Neurobehavioral Impact of Disease-Associated Variation in the Dopamine Transporter

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This thesis is dedicated to the hardest working man I know. One that provides endless support and love even when he is exhausted. He inspires me constantly and has always been my greatest role model. This thesis is dedicated to my Father.

Dr. Thomas Davis

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TABLE OF CONTENTS

Page

DEDICATION ii

ACKNOWLEDGMENTS iii

LIST OF TABLES vi

LIST OF FIGURES vii

LIST OF ABBREVIATIONS ix

Chapter

1 PAY ATTENTION: IT'S AN ADHD OVERVIEW

1.1 ADHD Overview 1
1.2 Dopamine Overview 5
1.3 DA-Associated ADHD Genetics 8
1.4 ADHD Models 9
1.5 DAT Val559 Mouse Model 20

2 IMPULSIVITY, ADHD, AND DOPAMINE

2.1 Introduction 23
2.2 Impulsive Action 25
2.3 Impulsive Choice 28
2.4 Cognitive Inflexibility/Compulsion 29
2.5 Neural Substrates of Impulsivity and Dopamine Associations 30
2.6 Specific Aims 34

3 EVALUATION OF COGNITIVE AND IMPULSIVITY TRAITS IN DAT Val559 MICE USING THE 5-CHOICE SERIAL REACTION TIME TASK

3.1 Introduction 35
3.2 Methods 36
3.3 Results 41
3.4 Discussion 48

4 ASSESSING IMPULSIVITY DOMAINS AND CONTRIBUTIONS FROM INTERVAL TIMING AND MOTIVATION IN THE DAT Val559 MOUSE

4.1 Introduction 50
4.2 Methods 51
4.3 Results 56

4.4 Discussion....65

5 PERSISTENT CHECKING BEHAVIOR FORMATION IN DEVALUED GOAL-DIRECTED AND HABITUAL SCHEDULES IN THE DAT Val559 MOUSE

5.1 Introduction....70
5.2 Methods....73
5.3 Results....77
5.4 Discussion....81

6 ALTERATIONS IN AMPHETAMINE-INDUCED ERK1/2 PHOSPHORYLATION IN DAT Val559 MOUSE

6.1 Introduction....86
6.2 Methods....88
6.3 Results....90
6.4 Discussion....95

7 CONCLUSIONS AND FUTURE DIRECTIONS....99

Appendix

1. Aging Effects on Passage of DAT Val559 Risk Allele....104

2. Open Field Experiments....114

3. Motivation and Early Life Stress....119

4. Perseverative Checking Behavior Rescue Experiments....122

5. Alternative Normalization of ERK1/2 Data....127

REFERENCES....130

LIST OF TABLES

Table **Page**
1. Ensure Consumption Comparisons 126

LIST OF FIGURES

Figure **Page**

1. Symptom Domains of ADHD 2

2. Dopamine Pathways of the Brain 6

3. 5-CSRTT Training Schedule and Paradigm…….....38

4. 5-CSRTT Acquisition…….....42

5. 5-CSRTT Baseline Performance…….. 44

6. Attentional Measures in 5-CSRTT…….. 45

7. Impulsivity Measures in 5-CSRTT…….. 47

8. Go/NoGo Paradigm Schematic……. 54

9. Interval Timing in DAT Val559 Mice…….....57

10. Go/NoGo Acquisition…….....59

11. Go/NoGo Behavioral Measures…… 60

12. Motivational Measures with Progressive Ratio…….....62

13. Sucrose Preference Test…….. 64

14. DAT Val559 Imaging Data……. 72

15. Within Lever Pressing Paradigm Schedule and Schematic…….....76

16. Assessing Goal-Directed vs Habitual Behavior…….. 78

17. Devalued Progressive Ratio…… 80

18. Phosphorylated ERK1/2 in the Inactive Phase 91

19. Phosphorylated ERK1/2 in the Active Phase 93

20. Allelic Distribution of Pups from Young vs Old Sires.....107

21. Assessment of Sperm in Young vs Old Sires 110

22. 3 mg/kg Methylphenidate Open Field Locomotor Data……. 115

23. Amphetamine Open Field Locomotor Data……. 116

24. D2 Antagonist Rescue of Amphetamine Locomotor Data……. 117

25. Effects of Early Life Stress on Motivation……..... 120

26. Amphetamine Rescue of Perseverative Checking Behavior……... 124

27. Inactive Phase Phosphorylated ERK1/2 Alternative……. 128

28. Active Phase Phosphorylated ERK1/2 Alternative……. 129

LIST OF ABBREVIATIONS

ADE	Anomalous dopamine efflux
ADHD	Attention Deficit Hyperactivity Disorder
AMPH	Amphetamine
ASD	Autism Spectrum Disorder
BPD	Bipolar Disorder
cAMP	Cyclic adenosine monophosphate
COC	Cocaine
COMT	Catechol-O-methyltransferase
CRF	Continuous Reinforcement
DA	Dopamine
DAT	Dopamine Transporter
D1	Dopamine 1 Receptor
D2	Dopamine 2 Receptor
ERK1/2	Extracellular signal-regulated protein kinase 1 and 2
FI	Fixed interval
FR	Fixed ratio
HET	Homozygous
HOM	Heterozygous
ITI	Inter-trial interval
KO	Knock-out
NK1R	Neurokinin 1 receptor
PFC	Prefrontal Cortex
PNE	Prenatal Nicotinic Exposure
PPI	Prepulse inhibition
PR	Progressive Ratio
RI	Random Interval
RR	Random Ratio
SHR	Spontaneously hypertensive rat
SN	Substantia nigra
SSRT	Stop-signal reaction time
VTA	Ventral tegmental area
VNTR	Variable number tandem repeat
WT	Wildtype
5-CSRTT	5-Choice serial reaction time task

CHAPTER 1

PAY ATTENTION: IT'S AN ADHD OVERVIEW

1.1 ADHD OVERVIEW

Attention-Deficit/Hyperactivity Disorder (ADHD) is one of the most prevalent neurobehavioral disorders diagnosed in children. ADHD affects approximately 5-7% of children and adolescents and 2.5-5% of adults worldwide[1-3]. ADHD appears to be present in boys more frequently than girls with an average of a three-to-one bias[4]. However, this gender ratio is fluid within subtype such that this bias can range anywhere from 2:1 to 9:1 [5]. ADHD can be considered a spectrum disorder with a wide range of possible phenotypes. The current diagnostic criteria for ADHD states that symptoms must be present before age 7 and that they are present for at least 6 months in a manner that is "maladaptive and inconsistent with developmental level." The core symptoms of ADHD are related to inattention, hyperactivity, and impulsivity. These symptoms can range from difficulties in sustaining attention, organizational problems, excessive fidgeting or movement, impatience, risky behaviors, and many other symptoms[6]. ADHD diagnosis can be divided into three sub-categories: predominantly hyperactive/impulsive type, predominantly inattentive type, and combined type (Figure 1)[6]. It is important to note that currently ADHD is only diagnosed through behavioral measures, with no concrete biomarker available for diagnostic use.

