified as a heterozygote in the proband (for patient genetic information and clinical evaluation, see supplemental material of Hamilton et al. 2013[240]. We found no evidence of behavioral or biochemical differences between WT and DAT T356M$^{+/-}$ mice in our study. While much conservation of functional DNA sequences does exist between humans and mice, the sequences involved in transcriptional regulation in mice do diverge from their human orthologues and thus could provide a basis for differential expression profiles of various genes in mice[337], including the DAT. Differential (or preferential) expression profiles of the DAT T356 allele vs. the DAT M356 allele could serve as the foundation for the discrepancy between the observed human genotype-phenotype correlation and the one reported here. Alternatively, the possibility exists that the proband harbored a unique set of common variants that acted in concert with the DAT M356 variant allele to produce the observed phenotype and presents an exciting new avenue for future research.

The behavioral and neurophysiological characteristics of the DAT T356M mouse presented here provide new evidence for a role of DAT dysfunction (specifically anomalous DA efflux and reduced reuptake of released DA) in the behavioral deficits typically associated with ASD and ADHD. This work supports and extends previous in vitro work that demonstrated anomalous DA efflux and reduced uptake of DA resulting from this mutation. Our model suggests that blockade of the DAT may improve behavior through reduction of non-vesicular, DAT-mediated DA efflux. Given the number of roles of DA in behavioral learning, reward processing, and action selection, this work presents the exciting possibility of a potential mechanistic underpinning (in at least a subset of patients) for the altered behaviors observed in ASD and ADHD. Future work should seek

to determine to what degree DA dysfunction exists across patients with these neuropsychiatric disorders and whether striatal dysfunction plays a central role in the pathology of these conditions.

CHAPTER IV

IMPACT OF THE DAT T356M VARIANT ON SENSORY BEHAVIORS AND SENSORIMOTOR GATING

Introduction

Sensory processing is known to be altered in individuals with ASD. In one study, up to 95% of the children with ASD sampled exhibited sensory processing dysfunction to some degree. More than 90% of this sample were reported to seek sensory stimulation, 77.6% were noted to exhibit auditory filtering, and 60.9% were reported to have tactile hypersensitivity[338]. At ages as young as 6 months, children who will go on to be diagnosed with ASD demonstrate hyporesponsiveness to social stimuli[339]. Such sensory symptoms typically precede social symptoms and are predictive of later diagnosis[340,341]. Neuroimaging studies provide evidence that these observed changes in sensory processing are likely due to changes in sensory processing in early sensory cortex[69]. Many hypothesize that early changes in sensory processing likely cascade into deficits in multisensory processing (also common in ASD[70,72]).

Studies have also demonstrated deficits in sensorimotor gating in individuals with ASD[342,343]. Sensorimotor gating is the process by which the central nervous system (CNS) regulates incoming sensory information (in combination with internal information from higher-order, cognitive processes) to suppress a motor response. This process is thought to support maximally efficient processing of the most significant features of the sensory environment and to facilitate behavioral integration with cognitive processes[344].

One method to assess sensorimotor gating relies on measuring the acoustic startle response (ASR). This response, a reflexive contraction of body musculature in response to sudden and intense auditory stimulus, is present across species and represents an integrated reflexive response to strong external stimuli. In humans, this reflex is measured as the eye-blink component of startle, which can be quantified by eyelid displacement via electromyography (EMG) of facial muscles. In mice, the reflex can be measured by measuring the amplitude of an animal's "jump" in response to an acoustic stimulus[345]. The basic reflex arc that mediates this reflex is located subcortically (within the brainstem). However, this reflex can be modulated by top-down influences descending from limbic and cortical regions of the brain. Such modulation of the auditory startle reflex is considered evidence of sensorimotor gating. Sensorimotor gating can be assessed by measuring the degree to which a weak prestimulus inhibits the startle reflex (i.e. prepulse inhibition, PPI)[346].

PPI is a ubiquitous across species and is particularly useful for understanding how neurological and psychiatric disorders affect stimulus reactivity and reflex excitability. While the acoustic startle reflex is mediated at the brainstem level, PPI is regulated by forebrain regions including limbic cortex, striatum, ventral pallidum, and hippocampus[347]. Since weak prestimuli induce centrally-controlled inhibition of startle, deficits in PPI may reflect a failure of central inhibitory processes that may be due to functional or anatomical changes in these brain regions[346]. That is, changes in PPI are reflective of underlying changes in neurobiology that prevent the nervous system from adapting to strong sensory stimuli. Measuring PPI can therefore give us insight into how to brain regulates incoming sensory information and responds to the environment.

These studies show significantly reduced PPI of the ASR in individuals with ASD as measured by EMG of the orbicularis oculi muscle. Evidence suggests this altered sensitivity to sensory stimulation may result from differences in brain development (including differences in cortico-striatal connectivity)[342]. Indeed, greater sensorimotor gating deficits have been shown to positively correlate with increased restricted and repetitive behaviors in individuals with ASD[343]. which are in turn associated with altered development of the striatum[184,348]. At least one study reports increases in the amplitude of the ASR in individuals with ASD[349], while others report no difference[343,350].

While the exact circuitry subserving PPI is unknown, we know that the VTA (a dopaminergic region of the brain) is active during PPI paradigms. Previous studies demonstrate that manipulations to the DA system may alter PPI. Ralph and colleagues demonstrated that mice lacking the DAT (DAT (-/-) mice) exhibit deficits in sensorimotor gating as measured by PPI of the startle response when compared to mice with two functional copies of the DAT (DAT (+/+) mice). Treating DAT (-/-) mice with raclopride, a D2 receptor antagonist, significantly increased PPI, while having no effect on PPI in DAT (+/+) mice. In this study, D1 receptor antagonism with SCH23390 was ineffective at increasing PPI in DAT (-/-) mice. In another study, rats treated with amphetamine (a DAT substrate that elevates extracellular DA) have significant loss of PPI[351]. Taken together, these findings suggest that elevated extracellular DA (via DAT blockade or loss of DAT) reduces PPI and that D2 receptors, but not D1 receptors, are necessary for the dopaminergic modulation of PPI[352].

We have shown previously that mice homozygous for the DAT T356M variant (a variant of the DAT found in an individual with ASD), demonstrate repetitive behaviors and

other ASD-associated behaviors (i.e. social deficits and hyperactivity). Additionally, these animals have dopaminergic abnormalities including elevated levels of extracellular DA and reductions in D2-mediated intracellular signaling cascades. Given the known role of both the D2 and DA in modulating PPI, in combination with the deficits in sensorimotor gating observed in humans with ASD, we hypothesized that DAT T356M$^{+/+}$ mice would display deficits in sensorimotor gating as measured by PPI of the ASR.

We further endeavored to understand the impact of this mutation on lower-level sensory behaviors. We chose to explore olfaction due to the critical importance of this particular sensory system in mouse behavior (e.g. detecting social partners, avoiding predators, and locating food) and because dopaminergic signaling it the olfactory bulb is known to modulate odor discrimination in rodents[353]. We therefore hypothesized that DAT T356M$^{+/+}$ mice would demonstrate deficits in olfactory discrimination and the ability to detect volatile odors as a result of DAT dysfunction.

We also chose to explore the impact of this mutation on mechanical nociception. Changes in tactile sensitivity have been report in individuals with ASD, including increased sensitivity to light touch[354] and behavioral avoidance of tactile stimulation[355]. Recent studies in transgenic ASD mouse models also demonstrate somatosensory neuron dysfunction in these models[356]. We therefore hypothesized the DAT T356M$^{+/+}$ mice would exhibit increased tactile sensitivity to pain-inducing stimuli.

Methods & materials

All behavioral experiments were performed in the Vanderbilt University Neurobehavioral Core Facility. All behavioral tests were performed at the same time of day (13:00-17:00) using mice 8-10 weeks of age. Mice were group housed (3-5 mice/cage) on a 12:12-h light-dark cycle with food and water available ad libitum. Mice were housed on paper bedding. Unless otherwise stated, each apparatus used was cleaned with a 10% ethanol solution between each animal or trial to provide a standardized testing environment.

Acoustic startle response & pre-pulse inhibition

Prepulse inhibition of the startle response is measured using the same sound-attenuated chambers used for measuring the startle response. Startle response was measured using commercially-available startle chambers (Med Associates). Mice are placed in a clear plastic cylinder (~5 cm diameter) within a ventilated, sound-attenuating enclosure. Sessions were preceded by a 5-minute acclimation period. Each session consisted of 54 trials over a 20-minute period. In the acoustic startle version of the prepulse inhibition task, the startle stimulus is a 40 ms, 120 dB burst of white noise. The prepulse stimuli are 20 ms bursts of 70-88 dB white noise, presented 50 ms before the startle stimulus. Acoustic stimuli of these intensities do not produce a startle response alone. The intertrial interval ranged from 10-20 sec. The maximal startle amplitude recorded during the 65-ms sampling window is used as the dependent variable. Prepulse inhibition was calculated as the percentage of inhibition of the startle amplitude evoked by the 120 dB pulse alone: ((Response on pulse trial – Response on prepulse

trial)/Response of pulse trial)x100. Trials were presented in a pseudorandom order. All acoustic stimuli were presented through a loudspeaker mounted 28 cm above the animal. If a mouse was observed to be abnormally fatigued, distressed, or injured, this mouse was immediately removed from the apparatus.

Buried food test

The buried pellet test was used to assess the ability of the animals tested to detect a volatile olfactory cue and to use this cue for foraging[357]. Briefly, mice were food restricted to 90% of their pre-test weight. Food restriction consisted of a diet of regular chow equal to 75% of their typical daily intake (or 90 g/kg of body weight per day). Mice were additionally provided 1 piece of frosted cereal (here, Frosted Cheerios) each night until they reached 90% of pre-test weight to establish odor familiarization and ensure palatability of the frosted cereal. At the start of the test, cages (189 mm x 297 mm x 128 mm) were prepared by filling clean, empty cages with 4.5 cm of Diamond Soft Bedding (Envigo Teklad 7089). Mice were placed individually into filled cages for a 5-minute acclimation period. Mice were then removed from the test cage and one frosted cheerio was buried ~1 cm below the surface of the bedding in a random corner of the cage. Mice were then reintroduced to the cage. Latency to uncover the buried cereal and latency to consume the cereal were recorded. This procedure was performed once per day for each mouse for 2 days.

Novel odor test for odor discrimination, habituation, and dishabituation

This test consists of sequential presentation of various odors. For our test, we presented the following odors (in this order): water once, dilute almond extract (1:100)

three times, and dilute lemon extract (1:100) once. Each odor was presented for 1 minute. The inter-trial interval was at least 5 minutes. Animals were acclimated to a clean, dry cotton tipped applicator for 10 minutes. After this acclimation period, mice were presented with the odors as described above. Time sniffing the cotton tipped applicator was recorded for each odor presentation. Habituation was defined as a reduction in olfactory exploration towards a repeated presentation of the same odor (here, almond). Dishabituation was defined as reinstatement of olfactory exploration in response to a novel odor (here, lemon).

Block test for social odor discrimination

This test relies on the innate preference of mice to explore non-familiary social odors over self-odors. Mice were single-housed overnight with three clean wooden blocks. On the day of the test, blocks were placed in sealed plastic bags with a handful of bedding. Mice were presented with two combinations of blocks: one trial of blocks from their own cage and one trial consisting of one block from their own cage and one block from the cage of an unfamiliar mouse. The unfamiliar mouse was age and sex matched to the test mouse. The order these trials were presented in was randomized and counterbalanced across mice. Time spent exploring each block was recorded.

von Frey filament test

Mechanical nociception was assessed using von Frey filaments. Filaments of various diameters were pressed against the plantar surface of the mouse's foot. The filaments bend, producing a constant force of application from 0.01 to 10 mN. Filaments

were presented in ascending order until a foot withdrawal response was observed. The force exerted by the filament that elicited the response was taken as the threshold.

Statistics

All statistical analyses were performed using GraphPad Prism software (version 8.3.0). Statistical methods are indicated in the figure legends and the results section. Data are presented as mean ± SEM. Differences are considered statistically significant at $P < 0.05$. Unpaired 2-tailed Student's t-test was used for 2-group comparisons, unless stated otherwise. Either 1- or 2-way ANOVA with appropriate post-hoc testing was used for multiple comparisons.

Study approval

All experiments were performed under a protocol approved by the Vanderbilt University Animal Care and Use Committee.

Results

DAT T356M$^{+/+}$ mice have reduced ASR amplitude and reduced PPI

Using the acoustic startle task, we measured the degree to which a sudden, intense acoustic stimulus elicited the startle reflex in WT mice, mice heterozygous for the T356M variant (DAT T356M$^{+/-}$ mice), and mice homozygous for the T356M variant (DAT T356M$^{+/+}$ mice). We found there was a significant effect of genotype on startle amplitude by one-way ANOVA ($F(2,29) = 3.441$, $p = 0.0456$). Post-hoc comparisons using Tukey's multiple comparisons test revealed that startle amplitude is significantly reduced in DAT

T356M$^{+/+}$ mice, but not DAT T356M$^{+/-}$ mice, compared to WT mice (Figure 15A; WT = 1356 ± 163.2 Startle Response Units [SRU], DAT T356M$^{+/-}$ = 945.3 ± 125.5 SRU, DAT T356M$^{+/+}$ = 811.7 ± 137.9 SRU, n = 9 WT, 13 DAT T356M$^{+/-}$, 12 DAT T356M$^{+/+}$, p = 0.0370). Startle amplitude did not vary according to genotype when a prepulse stimulus (70 – 88 dB) was presented (Figure 15B).

The pre-pulse inhibition task was used to measure the degree to which a weak auditory pre-stimulus could attenuate the startle response in WT, DAT T356M$^{+/-}$, and DAT T356M$^{+/+}$ mice. We performed a repeated measures two-way ANOVA to examine the effect of genotype and pre-stimulus intensity on PPI. Dunnett's multiple comparisons test revealed a significant deficit in PPI in both DAT T356M$^{+/-}$ and DAT T356M$^{+/+}$ mice at 70 dB and a significant deficit in PPI in DAT T356M$^{+/+}$ mice at 82 and 88 dB (see Table 9).

Previous studies in WT mice have demonstrated that PPI is correlated with startle amplitude. That is, mice who have a greater startle response also typically have greater PPI. Given that the DAT T356M$^{+/+}$ mice have a lower startle amplitude, we sought to determine whether there were differences in percent PPI across the tested prepulse intensities when controlling for basal startle amplitude. To examine this relationship, we conducted a repeated measures analysis of covariance. Results of this analysis reveal that basal startle amplitude was not significantly related to percent PPI ($F(1,30) = 1.703$, $p = 0.202$). This analysis also revealed there was a significant effect of genotype on percent PPI in after controlling for basal startle amplitude ($F(2,30) = 4.623$, $p = 0.018$). Post-hoc testing revealed a significant difference in percent PPI between WT and DAT

T356M$^{+/+}$ mice (p = 0.034), but not between WT and DAT T356M$^{+/-}$ mice or DAT T356M$^{+/-}$ mice and any other genotype.

DAT T356M$^{+/+}$ mice have no significant differences in odor discrimination, habituation, or detection

For the buried food test, latency to uncover the buried food was analyzed by fitting a mixed model as implemented in GraphPad Prism 8.0. This model uses a compound symmetry covariance matrix and is fit using Restricted Maximum Likelihood (REML) with Geisser-Greenhouse correction. There was not a significant interaction of genotype and day ($F(1,12) = 1.807$, $p = 0.20237$), however there was a significant main effect of day on latency to uncover the pellet ($F(1,12) = 6.729$, $p = 0.0235$). There was no main effect of genotype on latency to uncover the buried food ($F(1,13) = 0.1272$, $p = 0.2.665$). Post-hoc testing using Sidak's multiple comparisons test revealed no significant difference when time to uncover on day 1 vs. day 2 for either WT or DAT T356M$^{+/+}$ mice (Figure 17; WT Day 1 = 160 s ± 40.89, Day 2 = 26.80 s ± 8.924, $p = 0.0573$; DAT T356M$^{+/+}$ Day 1 = 197.8 s ± 36.81, Day 2 = 158.0 s ± 45.30, $p = 0.5423$; n = 5 WT, 9 DAT T356M$^{+/+}$).