ADHD diagnosis is steadily increasing, with a 22% increase of diagnoses observed between 2003 and 2007,[7] and an additional 18 % increase in adolescent diagnosis seen between 2009 and 2013[8]. Additionally, ADHD has a major effect financial impact on individuals and their families.

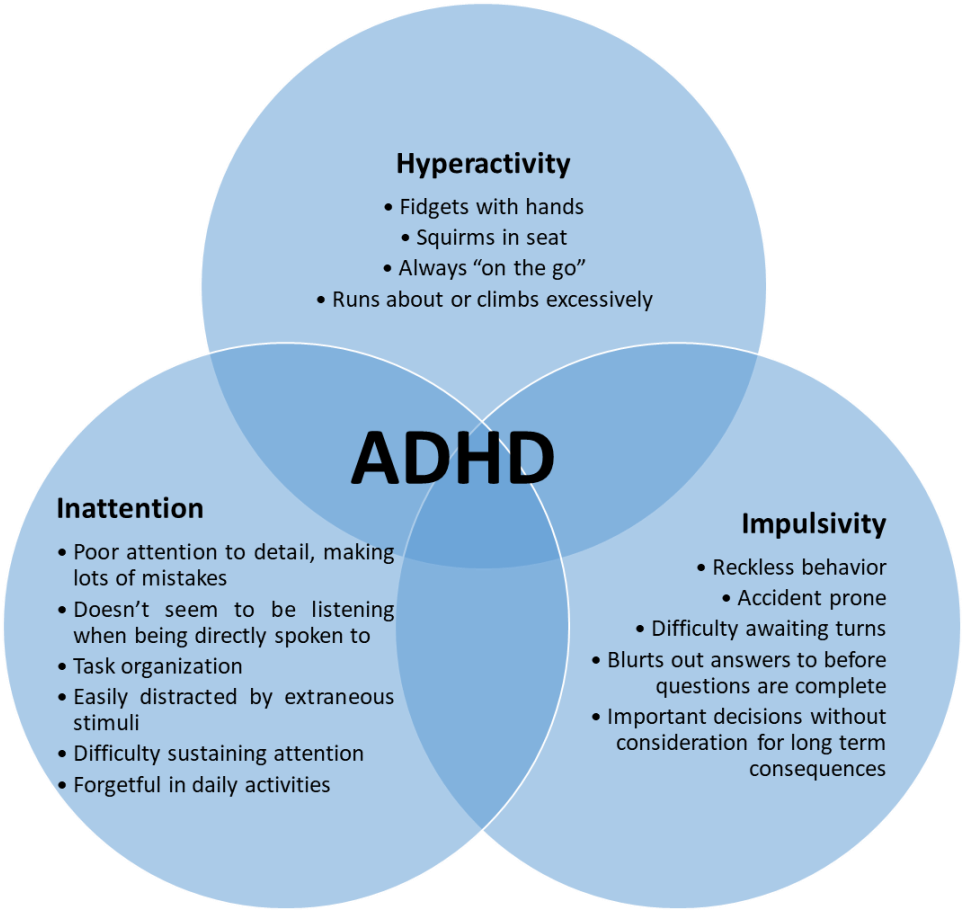


Figure 1. Classification and symptom domains of ADHD

In 2005 the economic cost of ADHD was between 36 and 52 billion dollars, assuming that the rate of prevalence is 5%[9]. Additionally, those with ADHD have a harder time in adulthood maintaining jobs and have decreased work performance, with a 4-5% reduction in work productivity[10, 11]. ADHD is also associated with an increased risk for other negative outcomes, such as an increased incidence in needing government support or being on welfare, and higher instances of substance abuse[12-15]. Specifically, those who are not on medication have an increased risk of having worse outcomes in terms of illicit poly-substance abuse[15]. As of 2017, 70% of those diagnosed with ADHD between ages 1-24 were reported to be on some sort of medication.

Psychostimulants such as methylphenidate (MPH, Ritalin) and Amphetamine (AMPH, Adderall) are the most prevalent pharmacotherapies used as a first line treatment for ADHD[16, 17]. In fact, the most widely prescribed medication to people under the age of 20 are psychostimulants[18]. Psychostimulants have been shown to be effective in approximately 70% of the clinical population treated, showing almost immediate positive responses in domains of conduct, attention, and academic performance[19-22]. Importantly, psychostimulants have been shown to be efficacious as a long term treatment option for ADHD symptoms[23, 24], with improved outcomes for those who maintain treatment[25, 26]. It should be noted, however, that there are some studies that indicate that long-term treatment with psychostimulants provide no prolonged benefit and that psychostimulant symptom reduction diminishes with time[27-29]. Even though psychostimulants show a higher far reaching efficacy for those with ADHD compared to non-stimulant options, both pediatricians and parents associate treatments with stimulant medications as a greater burden since stimulant medications are classified as a controlled medication[30]. In addition, stimulant medications can result in some serious side effects including risk of developing hypertension or other related cardiac issues, suppressed appetite, insomnia, and stunting of

growth[28, 31].

Indeed, diversion of prescribed stimulants to non-medical users for recreational and academic use, especially in universities, is one of the biggest concerns associated with stimulant prescriptions. An increasing number of studies show that students are purchasing Adderall (AMPH) and Ritalin (MPH) for increased focus and cognitive enhancement in academic settings, [32-34] as low doses of these substances are able to provide the working and declarative memory enhancements in those without ADHD[35, 36]. The result is that students are taking doses that are not titrated for the individual physiological response and in some cases resulting in the need for an emergency response[37]. The neurochemical changes that occur in the brain in response to MPH and AMPH result in an increase of extracellular dopamine (DA) in the brain, though through different mechanisms. While it is known that natural rewards elevate DA levels in the brain, this system can be hijacked by synthetic agents that produce the same or larger increases in DA. As such, students partaking in non-prescribed consumption of ADHD stimulant medication could predispose these students for substance abuse and addiction[38]. Interestingly, studies have shown that those with ADHD do not show this same increased risk for abuse potential and as alluded to before is shown to actually result in a reduction of risk for substance abuse[39, 40]. The protective effect of stimulant medication on substance abuse risk in the ADHD population likely reflects differences in neurochemistry and drug interaction in those with ADHD.