We analyzed the novel odor test results (i.e. time spent sniffing each odor presented, Figure 18) by was analyzed by fitting a mixed model as implemented in GraphPad Prism 8.0 (as above). Results of this model demonstrate a significant effect of scent presentation number ($F (3.161, 70.33) = 8.736$, $p < 0.0001$), but no significant fixed effect of genotype ($F (1,25) = 1.945$, $p = 0.1754$) or interaction between genotype and scent ($F (4, 89) = 2.234$, $p = 0.0717$). Post-hoc testing using Sidak's multiple comparisons test revealed no significant differences in time spent exploring each olfactory cue between

WT and DAT T356M$^{+/+}$ mice (See Table 10 for a summary of results). Post-hoc testing additionally revealed no significant differences in the time spent exploring water vs. the first presentation of almond scent, the first presentation of almond scent vs. the second, the second presentation of almond scent vs. the third, or the third presentation of almond scent vs. the first presentation of lemon for WT mice (Table 11). This analysis found only a significant difference between time spent sniffing the first presentation of the almond scent and the second for DAT T356M$^{+/+}$ mice (Table 12).

Finally, we explored social odor discrimination in WT and DAT T356M$^{+/+}$ mice. As described in the methods, mice were presented with two trials consisting of two blocks each. In one of the trials, both blocks were both self-scented. In the other, one block was self-scented and one contained the scent of a non-familiar mouse. The non-familiar mouse was always age and sex matched to the test mouse. The order of the trials was randomized and counterbalanced across mice. A three-way ANOVA was used to analyze the effect of genotype, block combination, and trial on time spent exploring with the blocks. Results indicate only a significant main effect of block combination ($F(1,20) = 5.576$, $p = 0.0285$). Post-hoc analysis using Sidak's multiple comparison's test demonstrated no significant difference in either WT or DAT T356M$^{+/+}$ mice in time exploring either block in the self-self block pair or either block in the self-novel block pair (see Table 13 for a summary of results; n = 5 WT, 7 DAT T356M$^{+/+}$).

Von frey filament testing revealed not significant difference in mechanical nociception between WT and DAT T356M$^{+/+}$

The von Frey filament test was used to assess the tactile sensitivity of WT, DAT T356M$^{+/-}$, and DAT T356M$^{+/+}$ mice. Each mouse was tested three time with at least 2

minutes between each trial. The force exerted by the filament eliciting a withdrawal reflex was recorded for each trial and then averaged to generate the average force required to generate a withdrawal response (i.e. the withdrawal threshold; Figure 20). There were no statistically significant differences in withdrawal threshold between genotypes as measured by one-way ANOVA ($F(2,35) = 2.384$, $p = 0.107$; WT = 12.76 mN ± 3.729, DAT T356M$^{+/-}$ = 11.46 ± 1.747, DAT T356M$^{+/+}$ = 14.74 ± 3.020; n = 4 WT, 6 DAT T356M$^{+/-}$, 4 DAT T356M$^{+/+}$).

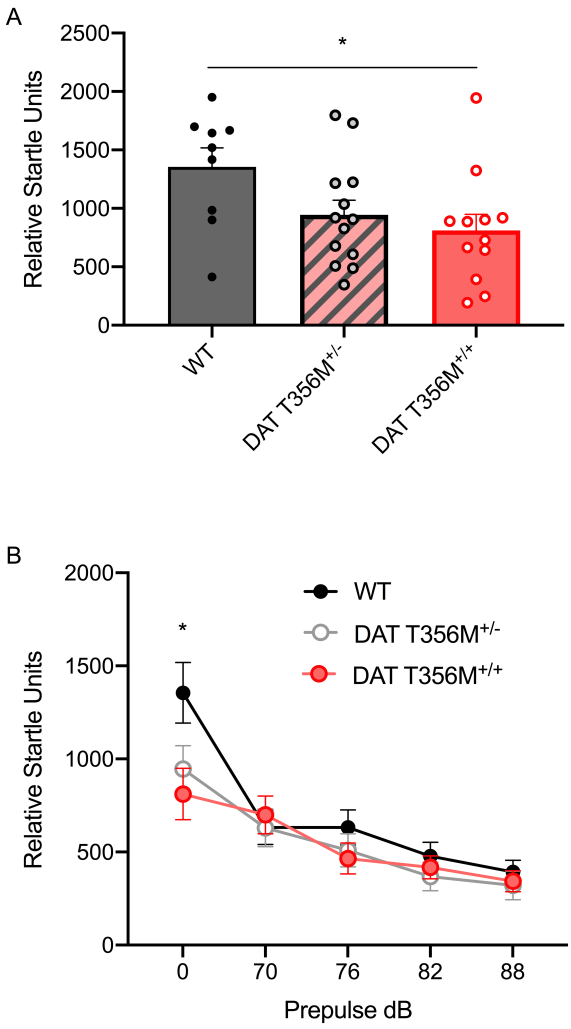

Figure 15. *Startle amplitude and PPI are reduced in DAT T356M$^{+/+}$ mice.*

(A) DAT T356M$^{+/+}$ mice have a significant reduction in the amplitude of their acoustic startle response as compared to WT mice (WT = 1356 ± 163.2 SRU, DAT T356M$^{+/-}$ = 945.3 ± 125.5 SRU, DAT T356M$^{+/+}$ = 811.7 ± 137.9 SRU, n = 9 WT, 13 DAT T356M$^{+/-}$, 12 DAT T356M$^{+/+}$; * $p < 0.05$ by one-way ANOVA followed by Tukey's multiple comparisons test). (B) Startle amplitude did not vary according to genotype when a prepulse stimulus was presented (70 – 88 dB).

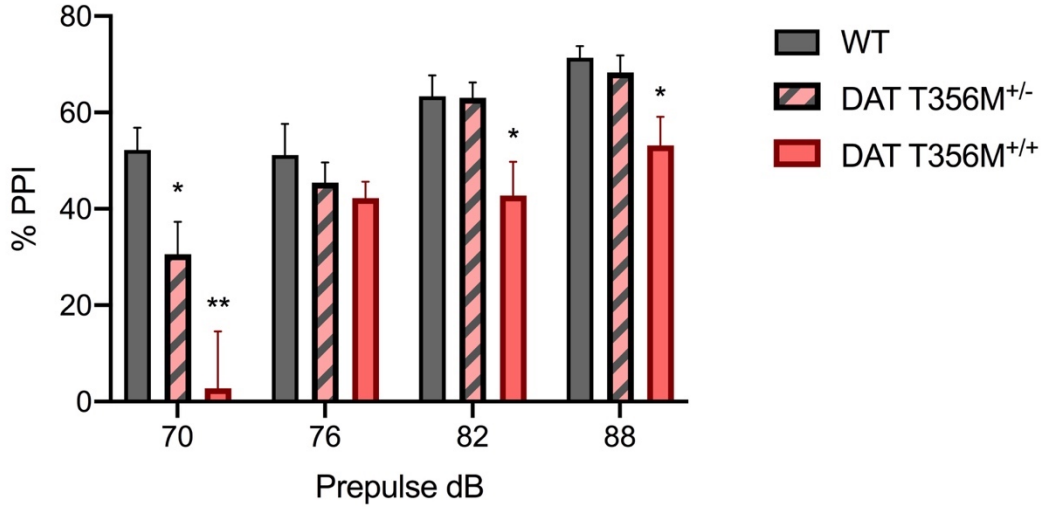

Figure 16. *Percent PPI is reduced in DAT T356M$^{+/+}$ mice.*

Percent PPI was used to determine the degree to which a pre-pulse stimulus inhibited the ASR. Both DAT T356M$^{+/-}$ and DAT T356M$^{+/+}$ mice show deficits in PPI at a prepulse stimulus intensity of 70 dB, while only DAT T356M$^{+/+}$ mice have significant deficits in PPI in at prepulse stimulus intensities of 82 and 88 dB (n = 9 WT, 13 DAT T356M$^{+/-}$, 12 DAT T356M$^{+/+}$; * $p < 0.05$, ** $p < 0.005$; see Table 9).

Table 9. Effect of prepulse intensity on percent PPI.