Since AMPH and MPH affect the (DA) system, many have postulated that it is alterations in the DA system that produce the behavioral perturbations seen in ADHD. As such, therapeutic actions of AMPH and MPH are thought to occur by restoring an already disrupted homeostasis in DA signaling. To further understand the role that DA could be contributing to the pathology of ADHD, I will first discuss the basics of the DA system, then look at genetic and imaging studies

that further implicate DA's role in ADHD, and end with a discussion of current animal models of ADHD and what has been learned from them.

1.2 DA OVERVIEW:

DA is a neuromodulator that is implicated in reward and locomotor circuitry. DA also contributes to memory, learning, and cognitive performance[41, 42]. The DA system is typically divided into four main pathways; the mesocortical, mesolimbic, nigrostriatal, and tuberoinfundibular pathways (Figure 2). The mesocortical and mesolimbic dopaminergic cells bodies are located in the ventral tegmental area (VTA) of the midbrain with projections to the frontal cortex and nucleus accumbens[43]. These pathways are traditionally associated with cognition and reward processes. The nigrostriatal dopaminergic cell bodies are located in the substantia nigra (SN) and project into the dorsal striatum (caudate and putamen). The nigrostriatal pathway is typically associated with locomotor behavior. The dopaminergic cell bodies of the tuberoinfundibular pathway originates in the hypothalamus and projects to the pituitary, where DA modulates prolactin release from the pituitary gland[44].

DA is derived by a two-step enzymatic process from the amino acid tyrosine. Tyrosine is converted to L-DOPA by tyrosine hydroxylase, which is then converted to DA by dopa dexcarboxylase[43]. Upon synthesis, DA is then packaged into vesicles until it is released[45]. Once DA release is stimulated it can interact with a variety of pre- and post-receptors that are classified into two families, the D1-like and the D2-like receptors. The D1-like receptors contain the D1 and D5 DA receptors which are coupled with stimulatory G-proteins and whose activations results in elevations of cyclic adenosine monophosphate (cAMP), a key second messenger in neuronal signaling. The D2 like family contains the D2, D3, and D4 DA receptors, which are coupled to the inhibitory Gi/o proteins, resulting in inhibition of cAMP. It should be noted that the D2-like

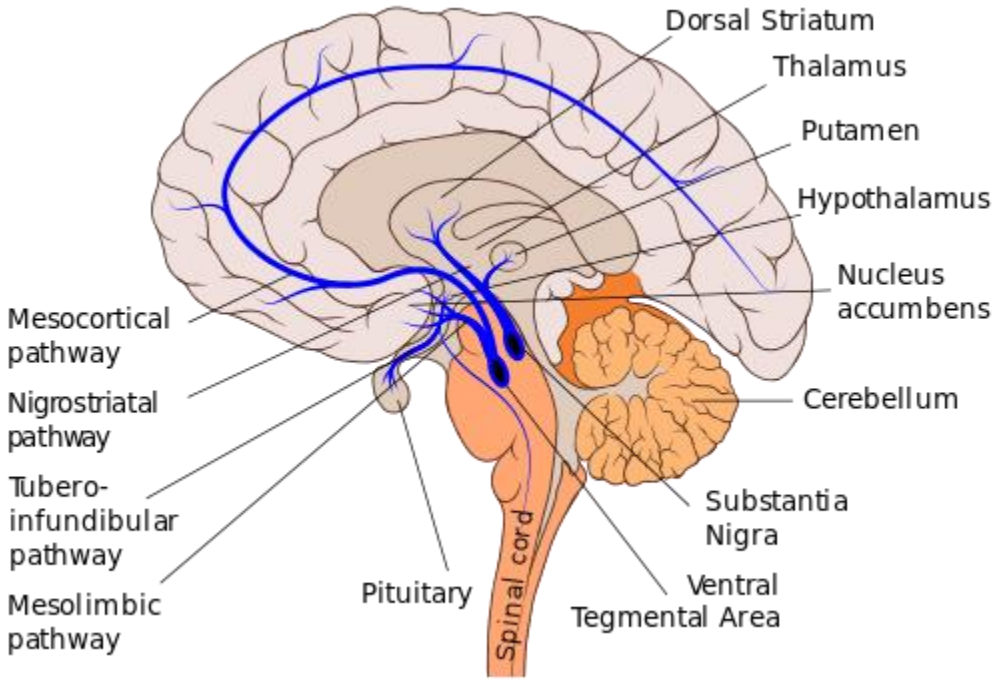


Figure 2. Four DA pathways of the brain. By User:Slashme; Patrick J. Lynch; User:Fvasconcellos (self-made; re-use File:Brain bulbar region.svg) [GFDL (http://www.gnu.org/copyleft/fdl.html) or CC BY-SA 4.0-3.0-2.5-2.0-1.0 (https://creativecommons.org/licenses/by-sa/4.0-3.0-2.5-2.0-1.0)], via Wikimedia Commons

receptor activation also stimulates Gβγ signaling activity to modulate intracellular calcium levels, as well as, G-protein inwardly rectifying potassium channel mediated-membrane potential[46]. The D2 receptor has two isoforms, short and long, with the short form classified as the autoreceptor. Expression of DA receptors on a given neuron allows DAergic nerve terminals to modulate the neurons it synapses onto in order to either increase (D1-like activation) or decrease (D2-like activation) the post-synaptic neuron's excitability depending upon the type of DA post-synaptic receptor present[46]. Additionally, DA neurons can modulate their own excitability as well as modulate excitability of neurons sending inputs into the VTA and SN through cell body and dendritic release (somatodendritic release) of DA[47]. For an extensive review of DA receptor signaling please see the excellent review by Beaulieu and Gainetdinov, 2011.

An important and necessary component of DA signaling and for maintaining the homeostasis of DA signaling is the dopamine transporter (DAT) encoded by the *SLC6A3* gene. DAT is a sodium/chloride-dependent 12-transmembrane domain transporter found on DAergic neurons near the synapse. This transporter regulates the amount of DA present in the extracellular space by transporting it out of the synaptic cleft and back into the cytosol of the pre-synaptic neuron for re-use or degradation[48]. Degradation of DA occurs through its enzymatic breakdown by catechol-O-methyl transferase and monoamine oxidase[43].