	Prepulse Intensity			
Genotype	**70 dB**	**76 dB**	**82 dB**	**88 dB**
WT	52.22 ± 4.588	51.16 ± 6.483	63.34 ± 4.372	71.37 ± 2.362
DAT T356M$^{+/-}$	30.60 ± 6.734 *	45.41 ± 4.251	63.04 ± 3.172	68.31 ± 3.542
DAT T356M$^{+/+}$	2.746 ± 11.8 **	42.23 ± 3.393	42.75 ± 6.983 *	53.17 ± 5.910 *

Values represent % PPI ± SEM.

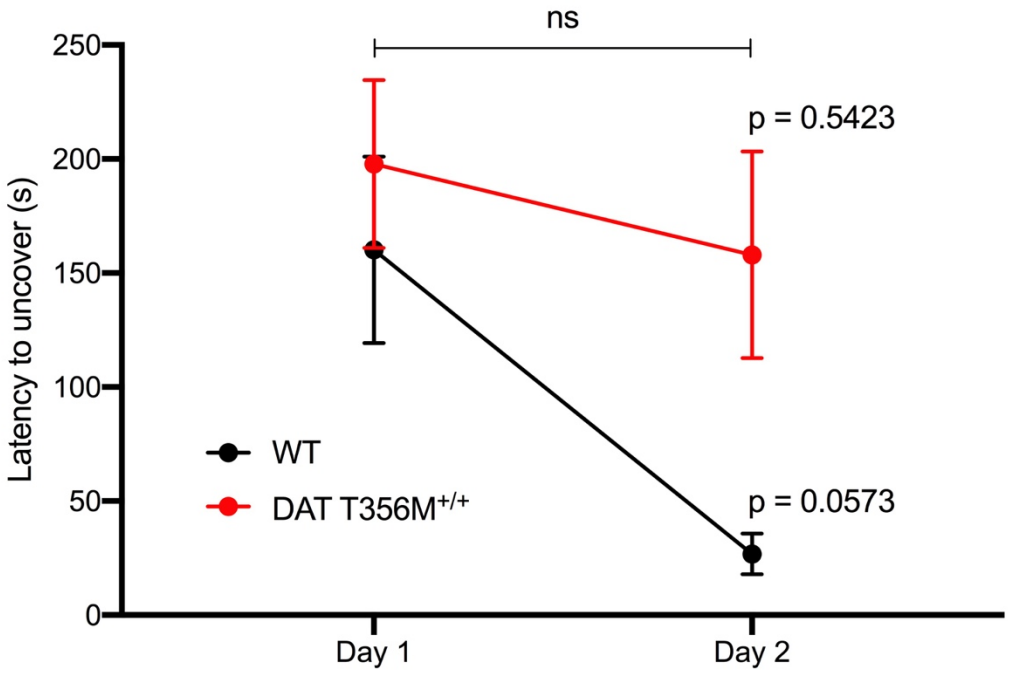

Figure 17. Latency to uncover buried food.

Latency to uncover a buried pellet was used to assess the ability of WT and DAT T356M$^{+/+}$ mice to detect a volatile odor and to use that cure for foraging. There was not a significant interaction of genotype and day ($F(1,12) = 1.807$, $p = 0.20237$), however there was a significant main effect of day on latency to uncover the pellet ($F(1,12) = 6.729$, $p = 0.0235$). There was no main effect of genotype on latency to uncover the buried food ($F(1,13) = 0.1272$, $p = 0.2.665$). Post-hoc testing using Sidak's multiple comparisons test revealed no significant difference when time to uncover on day 1 vs. day 2 for either WT or DAT T356M$^{+/+}$ mice (Figure 17; WT Day 1 = 160 s ± 40.89, Day 2 = 26.80 s ± 8.924, $p = 0.0573$; DAT T356M$^{+/+}$ Day 1 = 197.8 s ± 36.81, Day 2 = 158.0 s ± 45.30, $p = 0.5423$; n = 5 WT, 9 DAT T356M$^{+/+}$).

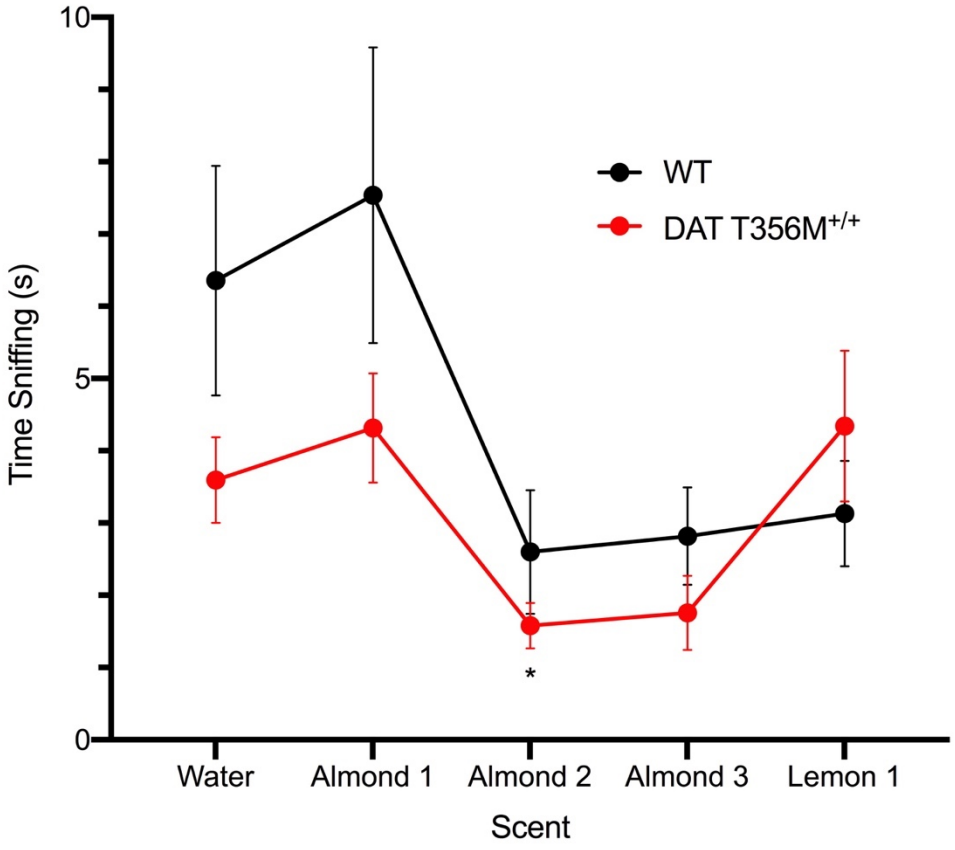

Figure 18. Time spent exploring each odor presentation.

Time spent sniffing each odor presented was analyzed by fitting a mixed model that uses a compound symmetry covariance matrix and is fit using Restricted Maximum Likelihood (REML) with Geisser-Greenhouse correction. Results of this model demonstrate a significant effect of scent presentation number ($F(3.161, 70.33) = 8.736$, $p < 0.0001$), but no significant effect of genotype ($F(1,25) = 1.945$, $p = 0.1754$) or interaction between genotype and scent ($F(4, 89) = 2.234$, $p = 0.0717$). Post-hoc testing using Sidak's multiple comparisons test revealed no significant differences in time spent exploring each olfactory cue between WT and DAT T356M$^{+/+}$ mice (See Table 10 for a summary of results). Post-hoc testing additionally revealed no significant differences in the time spent exploring

water vs. the first presentation of almond scent, the first presentation of almond scent vs. the second, the second presentation of almond scent vs. the third, or the third presentation of almond scent vs. the first presentation of lemon for WT mice (Table 11) This analysis found only a significant difference between time spent sniffing the first presentation of the almond scent and the second, but not for any other scent trial pairs analyzed for DAT T356M$^{+/+}$ mice (see Table 12).