Because the psychostimulant therapies that are effective in treating ADHD are known to directly target DAT, several imaging studies have focused on the potential difference of DAT in ADHD populations[49]. These studies have had mixed results, with the most consistent result across studies indicating increased DAT availability[50]. In fact DAT availability was demonstrated to correlate with therapeutic effectiveness of MPH[51]. Additionally, imaging studies have demonstrated that those with ADHD have higher levels of DAT availability compared to control

subjects and that this increased DAT availability then demonstrates a reduction of availability post-treatment[52]. However, Volkow and colleagues demonstrated a decrease in DAT binding in the left caudate of drug naïve ADHD patients, implying either reduced DAT expression or reduced DAT availability[53]. Other studies have indicated that long term treatment with MPH actually induces an increase in DAT availability in those with ADHD[54]. Despite the mixed results, these imaging studies do indicate that DAT seems to be altered in those with ADHD, spurring further investigations into the role of the DA system in ADHD.

1.3 DA-ASSOCIATED ADHD GENETICS

ADHD is reported to be one of the most heritable psychiatric disorders with reports of heritability in twin studies ranging from 0.76 to 0.9[55]. Genetic perturbations in the DA system have long been associated with or linked to ADHD. One that seems to be consistent throughout the literature is the presence of a variable number tandem repeat (VNTR) in the non-coding region of the 3' end of the DAT-1 gene. First prompted examine DAT because of the manner in which ADHD is treated, Cook et al. identified a significant association between ADHD and a 10-copy VNTR[56]. Since the initial association of the 10-copy VNTR region of the DAT1 gene with ADHD, several other studies have confirmed its link with ADHD[56-58]. Many of the genes encoding the various DA receptors are implicated in risk for ADHD. Variations in the DRD4 gene, which encodes for the D4 receptor, is steadily implicated in ADHD[59-61], being cited as the most consistent and replicated genetic findings in linking ADHD with the DA system[60]. The main variation in the DRD4 gene that is indicated as a risk factor for ADHD is a seven-repeat VNTR in the third exon of the gene, affecting the third cytoplasmic loop of the receptor[62]. Although there are some mixed accounts reported, commonly variations in the DRD4 gene result in poorer cognitive performance on tasks of executive function[63-66]. Additionally, this genotype produces decreased activation and

less coupling of neural networks during cognitive tests[67]. In addition, variants in both the DRD1 and DRD2 genes are implicated in ADHD[55, 68]. Studies have also indicated that differences in DA-associated proteins can increase risk for ADHD, such that one's COMT genotype can affect performance on working memory tasks in an ADHD population[69]. The repeated association of DA and DA-related genes and ADHD has led to the use of several rodent models that have perturbations in their DA-systems as models for ADHD.

1.4 ADHD MODELS

DAT knock-out mouse: One of the most widely used models of ADHD is the DAT knock-out mouse. First reported in the mid-1990s, these mice were created to obtain a better understanding of the role that DAT plays in DA homeostasis, but these mice were rapidly adopted as an ADHD model offering face and predictive validity, meaning that the model demonstrates several phenotypes associated with ADHD and some of these phenotypes are ameliorated with psychostimulants[70],[48]. The most overt of these phenotypes is its extreme hyperactivity in a novel environment—the overall activity level of DAT-KO animals is 5-6 times higher than that of WT mice in both the inactive and active phase of the behavioral cycle. In addition, DAT-KO mice also lack the ability to habituate to novel environments.

Studies indicate that the hyperactivity of DAT-KOs is supported by the abnormally high levels of extracellular DA found in the striatum of this animal[71]. DAT KO mice have a 5 fold increase in basal extracellular DA levels, though a 100 fold decrease in total tissue DA concentration compared to WT animals[71]. The high extracellular DA level is thought to be caused by the slow DA clearance that occurs at 100 seconds versus 1 second, allowing extended time in the synapse[48]. Interestingly, the overall levels of both DA and tyrosine hydroxylase are drastically reduced to less than 5% and 10% respectively compared to wildtype (WT) [71]. This decrease is not due to any

structural anomalies as the terminals are intact and the number of DA neurons between genotypes is equivalent[71, 72]. In addition, there is a large reduction in both D1 and D2 receptors[48, 73].

There are profound behavioral effects that accompany these and the many other intricate biochemical changes found in the DAT KO. In addition to the hyperactivity and increased activity levels, the DAT KOs show impulse control deficits in a variety of situations. In one measure of impulsivity, in the cliff avoidance test, DAT KOs showed an impaired cliff avoidance reaction[74]. They had an increased incidence of approaching the edge of an elevated platform and extending out far enough such that they fell off, demonstrating an increase in impulsivity and risk-taking behaviors. Interestingly, injection of MPH ameliorated this impulsivity measure. Pre-adolescent DAT KOs also showed increased impulsivity and risky behavior demonstrated by the number of times they dip their heads over an unprotected edge in an elevated plus-maze compared to their WT counterparts[75].

DAT KO mice have also demonstrated some cognitive impairment, specifically in spatial memory, though this cognitive deficit seems to appear later in life, not being present in pre-adolescent DAT KOs[75-77]. Social impairment is also present, spending less time in engaging in social investigation[78]. When they are being social it tends toward being aggressive. Additionally, DAT KO mice spend more time performing stereotyped and perseverative behaviors, suggested to result in a restricted and inflexible behavioral repertoire. This is interesting because social problems have been reported in the ADHD community, [79] And many subjects with ASD meet clinical criteria for ADHD.

DAT KO mice have also demonstrated deficits in sensorimotor gating using pre-pulse inhibition as a measure, [74, 80] which can be corrected with atypical antipsychotics[80]. To an extent, these aspects of the DAT-KO model seem more akin to a schizophrenia model than an ADHD

model[81, 82]. Importantly, schizophrenia is commonly treated with DA receptor antagonists and imaging studies support elevated release of DA in the basal ganglia, reminiscent of changes in the DAT KO model. Additionally, in a few instances this model has even been cited as a depression model, showing impairment in the forced swim test[82].

Having such an extreme disruption in the DA system results in behavioral deficits that extend beyond the scope of what one could reasonably link or associate with ADHD. Complete functional ablation of DAT does not allow for a fine-tuned in-depth dissection of the disorder. A major caveat with this mouse model is that when there is a homozygous loss of function of DAT in humans results in infantile parkinsonism-dystonia, a severe and early-onset neurological disorder[83]. That being said, the DAT-KO mouse is used as both a face- and predictively-valid model of ADHD in that it shows some ADHD-like behaviors, and these behaviors can be mediated by the therapeutics used to treat ADHD.