Table 10. *Results of analysis of difference between WT and DAT T356M$^{+/+}$ for time exploring each scent.*

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
WT - DAT T356M$^{+/+}$			
Water	2.760	-2.441 to 7.961	0.5037
Almond 1	3.221	-3.469 to 9.910	0.5977
Almond 2	1.020	-1.777 to 3.818	0.8141
Almond 3	1.063	-1.359 to 3.484	0.7195
Lemon 1	-1.210	-4.800 to 2.380	0.8860

Test Details	Mean 1	Mean 2	Mean Diff.	SE of Diff.	N1	N2	t	DF
WT - DAT T356M$^{+/+}$								
Water	6.355	3.595	2.760	1.697	10	16	1.627	11.55
Almond 1	7.535	4.314	3.221	2.180	10	16	1.477	11.49
Almond 2	2.599	1.579	1.020	0.9113	10	15	1.120	11.45
Almond 3	2.819	1.756	1.063	0.8450	10	14	1.257	18.19
Lemon 1	3.130	4.340	-1.210	1.272	8	15	0.9513	21.00

Table 11. *Results of analysis for WT mice of difference in time spent exploring each scent vs. the preceding scent.*

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Water vs. Almond 1	-1.180	-6.220 to 3.860	0.9305
Almond 1 vs. Almond 2	4.936	-0.4793 to 10.35	0.0772
Almond 2 vs. Almond 3	-0.2200	-3.359 to 2.919	0.9992
Almond 3 vs. Lemon 1	-0.3110	-2.466 to 1.844	0.9843

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Water vs. Almond 1	6.355	7.535	-1.180	1.626	10	10	0.7255	9
Almond 1 vs. Almond 2	7.535	2.599	4.936	1.747	10	10	2.825	9
Almond 2 vs. Almond 3	2.599	2.819	-0.2200	1.013	10	10	0.2172	9
Almond 3 vs. Lemon 1	2.819	3.130	-0.3110	0.6488	10	8	0.4794	7

Table 12. *Results of analysis for DAT T356M$^{+/+}$ mice of difference in time spent exploring each scent vs. the preceding scent.*

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Water vs. Almond 1	-0.7194	-2.848 to 1.409	0.8226
Almond 1 vs. Almond 2	2.736	0.6673 to 4.804	0.0085*
Almond 2 vs. Almond 3	-0.1778	-1.221 to 0.8652	0.9797
Almond 3 vs. Lemon 1	-2.584	-5.572 to 0.4048	0.1019

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Water vs. Almond 1	3.595	4.314	-0.7194	0.7457	16	16	0.9647	14
Almond 1 vs. Almond 2	4.314	1.579	2.736	0.7167	16	15	3.817	13
Almond 2 vs. Almond 3	1.579	1.756	-0.1778	0.3511	15	14	0.5062	11
Almond 3 vs. Lemon 1	1.756	4.340	-2.584	1.022	14	15	2.528	12

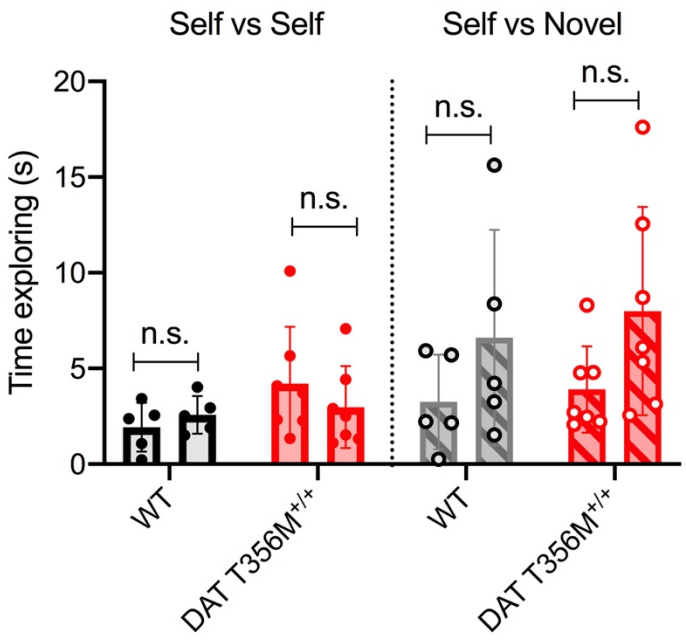

Figure 19. *Social odor discrimination test.*

A social odor task was used to determine the ability of WT and DAT T356M$^{+/+}$ to detect and discriminate the scent on a novel mouse from their own scent. Results were analyzed using a three-way ANOVA. Results indicate only a significant main effect of block combination (i.e. whether the pair was self-self or self-novel; $F(1,20) = 5.576$, $p = 0.0285$). Post-hoc analysis using Sidak's multiple comparison's test demonstrated no significant difference in either WT or DAT T356M$^{+/+}$ mice in time exploring either block in the self-self block pair or either block in the self-novel block pair (see Table 13 for a summary of results; n = 5 WT, 7 DAT T356M$^{+/+}$).

Table 13. *Summary of the results of the social odor discrimination task.*

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Adjusted P Value
Self Block 1:WT vs. Self Block 2:WT	-0.6520	-6.185 to 4.881	0.9967
Self Block 1:DAT T356M$^{+/+}$ vs. Self Block 2:DAT T356M$^{+/+}$	1.234	-3.442 to 5.910	0.9351
Self Block: WT vs. Novel Block:WT	-3.350	-8.883 to 2.183	0.4063
Self Block:DAT T356M$^{+/+}$ vs. Novel Block:DAT T356M$^{+/+}$	-4.093	-8.769 to 0.5833	0.1068

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Self Block 1:WT vs. Self Block 2:WT	1.928	2.580	-0.6520	2.121	5	5	0.3073	40.00
Self Block 1:DAT T356M$^{+/+}$ vs. Self Block 2:DAT T356M$^{+/+}$	4.216	2.981	1.234	1.793	7	7	0.6884	40.00
Self Block: WT vs. Novel Block:WT	3.250	6.600	-3.350	2.121	5	5	1.579	40.00
Self Block:DAT T356M$^{+/+}$ vs. Novel Block:DAT T356M$^{+/+}$	3.904	7.997	-4.093	1.793	7	7	2.283	40.00

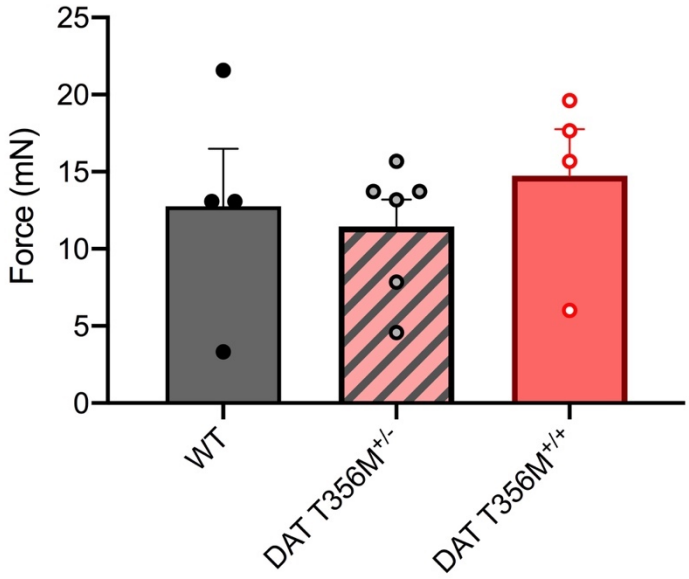

Figure 20. *Withdrawal threshold does not vary according to genotype.*

The von Frey filament test was used to determine the force required to elicit a withdrawal response. Results shown here at the average of three trials for each mouse tested. There were no statistically significant differences in withdrawal threshold between genotypes as measured by one-way ANOVA ($F(2,35) = 2.384$, $p = 0.107$; WT = 12.76 mN ± 3.729, DAT T356M$^{+/-}$ = 11.46 ± 1.747, DAT T356M$^{+/+}$ = 14.74 ± 3.020; n = 4 WT, 6 DAT T356M$^{+/-}$, 4 DAT T356M$^{+/+}$).