NK1R KO Mouse: Another knock-out model that is utilized to study ADHD is the neurokinin 1 receptor (NK1R) KO mouse. NK1R is the endogenous receptor for substance P, a neuropeptide associated with a wide range of functions from pain and inflammation to mood and anxiety[84]. Interestingly, this KO model was originally created to study the role of NK1R in pain[85]. However interest shifted towards using it as a model to study anxiety and depression, as it was demonstrated to be resistant to anxiety and depressive-like behaviors, similar to animals treated with SSRIs. One of the groups researching the anxiolytic effects of the NK1R KO realized that the mouse was hyperactive, and that this hyperactivity could be ameliorated by AMPH and MPH[86]. So although ADHD seems to be correlated with an enrichment of anxiety related issues[87], a model previously used because of its anxiolytic characteristics, the NK1R KO mouse, began to be touted as an ADHD model.

Interestingly, coding variations in the gene for the NK1R appear to confer risk for ADHD, BPD, and ASD[88, 89]. Additionally, NK1R expression occurs in key regions that intersect with the DA system including the striatum,[90] and can modulate DA-associated behavioral effects[91]. Microdialysis in the NK1R KOs further reveals a connection between NK1R and DA, as NK1R KOs have a reduction in basal DA efflux in the motor cortex 2 region of the prefrontal cortex with no DA-efflux occurring in the dorsal striatum in response AMPH administration (counter to AMPH normal actions). Additionally, pre-treating the WT animals with a NK1R antagonist prior to AMPH administration recapitulated the abnormal dorsal striatum efflux response to AMPH seen in the NK1R KO in WT mice, demonstrating that it is specifically the NK1R receptor that is mediating the alterations in the DA system of this model[89].

At first glance, NK1R KO mice display several interesting deficits associated with the core clinical features of ADHD. Utilizing the 5-choice serial reaction time task, NK1R KOs demonstrated increases in perseveration, premature responses, and omissions. However, low doses of AMPH failed to rescue any of these deficits and actually seemed to exacerbate the premature responses, significantly increasing the impulsivity measure in the NK1R KOs only and not their WT counterparts. Additionally, there was a rather dramatic effect of testing time on performance of the task. NK1R KO were significantly less impulsive compared to WT in the morning, but more impulsive when tested in the afternoon[92]. Though the mechanism contributing to the differential impact of testing time on impulsivity measures in the NK1R KOs remains unknown, identifying the mechanism might provide insight into pathogenic alterations in seen in ADHD subjects as some studies suggest that ADHD behavioral phenotypes fluctuate in their severity with time of day, with symptom severity being greatest during early morning and evening time frames[93, 94].

Additional studies done in the NK1R KO model show that the design of the behavioral

paradigm also has a strong effect on the behavioral phenotypes observed. The effects of paradigm choice on behavioral phenotypes was demonstrated through use of the 5-choice continuous performance test, a task similar to 5-choice serial reaction time task, except with an additional component of a no-go cue where the animal is signaled to withhold a response in order to receive a reward. This adds an additional measure in the impulsivity domain. In this particular paradigm, there is no longer an increase in omissions (an index of inattentiveness) regardless of time of day in the NK1R KOs. Additionally, NK1R KOs have fewer false alarms and only display a mild increase in premature responses in afternoon test sessions[95]. The only consistently stable phenotype is the increased perseverative responses of the NK1R KO mice. It should also be noted that both AMPH and MPH both decreased perseverative behaviors in these animals[96, 97].

Unfortunately, the bulk of the work done on these animals in regards to ADHD research occurred with animals produced from two separate colonies, such that, their WT animals and NK1R KOs were produced from homozygous breeding techniques, rather than heterozygous breeding. When NK1R KO mice from heterozygous breedings were tested several important phenotypes disappeared, including measures indicating increased impulsivity (perseveration and premature responses) and inattention (omissions) [98]. NK1R KOs do, however, maintain increased activity relative to controls in the home-cage during the active phase, though it was not confirmed if psychostimulants still have their calming effect on this behavior[98].

Spontaneously Hypertensive Rat: Another well-studied ADHD model is the spontaneously hypertensive rat (SHR). An advantage to this model is the expansion of the behavioral paradigms that may be performed with this model. Compared to mice, rats are able to undergo more complex behavioral tests in a shorter training time period that allow for the observation of more cognitive-related tasks. This model is a rat strain derived from selective inbreeding of Wistar rats[99]. Similar

to the DAT-KO mouse, these animals are hyperactive[100, 101]. They also demonstrate impulsivity, attention impairments, and cognitive deficits[102-105]. As a result the SHR is lauded as the most validated model of ADHD[102]. There are some major caveats to this statement. The SHR does display a number of symptoms of ADHD but these seems to be inconsistent depending on the task being performed and which strain of rat is being used to represent the control. In addition to these inconsistent findings, the SHR has mixed results as a predictive model of ADHD and also lacks construct validity.

In some studies, psychostimulants seem to help the SHR in performances on attention-related tasks, where in others it does not[102, 104, 106, 107]. Additionally, psychostimulants do not have a calming effect on the hyperlocomotion aspect of this model but rather potentiate the behavior[108]. Similar to the DAT-KOs this model also displays sensorimotor deficits as measured by prepulse inhibition (PPI) studies[109, 110]. The observance of this disruption is interesting because of the literature examining PPI in the ADHD population presents with mixed results at best, but the overall tenor of that body of literature trends toward demonstrating that the ADHD population does not suffer from PPI deficits[111-113]. This seems especially true when the paradigm is set up so that it does not require sustained attention of the test subjects[113]. So these PPI deficits seem more indicative of a translational measure for schizophrenia, not ADHD.

What is most disconcerting about using the SHR as a model for ADHD is the lack of a solid control comparison. Unlike mouse research, one does not have a control WT rat and an ADHD rat. That leaves comparing the SHR to other strains that are deemed as normal. The Wistar-Koyoto (WKY) rat oftentimes is used as the main comparison in behavioral studies of the SHR as both lines were selectively bred from the same outbred Wistar rat colony, with the SHR selectively bred for high blood pressure and the WKY rat selectively bred for normal blood pressure[114]. When this

is done strain differences are seen in performance tasks involving attention and impulsivity, with deficits arising in the SHR[102-104].In some instances, these deficits are ameliorated by the application of MPH or AMPH[102, 104]. When third strains are added, however, the apparent face validity drastically changes. A good example of this would be studies where the Sprague-Dawley rats were included with WKY and SHR in behavioral tasks of temporal processing to probe timing and motivation. The results indicated that the WKY strain was behaviorally similar to the SHRs, and none of the strains showed differential sensitivities to MPH or AMPH[106]. Interestingly, in a different study comparing attention and impulsivity between SH, WKY, and Wistar rats in a differential reinforcement of low-rate responding paradigm, SHRs were out-performed by the WKY but not by the Wistars. Additionally, in a 5-choice serial reaction time test (5-CSRTT), the attentional performance of all three strains were equivalent, but as the task proceeded the Wistars actually made more impulsive choices that were then attenuated by MPH[107]. Certain studies have also implicated that AMPH potentiates some of the ADHD behaviors seen in SHRs, including hyperlocomotion. In these same studies, SHRs were demonstrated to have social deficits that were exacerbated by AMPH in conjunction with an increase in stereotypic behavior, [108] whereas atypical antipsychotics ameliorated these symptoms. Additional independent studies with the SHR strain have demonstrated that AMPH also worsens PPI deficits in SHRs, whereas atypical antipsychotics improve deficits[110].

A major issue with this particular model is the lack of understanding as to what is causing these behavioral changes. Studies have shown that SHRs exhibit a down-regulation in the D4 receptor in the prefrontal cortex, which is intriguing because of the genetic studies linking the D4 receptor to ADHD[109]. Additionally, these rats have been demonstrated to have an increase in DA efflux in the striatum with increased levels of DAT, although there is a decrease in basal norepinephrine

efflux in the prefrontal cortex[115]. These microdialysis data has led to the proposal that the SHR strain has a hypernoradrenergic and hypodopaminergic system in the prefrontal cortex, leading to the behavioral deficits observed. However, what is not known currently is how these neurochemical changes originate. Is the initial insult in the DA system, the norepinephrine system, or somewhere else entirely? Are these changes exhibited by subjects with ADHD? At the least, there seems to be a slight problem in using this rat strain in understanding the underlying etiology of ADHD, bringing into question the construct validity of the SHR as a model.

High-Activity Selective Breeding Model: A new and rather interesting method for modeling ADHD is through a mouse breeding scheme that selects and breeds for high activity animals relative to a control line that is maintained by random breeder selection. This particular strategy argues that the existent single-gene mutant models do not account for the "multifactorial, genetic foundation of ADHD," denoting that a single-mutation method fails to account for broader gene network changes that occur in the disorder and thus fail to provide a pathway for improved, viable treatments[116]. Although it could be argued that the above reasoning for this breeding model undervalues the merit of a *good* single-gene mutant model, this particular strategy warrants evaluation of its merits when considering the many models of ADHD. This high activity selective breeding line rings similar to the several selectively bred rat models, but with the argument that they provide a control line, an oft cited critique of the strain-selective rat lines. Although this strategy is still relatively new, a few interesting measures have been evaluated that argue for both predictive and face validity for this model. As previously mentioned, this line is selected for high activity in the home cage setting and as such called the high activity selective breeding line. Unsurprisingly, they also have elevated activity both in open field tests as well as wheel-running[117]. Chronic low doses at 0.25 mg/kg and 0.5 mg/kg of AMPH administration produce a

calming effect on the home-cage hyperactivity, while producing elevated home cage activity in the control line. This calming effect was lost at a 2mg/kg does of AMPH[117]. It is unknown what a single acute dose does for these animals, however, as the locomotor data presented averages morning and afternoon injections given to animals singly housed in home cages, potentially masking valuable locomotor drug response information. An additional caveat to this otherwise interesting data set is the extremely high variability, and very low N per group (only 4/condition/mousseline). Of an interesting note, although both males and females of this selectively bred high activity line display increased activity levels compared to their control line, the females display an even higher level of activity than their male siblings, diverging from the human ADHD literature where male/female bias is high.

This difference in sex activity levels is also reflected in measures of impulsivity in this high-activity selective breeding model as well. Some tenuous data demonstrates increased measures of impulsivity in the Go/NoGo paradigm as measured by an increased number of false alarms to no-go cues as well as increases in premature responses pre-cue (see chapter 2, page 25 for an in depth paradigm explanation). This response is most striking in adult female mice of this high-activity model. However, the phenotype is much milder in adolescent male mice of the high activity line. Additionally, administration of a low dose of AMPH demonstrated to rescue hyperlocomotion failed to rescue these impulsivity measures in both females or males[116]. However, this could be due to the remarkably low number of animals and extremely high variability. Although this could be an illuminating new strategy, the current work is rather lacking, with limited comparisons being made across genders and age-groups, overall lacking the necessary power and cohesiveness to draw any real conclusions from this new model.

Further, although it is argued that the behavioral changes seen in this model are due to the

careful breeding selection strategy, as opposed to random genetic drift, as of yet, no efforts have been made to identify altered gene networks underlying the behavioral phenotypes. This seems to somewhat negate its supposed superiority to a more controlled single-gene approach, which can arguably also provide insight into how gene networks can change, giving insight into other nodes that could also result in a diagnosis of ADHD or related disorders.

Prenatal Nicotinic Exposure Model (PNE): In addition to the current transgenic models and selective breeding models, there are also several models of ADHD centered on environmental insult[118-120]. I will focus on one of particular interest, the prenatal nicotinic exposure model (PNE). Although it is definitely plausible that environmental factors can have a profound impact on the expression of ADHD phenotypes, it is unlikely that an environmental factor is a causative factor to ADHD, considering the significant heritability of ADHD. Additionally, there have been mixed accounts in the human literature linking prenatal nicotinic exposure and ADHD conditions, [121] though it certainly does not rule prenatal nicotinic exposure out as a very serious risk-factor. That being said, recent research has demonstrated that the altered behavioral phenotypes seen in the PNE model (discussed below) can be passed down trans-generationally through the maternal line, indicating a strong epigenetic component to PNE[122].

The PNE model demonstrates a variety of altered behaviors associated with ADHD. These mice, compared to untreated animals, display hyperactivity in the form of increased locomotor levels that can be ameliorated with MPH[122, 123]. Additionally, PNE mice have elevated levels of DA in the frontal cortex with a significantly reduced DA turn-over rate. Although no basal differences in DA levels were seen in the striatum of these mice, they did display a significantly reduced DA turn-over rate similar of that seen in the frontal cortex[123]. Interestingly, when PNE mice were administered a low dose of MPH, the DA turnover rate increased significantly, partially

rescuing the DA turnover rate to levels more similar to non PNE animals. These animals also displayed alterations in the attentional and impulsivity domains. Specifically, PNE mice (males only) showed reductions in spontaneous alternations in the Y-maze task, a measure of working memory and attention. The reduced number of spontaneous alternations in the PNE mice is reversed to WT performance levels with administration of MPH[124]. Additionally, both males and females score significantly poorer on attentional measures as determined by the object-based attention test, a test similar to novel object, which measures time spent exploring a previously explored object and a new object. PNE mice spent significantly less time exploring the novel object, interpreted as a deficit in attention. These differences were also ameliorated with MPH. MPH also reduced the increased measures of impulsivity seen in the PNE mice in the cliff avoidance task[124].