Discussion

This work demonstrates that DAT T356M$^{+/+}$ mice have reduced ASR amplitude in response to a 120 dB stimulus and may also have reductions in PPI at multiple prepulse stimulus intensities as compared to WT mice when controlling for ASR amplitude. However, based on the observed reduction in startle amplitude, it is not possible to definitively state that PPI is reduced given the current evidence. Previous work reveals mixed results regarding the amplitude of the ASR in individuals with ASD. Some studies report increased amplitude of the ASR, indicating a hypersensitivity to these auditory cues (and possibly to other sensory modalities)[349]. Here we show the opposite: that DAT T356M$^{+/+}$ mice have reduced startle amplitude in response to an auditory cue. This finding could result from a number of factors. First, it is possible the DAT T356M$^{+/+}$ have hearing loss not seen in their WT littermates. This is unlikely given that a startle response, though attenuated, is observed in response to the 120 dB acoustic stimulus. Moreover, the ASR is attenuated by stimuli lower in intensity than 120 dB. It is also possible, given the known role of DA in motor behaviors, that some defect exists in the DAT T356M$^{+/+}$ mice which attenuates the motor component of their ASR. Again, this is unlikely as our previous research suggests the DAT T356M$^{+/+}$ have no deficits in strength or coordination. These potentially confounding factors complicate our interpretation of the reduced ASR in the DAT T356M$^{+/+}$ mice, but suggests either a hyposensitivity to reflex-inducing acoustic stimuli or a deficit in the initiation of the motor component of the ASR. To investigate these potential confounds, future studies should aim to determine whether the force exerted by the DAT T356M$^{+/+}$ mice is indeed reduced or if hearing loss is present in these animals.

What is perhaps of more value is considering the degree to which a weak pre-stimulus can inhibit the ASR (i.e. PPI) in these animals. We found that DAT T356M$^{+/+}$ mice had significant reductions in PPI as compared to WT controls. That is, increasing intensity of the prepulse stimulus failed to attenuate the ASR to the same degree in DAT T356M$^{+/+}$ mice as it did in WT mice. If the DAT T356M$^{+/+}$ mice had an impairment in their motor output, the prepulse should still attenuate the ASR to the same degree in these animals if there was no effect of genotype. This is because this measure relies on the magnitude of the startle on prepulse trials relative to the magnitude on pulse trials. If the magnitude of the startle is low on pulse trials due to a motor defect, the magnitude of the startle should be similarly low on prepulse trials, but would be proportionally inhibited. However, if there is a difference in the degree to which a prepulse inhibits the startle response, this indicates a failure of the weak prestimulus to activate central inhibitory processes and thus a failure of sensorimotor gating. Indeed, this is the pattern observed in the DAT T356M$^{+/+}$ mice. The magnitude of their startle, though lower on average than the WT for basal startle trials, does not attenuate to the same degree as the startle reflex of the WT mice or mice heterozygous for the T356M variant. This suggests a failure of central gating of the ASR.

To further address the possibility that basal startle amplitude had an effect on percent PPI, we performed statistical analyses to determine whether percent PPI varied according to genotype when controlling for basal startle amplitude. We found no evidence that basal startle amplitude covaries with percent PPI and that significant differences in PPI exist for DAT T356M$^{+/+}$ mice compared to WT mice when controlling for basal startle

amplitude. Taken together, these results suggest that percent PPI is also decreased in DAT T356M$^{+/+}$ mice.

Reduced startle amplitude has been reported in several mouse models with genetic manipulations of the central nervous system[358–360]. These models provide important clues and insights to guide our exploration of the possible causes for reduced startle amplitude in the DAT T356M$^{+/+}$ mouse. One such model is a mouse with a loss-of-function mutation in the PARK2 gene, which encodes the protein parkin. Loss-of-function of this protein results in neuronal degeneration that manifests as autosomal recessive juvenile parkinsonism (ARJP)[361]. Parkin null mice have significantly fewer dopaminergic cells in the locus coeruleus (LC), but do not have differences in levels of DA or its major metabolites. These animals do have reduced concentrations of NE in the olfactory bulb and the spinal cord (two major targets of axons projecting from the LC). This study confirms previous findings that demonstrate that loss of LC neurons leads to impaired startle[362]. It is important to note that there is much communication between the LC and dopaminergic midbrain neurons and that the activity of neurons in the LC can influence the activity of midbrain DA neurons. It is therefore conceivable that the DAT T356M variant impairs this communication by altering the ability of target dopaminergic neurons to appropriately respond to incoming signals from the LC. However, there are many other animal models with reduced startle responses, including 5-HT$_{1B}$ receptor knockouts[360], mice overexpressing corticotropin-release hormone[358], and mice lacking specific isoforms of creatine kinase[363]. The exact mechanism by which the ASR is impaired in these models is unclear, but does point toward the potential of multiple possible etiologies this impairment.

Much effort has been directed toward understanding the neural circuitry that mediates and regulates PPI. The brain regions implicated in the regulation of PPI include hippocampus, PFC, basolateral amygdala, NAc, striatum, VTA, ventral pallidum, globus pallidus, SN reticulata, thalamus, pedunculopontine nucleus, superior colliculus, and inferior colliculus. While a review of the roles of each of these regions is beyond the scope of this discussion, it is clear that PPI is mediated by a number of brain regions acting in concert. Therefore, experimental manipulations of PPI (either pharmacologic or as the result of genetic manipulations) can be used to determine the nature of the interactions of these regions and their roles in sensorimotor gating.

Here we show that one such manipulation, the DAT T356M variant, results in reduced PPI. These results, when considered in combination with our previous studies in the DAT T356M$^{+/+}$ mouse, suggest that leak of DA into the extracellular space via the mutant transporter result in deficits in PPI. These findings support previous work demonstrating mesolimbic DA overactivity as a means by which PPI can be disrupted[364] and provides evidence that genetic variants associated with ASD can promote changes in sensory-based behavioral tasks and sensorimotor gating. The interpretations of our findings are somewhat limited as this variant is expressed in all dopaminergic neurons and at all dopaminergic synapses in animals carrying this gene. Therefore it is not possible to say, without further genetic or pharmacologic manipulations, in what region or in epoch DA leak is required to produce the observed deficits in PPI. Future work should endeavor to expand upon and explore the mechanisms by which this variant disrupts the neurobiology of sensorimotor gating and to determine the impact of this variant on

sensorimotor gating as mediated by non-auditory sensory systems (i.e. tactile-based sensorimotor gating).

We cannot conclude from the work presented here that DAT T356M$^{+/+}$ mice have differences from WT mice in olfactory sensitivity or olfactory discrimination. The buried food test revealed no difference in time to uncover a hidden food source between WT and DAT T356M$^{+/+}$ mice. This result comes with important considerations. First, our sample size was limited (n = 5 WT, 9 KI). Typically, behavioral tests require, at the very minimum, 10 subjects per group before a given result can be fully interpreted. Therefore, we must increase the sample size to confidently ascertain the effect of genotype on ability to detect a volatile odor. Second, DAT T356M$^{+/+}$ mice are known to have changes in digging-related behavior. Specifically, DAT T356M$^{+/+}$ mice bury significantly fewer marbles than WT mice and dig less frequently when exposed to a novel environment (although not significantly less; Chapter III). This could indicate a general difference in willingness to engage in digging-related behaviors. We therefore propose a two-stage test to control for general digging behavior. Mice should undergo baseline observation as described in Chapter III, specifically for incidence of digging. The same mice can then be tested following the protocol here for the buried food test. An ANCOVA can then be performed to determine whether digging covaries with latency to uncover a hidden food source.

Our tests of olfactory discrimination, the novel odor test and the social odor discrimination tests, did not reveal any statistically significant differences in ability to detect a novel odor (social or otherwise) between WT and DAT T356M$^{+/+}$ mice. However, the pattern of results of this test limit the interpretability of the results. Neither WT nor DAT T356M$^{+/+}$ mice explored the novel almond scent more than the scent of water.

Further, only DAT T356M$^{+/+}$ mice demonstrated evidence of habituation to the novel odor. This is concerning as WT mice should habituate to the repeated presentation of the same odor cue[365]. While this could be due to the small sample size, particularly the small number of WT mice (n = 11 WT, 17 DAT T356M$^{+/+}$), there are a number of factors that could contribute to the pattern of results observed. The first is that, because the mice had free access to the scented cotton applicator, the exploration observed may be related to overall interest in this novel object and not a pure olfactory-driven exploratory drive. This is supported by the observation that mice did not explore the novel almond scent more than the scent of water. Alternative protocols to the one used here recommend using an olfactory source that the mice cannot access (e.g. a scented cotton ball inside a tissue cassette) to control for this potential confound. It is also possible that the scent of the almond extract or the lemon extract was innately aversive to the mice. This is somewhat unlikely, as previous studies have used the scents used here at the same dilutions used here. The failure of WT mice to dishabituation when presented with the second novel odor indicates these animals may be fatiguing after multiple trials. An alternative protocol wherein only two repetitions of the first novel odor are presented (as the maximum habituation occurred here for the DAT T356M$^{+/+}$ mice) before presenting the second odor may represent a solution to the issue of fatigue.