One caveat to the PNE model, however, is that it relies on pure nicotine exposure through drinking water, whereas cigarettes are composed of a variety of toxically unpleasant compounds. Another study recapitulating the PNE model did so by exposing the pregnant dams to cigarette smoke throughout the entirety of gestation[125]. In this cigarette smoke exposure study, the PNE pups also had an increase in locomotor activity, but only in the males, though MPH ameliorated the elevated locomotor activity, similar to nicotine exposure through drinking water. Contrary to the drinking exposure model method, smoke exposed animals showed significantly decreased levels of total tissue DA content in the striatum with reduced tyrosine hydroxylase levels as well[125]. However, these investigators did not do analysis on DA turnover or look at the prefrontal cortex. Overall, however, the PNE model is an extremely interesting model that may provide very insightful information on the environmental and epigenetic factors contributing to ADHD.

1.5 DAT VAL559 MOUSE MODEL

Although the mouse models discussed above provide various degrees of valuable information related to factors that could be contributing to ADHD pathology, and all seem to produce alterations to the DA system either directly or indirectly, they lack an important factor, construct validity. By using models with large disruptions, such as knockouts, or utilizing models where the basis of disruption in unknown, we are limited in the clinically relevant knowledge that can be gathered, resulting in potentially serious gaps in our knowledge in the underlying mechanisms of ADHD. Thus development and utilization of a model with construct validity, i.e. a model derived from a genetic variation associated with ADHD, is needed to connect underlying behavioral traits exhibited to mechanisms responsible for neuropsychiatric conditions.

With all the genetic studies linking components of the DA system to ADHD one is faced with the non-trivial challenge of deciding which component to focus on in the development of a construct valid model. One issue with the development of an ADHD model is that many of the common variants in ADHD are associated with non-coding regions of the genome, such as variations in the 3' and 5' UTRs. This leads to a variety of issues when considering a construct valid model of ADHD. A major issue is the uncertainty of how non-coding regions function. Additionally there is the lack of conservation in these regions between humans and mice. Where does this leave ADHD research? One strategy is to focus on mutations associated with ADHD found in the coding regions of genes as these regions are often conserved across species. Specifically of interest is DAT because of its implicated role in ADHD risk[126]. There are no common coding variants found in DAT associated with ADHD, but there are multiple documented rare coding variants[127]. Studying rare coding variants of DAT that have been identified in the ADHD population would provide unique insights about the contribution that perturbations in the

DA system make to the phenotypes seen in ADHD. . Making a construct valid model of ADHD with DAT mutation may also provide valuable insight into how the current pharmacotherapies of ADHD are having their therapeutic effect, a line of inquiry that has long been pursued in the ADHD field.

With this goal in mind, our lab pursued a screen of ADHD children, searching for evidence of functional, DAT coding variation, identifying four rare coding substitutions (A559V, R615C, L167F and V24M)[127]. Focusing on the Val 559 mutation, this variant is found in the juxtamembrane region of the 12th transmembrane domain and derives from a single nucleotide polymorphism that converts an alanine residue at the 559th amino acid position to a valine. Interestingly, the Val559 variant has also been found in a girl with bipolar disorder (BPD) and two unrelated boys with autism (ASD)[128, 129]. Notably, BPD and ADHD are sometimes comorbid, and approximately one third of subjects with ASD meet DSM-IV criteria for ADHD[130, 131] Therefore studies with the DAT Val559 mouse model provide a unique opportunity to understand the consequences of DA dysfunction in a construct-valid model that could provide invaluable information related to multiple neuropsychiatric conditions.

Initial investigations into the functional impact of DAT Val559 in transfected HEK 293 cells revealed normal total and surface DAT expression as well as normal DA uptake activity[132]. However, in studies of DA preloaded cells, amperometric measurements revealed that the transporter variant induced a spontaneous outward leak of DA not seen in cells that express the WT DAT. Furthermore, this anomalous DA efflux (ADE) was determined to be voltage-dependent and to be accompanied by a shift to higher sensitivity for intracellular Na+ that could further stimulate the efflux process[133]. The voltage-dependence of DAT Val559 ADE suggests, assuming similar properties exist *in vivo*, exacerbation of DA efflux with neuronal excitation. Another

striking finding emerged in these studies when DAT Val559 cells were exposed to AMPH. Whereas AMPH produces DA efflux in DA-preloaded cells that express WT DAT, AMPH fails to produce DA efflux in DAT Val559 cells, instead acting to attenuate tonic DA leak[133]. Studies with combined AMPH/MPH or AMPH/cocaine (COC) administration, revealed a lack of additive effects of these treatments that, along with an absence of DA leak from preloaded cells lacking the transporter, indicate that AMPH attenuation of ADE is DAT-mediated. To determine whether these features arise *in vivo*, the Blakely lab generated a DAT Val559 knock-in mouse via ES cell homologous recombination approaches. Initial characterization of the mouse revealed normal growth and survival rates compared to its WT counterpart, unlike the DAT KO mouse, which exhibits deficits in growth and long-term survival[48]. Striatal microdialysis in freely moving mice showed that the mutant mice exhibit significantly elevated extracellular DA, with elevations seen in both HET and HOM animals, consistent with *in vivo* ADE. Intrastriatal administration of 0.1μM AMPH revealed that the mutant animals also display a blunted capacity to elevate extracellular DA levels, which we theorize reflects loss of efflux generation yet maintenance of competitive uptake inhibition. Consistent with this idea, acute striatal slice studies demonstrated a significant loss of AMPH-induced DA release, mirroring the effects seen in cell studies. Unlike the deficits seen in the DAT KO model, radioligand binding studies and western blots revealed no changes in striatal D1 or midbrain/striatal D2 receptor levels in the DAT Val559 model compared to WT. Additionally, DAT Val559 animals lack the locomotor hyperactivity in the open field test seen in the DAT KO model. However, the mutant mice display a hyper-reactive motor response to imminent handling that we have termed "darting." This response shows a clear gene-dosage effect in reactivity in terms of speed and frequency in response to a "hand grab" stimulus. While this behavior is different from overt hyperactivity (the seeming standard for a face-valid model of

ADHD), one could argue that this hyper-reactive response may actually be more relevant to the clinical ADHD population, possibly representing a deficit in top-down inhibitory control. As with DA release in microdialysis studies, AMPH treatment of DAT Val559 mice results in a blunted locomotor response compared to WT animals[134].