Our third test of odor discrimination was the block test for social odor discrimination. We found no differences between genotype for time spent exploring self scent versus the scent of an unfamiliar mouse. However, we also found no significant preference for the novel social odor over self-scent in either WT or DAT T356M$^{+/+}$. This result is concerning as WT mice typically demonstrate a preference for novel social

odors[366]. Again, these experiments were limited by a small sample size (n = 5 WT, 7 DAT T356M$^{+/+}$), but serve as preliminary evidence that there may be no difference in ability to detect novel social odors and to explore those odors.

Together these results are suggestive of no overt impairment in olfactory ability in the DAT T356M$^{+/+}$, although further studies are warranted to determine the replicability of these experiments as many are limited by their small sample sizes. The possibility exists that no difference in odor detection or discrimination exists between DAT T356M$^{+/+}$ and WT mice. Such a result, while unexpected, would permit exploration of behaviors that require intact olfaction, such as associative olfactory learning. Such studies would allow us to dissect the contribution of DA dysfunction on olfactory learning or odor-induced motivated behaviors[367]. For example, WT mice and DAT T356M+/+ mice might be equally able to detect an odor, but might differ in their ability to learn an association between that odor and a rewarding stimulus (e.g. a certain odor paired with a food reward) or a negative reinforcer (e.g. foot shock or loud tone). A study by Murata and colleagues supports this hypothesis. In this study, the authors found that D1(+) and D2(+) neurons in the olfactory tubercle were activated by cues eliciting motivated behavior (i.e. odor paired with sugar reward) and in response to cues inducing aversive behaviors. These results suggest that DA neuron activation in the olfactory tubercle may be serve to mediate learning of odor-induced motivated behaviors rather than representing the odorants themselves[368].

Finally, we explored tactile sensitivity in DAT T356M$^{+/+}$ mice here. We found no evidence to suggest differences in tactile sensitivity in DAT T356M$^{+/+}$ mice as compared to WT or DAT T356M$^{+/-}$ mice. Again, interpretations of this result are limited by a small sample size. Future studies should seek to expand the number of animals tested to test

the replicability of this finding. While gross tactile sensitivity may remain intact in DAT T356M$^{+/+}$ mice, this does not rule out the possibility of deficits in other somatosensory domains, including thermal nociception, tactile sensitivity (e.g. to whisker stimulation), or tactile discrimination. Studies relate to differences in these areas (all of which are reported to be altered in ASD[355,369,370]) will help elucidate if the DAT T356M variant impacts somatosensation and therefore guide future studies related to the neural mechanisms behind any changes observed.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

The study of DA and the proteins that regulate this neurotransmitter system (i.e. the DAT) are of critical importance to elucidating the role of DA dysfunction in health and disease. DAT variants, dysregulation of DA, and altered developmental trajectory of dopaminergic structures have been associated with ASD and related neuropsychiatric disorders, such as ADHD[181,184,240–242,295,371]. By studying discrete variations of the DAT, we can not only further understand the impact of these variants on transporter function, but also determine how transporter dysfunction contributes to neurophysiological and behavioral alterations.

Here we present the first murine model of an ASD-associated de novo mutation to the DAT, the DAT T356M mutation. The DAT T356M model allows us to explore both the physiological and phenotypic consequences of this mutation, particularly as it pertains to DA homeostasis and behaviors known to be DA-dependent. Our work demonstrates that the T356M variant imparts impaired transporter function, leading to elevations in extracellular DA levels (Chapter III). We provide evidence that this extracellular elevation of DA leads to increased DA catabolism and ultimately reductions in total tissue content of DA, likely through D2-receptor desensitization and resulting downregulation of the synthetic pathway of DA (Chapter III). We show that DAT T356M$^{+/+}$ mice exhibit patterns of behavior typically associated with the ASD phenotype (altered social interactions and repetitive behaviors) as well as behaviors associated with ADHD (i.e. hyperactivity), a

common comorbidity of ASD (Chapter III). We also present evidence that mice homozygous for the T356M variant have reduced acoustic startle responses and deficits in sensorimotor gating (Chapter IV), directly replicating physiologic findings in individuals with ASD[349,372]. Our investigation thus links altered dopaminergic signaling due to DAT dysfunction caused by a genetic variant associated with ASD with behavioral alterations that reflect aspects of the human disorder in a murine model.

This work also provides evidence for a potential therapeutic target for at least a subset of individuals with ASD. We show that DAT blockade (with two different compounds: ACT-01 and GBR12909) results in reduction of locomotor activity in mice homozygous for the T356M variant. We hypothesize this effect is the result of a reduction of the ongoing anomalous DA efflux through the faulty transporter. We also demonstrate that hyperactivity mediates the effect of genotype on repetitive rearing. Therefore, it is possible that interventions targeting hyperactivity, such as DAT blockade, may also improve or reduce repetitive behaviors in this mouse model. While this hypothesis remains to be tested, such a finding would have important clinical implications as there is limited evidence to suggest the efficacy of currently available therapeutics and behavior-based interventions for reducing restricted and repetitive behaviors. Clinical trials in individuals with ASD found that stimulant therapy (specifically, methylphenidate) improved hyperactivity and possibly inattention, but have no impact on sterotypies[373,374]. It is possible that better sub-grouping of individuals with ASD will result in better response to such mediciations. Through this study we show how leveraging our current clinical ability to sequence the genome, detect a variant, and understand the impact of that variant may translate into viable, targeted therapeutic options.

Much remains to be understood in regards to the complex relationship between DA and ASD. Still, we feel this work expands our current neuroscientific knowledge in a number of ways. We have extended our understanding of the roles of DA in behavior, the intricacies of DAT function and its intracellular interactions, and one possible mechanistic underpinning for the behaviors associated with ASD. We know now, from this work in combination with that of others, that the DAT plays an integral role in regulating many complex behaviors and that DA dyshomeostasis can lead to behavioral symptoms we associate with ASD. A better understanding of the roles of DA in behavior and the complex neurophysiology of the DAT will further our ability to understand the role of DA dysfunction in neuropsychiatric disorders and to develop targeted therapies for these conditions.

This work leaves much to be explored. While we have demonstrated that the DAT T356M variant, when expressed globally and for the duration of an animal's lifespan, leads to multiple dopaminergic abnormalities in the striatum and to behavioral changes, we cannot say with certainty that these behavioral changes stem directly from the observed striatal changes in DA. The DAT is widely expressed not only in the brain, but also elsewhere in the body. DA is produced and release by various peripheral tissues including the pancreas[192], the adrenal medulla[193], the kidney[194], and peripheral leukocytes[195]. Indeed, DA is co-released with insulin from the beta cells of the pancreas and acts by feedback inhibition to regulate the secretion of insulin[375], while insulin acts in the brain to enhance striatal DA release[376]. Given the intricately intertwined roles of DA in the periphery and in the CNS, future research should seek to dissect the impact of DAT dysfunction in the brain vs. in the periphery. Such research might entail the generation of

a mouse that selectively expresses variant DAT only in targeted tissues (e.g. the brain only or the pancreas only), which maintaining the wildtype DAT in all other tissues. Such studies would allow us to determine the impact DAT dysfunction in a given organ system has on physiology, neurobiology, and behavior in isolation from confounding effects in other organ systems.

DAT is found on the presynaptic membrane of all dopaminergic nerve terminals, which form synapses in the cerebral cortex, the limbic system, the hypothalamus, and in the striatum. Given the complex roles of each of these brain regions in behavior and the complex interplay of these brain regions (with one another and with other subcortical structures), it is unclear if DAT dysfunction in the striatum alone is sufficient or necessary to drive the observed behavioral changes. I hypothesize that central DAT dysfunction, and specifically DAT dysfunction in the striatum, is indeed sufficient to drive these behavioral changes. To test this hypothesis, one could generate a mouse that selectively expresses the mutant form of the DAT only in nigrostriatal neurons. This would result in DAT dysfunction almost exclusively at striatal synapses. These mice could then undergo the same battery of behavioral, biochemical, and neurophysiological assays presented here to determine which, if any, differ between mice expressing mutant transporter throughout the body and mice expressing mutant transporter only in the striatum.

Similarly, if one were interested in determining the physiologic impact of the DAT T356M variant on a peripheral body function, such as insulin release, one could generate a mouse that exclusively expresses mutant transporter in the beta cells of the pancreas. I hypothesize that, given the known regulatory role of DA in the pancreas and of insulin in the brain and our findings of altered body weight in the DAT T356M$^{+/+}$ mice, such a

mouse would exhibit changes not only in peripheral insulin regulation, but also in brain DA, likely cascading into a number of behavioral changes. Likely these behavioral changes would not be as marked as those observed in mice expressing the mutant form of the transport throughout the body or would differ in their scope. However, the exact interplay between peripheral DA regulation and central processes remains unclear. Such a study would provide important mechanistic clues as to the role of peripheral dysregulation of both DA and insulin on the brain and behavior.

Another important consideration is the developmental effects of the variant transporter. ASD is, by definition, a neurodevelopmental disorder. Yet it remains unclear at what point during development genetic variants and environmental influences must act in order to drive the development of this disorder. Determining at what point during development a variant must arise to be pathologic is critical for expanding our understanding of the neurobiology surrounding this disorder. For example, if a given genetic variant must arise early in development to have pathologic effects in the brain, it is possible that variant is in a protein that has a critical role in brain development (for example, in the patterning of the nervous system), but may play a minor role once these foundational processes are complete. Alternatively, if a genetic variant can arise later in life and lead to disease, the gene impacted by this variant is almost certainly involved in maintaining ongoing processes rather than in foundational processes. To complicate matters, we know that some proteins are expressed only transiently during development in various regions of the brain. For example, the 5-HT transporter, while expressed throughout the brain in adulthood, is transiently expressed on thalamocortical neurons during development and serves to regulate cortical patterning[377,378]. A failure to express

the 5-HT transporter, a failure to downregulate the expression of the transporter after cortical patterning is complete, or a dysfunction of the transporter could all lead to disrupted thalamocortical projections. To begin to tackle the question of the role of the DAT T356M$^{+/+}$ in development, one could develop a mouse that expresses the variant form of the transporter only in response to externally provided stimuli (i.e. via an inducible gene expression system)[379,380], thus providing temporal control of the expression of the variant transporter. For example, one could test the impact of expression of the mutant transporter during early prenatal development vs. expression early after birth (e.g. postnatal day 1) vs. expression in adolescence vs. expression only in adulthood. I hypothesize that prenatal expression or early postnatal would be required to drive all the behavioral characteristics seen in the DAT T356M$^{+/+}$ mouse, while expression only during adulthood would have similar effects as chronic administration of a DAT antagonist.

One limitation of our study has been the exclusive study of the impact of this mutation on the neurobiology of the striatum. Critical to our understanding of how this variant (and others like it) impacts behavior is a more thorough understanding of how this mutant disrupts other brain structures, especially those implicated in ASD. At the most basic level, this would entail exploration of the impact of this variant on DA tissue concentrations in brain regions beyond the striatum (i.e. cerebral cortex, hippocampus, and cerebellum) and on DA release and reuptake in these regions. This is important as the biology of the DA system is known to differ according to brain region. For example, in the cerebral cortex, the norepinephrine transporter is the primary transporter of the DA[381]. It is conceivable, therefore, that a dysfunctional DAT would have little impact on DA release and clearance from the synapse in the cortex, where its expression is low.

This is not to say the DAT T356M variant would have no impact on ongoing cortical processes. For example, the striatum is known to receive input from sensory cortex and to project to thalamus, which in turn projects to sensory cortex. As such, DA dysregulation in the striatum due to the DAT T356M variant would likely disrupt the flow of information from thalamus to cortex or distorts its meaning. Given the critical importance of the sensory systems in shaping our interactions with the world, any change in sensory processing or perception is likely to cascade into higher-order deficits such as those seen in ASD. Indeed, much evidence exists suggesting a disruption in multisensory processing and sensory perception in individuals with ASD[70,72,160]. Yet we have limited understanding of the how the many genetic variants associated with ASD might impact sensory function in the brain and, as a result, behavior. New advances in electroencephalography (EEG), anatomical techniques, and optogenetics provide myriad options for probing the neurobiological impact of these variants on brain structure and function in animal models such as the DAT T356M mouse. Techniques that allow us to record and manipulate ongoing neural activity, such as optogenetics, can be successfully paired with behavioral tasks to dissect the impact of genetic variants on not only brain function in specific regions, but how these changes in brain function might translate into alterations in behavior. Recording ongoing sensory cortical activity simultaneously with the activity of dopaminergic neurons synapsing on corticostriatal neurons during a sensory-based task would provide great insight as to how altered DA signaling due to the T356M variant impacts ongoing processing of sensory information.

At the most fundamental level, future work should aim to further characterize sensory-based behaviors in DAT T356M$^{+/+}$ mice to determine whether this variant has an

impact on any sensory-based behaviors (i.e. olfactory discrimination, tactile sensitivity, tactile discrimination, etc.). While the findings presented here are suggestive of no olfactory differences or somatosensory differences, they are limited by their small sample size and potential confounds that exist (such as motivation). Understanding how such basic sensory behaviors are impacted by the DAT T356M variant will ultimately permit more complex analyses of the neurobiological mechanisms (central or peripheral) that may produce changes in these behaviors.

The results of our work characterizing the impact of the DAT T356M variant provides important and immediate considerations for future studies. We demonstrated that male mice homozygous for this mutation gain weight less rapidly and achieve lower adult weights than their WT littermates (Chapter III, Figure 8). This finding presents a number of new and intriguing questions and hypotheses. First, there is the question as to whether the observed change in body mass is due to an alteration in body composition (i.e. fat vs. lean body mass) and whether such a change in body composition can be accounted for by the increased locomotor activity observed in DAT T356M$^{+/+}$ mice. The next question arising from such a line of inquiry is, if there are changes in body composition that are not accounted for solely by increased activity, could this variant have an impact on the regulation of blood glucose and thus metabolism? As we know that the T356M variant DAT constitutively leaks DA through the transporter leading to central D2 receptor desensitization, I hypothesize that such transporter dysfunction in the pancreas of the DAT T356M$^{+/+}$ mice will result in D2 autoreceptor desensitization in the beta cells. A loss of this important negative feedback mechanism could lead to increased insulin release in response to a glucose challenge and may be protective against hyperglycemia.

Alternatively, constitutively increased insulin release could lead to peripheral insulin resistance[382] and ultimately poorer handling of blood glucose.

When considering variants in genes encoding proteins responsible for regulating major neurotransmitter systems (such as the DAT) and the possible role of such variants on metabolism, we must also consider the roles of such neurotransmitters in the gut-brain axis. The same neurotransmitters found in the central nervous system are found in the enteric nervous system and the cross-talk between these systems is becoming increasingly important to our understanding of brain and body physiology. Evidence suggests that gut-derived hormones and neurotransmitters can affect central neurotransmitter systems and, ultimately, behavior[383–385]. This has important implications for the work presented here. It is possible, and likely, that the DAT T356M variant drives changes in DA handling in the ENS, which may compound that centrally-mediated changes in DA homeostasis resulting from this variant.

Finally, at the broadest level, this work highlights the importance of better and more comprehensive behavioral and neurobiological testing of animal models of genetic variants associated with ASD and other neuropsychiatric disorders. These animal models are only as useful as the rigor and thoroughness of our studies. If we are to understand how a given variant might have overlapping effects with another, we must understand all the ways in which one variant impacts the function of the brain and the behaviors that result from this function. Too often our studies focus intensely on a narrow subset of neurobiological mechanisms or aspects of a behavioral profile and neglect to consider possible “off-target” effectives of the variants modeled. We must seek to define the same mechanisms (neurobiological and behavioral) in each model if we are to discover the

differences and commonalities driven by these variants. Such an approach will permit more comprehensive approaches to determining the subtypes that exist within conditions as heterogeneous as ASD and potential therapeutic targets that may exist for these individuals.

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