Our initial studies of the DAT Val559 model indicate that these animals provides a unique opportunity to investigate the *in vivo* impact of genetically-determined DAT dysfunction. My thesis will build on these initial observations to broaden the understanding of both behavioral and biochemical changes that attend lifelong DAT Val559 expression. Although there are good models to help understand what occurs when DA signaling is disrupted, currently, none of them are directly linked with ADHD, despite substantial evidence linking DA de-regulation to ADHD pathogenesis. A construct valid mouse model is critical to increase the understanding of the molecular and cellular perturbations present in ADHD and how such aberrations affect neural networks and behavior. Use of the DAT Val559 model allows for the identification of discrete phenotypes and biomarkers that arise in the context of etiologically relevant genetic or environmental insults, thereby enhancing opportunities for translation of preclinical research findings. Importantly, while our studies originated in a search for contributions of DAT dysfunction to ADHD, the discovery of DAT Val559 variants in BPD and ASD reminds us that multiple psychiatric conditions can arise from common mechanisms, with ultimate trajectories likely dictated by concomitant environmental and/or genetic factors. Moreover, using the mouse model, we are able to, for the first time, establish the *in vivo* consequences of non-vesicular neurotransmitter efflux, yielding both mechanistic and conceptual advances that could lead to novel diagnostics and therapeutics for multiple neuropsychiatric disorders.

CHAPTER 2

IMPULSIVITY, ADHD, AND DOPAMINE

2.1 INTRODUCTION

Deficits in inhibitory processes are a hallmark for a variety of psychiatric diseases. The study of "inhibition" or "inhibitory processes" represents a large field of work dedicated to understanding the distinct complex neural circuits and interactions that when altered or disturbed result in issues with impulsivity and compulsivity. These are two simple words to capture an entire sub-field of neuropsychiatric and neurobehavioral work. However, the sources of impulsivity and the resultant behavioral deficits in behavioral inhibition vary greatly.

A core diagnostic component of ADHD is an impulsivity domain, but given the variability within the inhibition research field, what does that really mean? According to the Diagnostic and Statistical Manual of Mental Disorders impulsivity in ADHD represents or is defined by:

> *". . . hasty actions that occur in the moment without forethought and that have high potential for harm to the individual (e.g., darting into the street without looking). Impulsivity may reflect a desire for immediate rewards or an inability to delay gratification. Impulsive behaviors may manifest as social intrusiveness (e.g., interrupting others excessively) and/or as making important decisions without consideration of long-term consequences (e.g., taking a job without adequate information).*[5]"

To provide further understanding, I will discuss how impulsivity is classified and measured, its common manifestations in the ADHD community, and look at the various neural substrates that

contribute with a specific focus on the DA system. This will not be an in-depth review of all domains of impulsivity, however, this review will be a primer necessary for understanding the rationale for paradigm choice and interpretation in later chapters.

Under the broad umbrella of inhibition, there is a division between behavioral and cognitive inhibition, corresponding to the ability to control ones actions versus ones thoughts. For the purpose of this review, I am specifically going to focus on behavioral inhibition. Behavioral inhibition can be broken down into three broad components; impulsive action, impulsive choice, and behavioral inflexibility/compulsivity[135]. Each sub-category of impulsivity is associated with a specific deficit, in relation to which a variety of behavioral paradigms have been developed that can be used in both model organisms and humans to reveal and test for these impulsivities.

Impulsive action is considered a deficit in motor inhibition. Specifically, impulsive action can be broken down into a deficit in the ability to wait (a waiting impulsivity), an inability to withhold a response, and finally, a disruption in the ability to stop an already initiated response[135]. These sub-categories of impulsive action all represent an inability to withhold a prepotent motor response, however, they occur within behaviorally distinct contexts and can be caused by distinct underlying mechanisms[136-138].

2.2 IMPULSIVE ACTION

Waiting impulsivity can be assessed by utilizing a behavior paradigm in which the animal or subject must wait a specific amount of time before the presentation of a cue. Presentation of the cue represents a conditioned stimulus to which the subject must accurately respond in order to obtain a reward or positive feedback. The delay between the last cue presentation and the next cue presentation can be a variable or a fixed timing component that can be lengthened or decreased to assess an individual's ability to withhold a motor response until the presentation of the cue. An

inability to inhibit one's behavioral response until the presentation of the cue represents a waiting impulsivity deficit. A concrete example of a behavioral paradigm that is often used in the literature to assess this is the 5-choice serial reaction time task (5-CSRTT)[137]. In this task a rodent or human is taught to respond to one of 5 stimulus windows after the start of a trial, either started by the participant, or indicated by a light or other stimulus cue indicative that the trial is starting. Normally, a subject is trained such that there is a set expected delay between initiation of the trial and presentation of the stimulus/cue. Deficits in impulsivity may be evident with the trained delay time, though further manipulations of delay time may also be necessary to reveal deficits. Common task manipulations to further probe waiting impulsivity in 5-CSRTT include lengthening the delay or randomizing the delay variable rather than a set duration. To identify a specific waiting impulsivity vs a more global behavioral inhibition issue, tests to assess deficits in stopping and restraint must be utilized[137]. If one does not see global impulsive deficits across multiple tests of impulsive action, 5-CSRTT and similar paradigms are a good mechanism to confirm issues with waiting. 5-CSRTT is often considered an excellent translational tool between rodent models and humans as it is very similar to the continuous performance task, used to help diagnose those with ADHD as the show both attentional and impulsivity deficits in this task[136, 139, 140].

Behavioral restraint is another measure of impulsive action, but distinct from waiting impulsivity. Behavioral restraint is the ability to make a response when cued and withhold a response as indicated by a separate cue. In other words, a subject must withhold prepotent motor response that is independent of a timing component, but based on differentiating between learned cues. This is often assessed with a Go/NoGo paradigm, where "go" cues and "nogo" cues are intermixed in their presentation across multiple trials within a session. An animal model or person has to learn which cue signifies making a response, and which signals response restraint. The two

main measures assessed in this paradigm are correct rejections and false alarms, although one can also collect information on premature responses, perseverative responses, accuracy, and omissions further informing on impulsive and attentional issues. Correct rejections are when a subject is able to withhold responding under the correct NoGo associated cue. False alarms, also called commission errors, are when there is a failure to withhold the motor response during a NoGo trial. Those with ADHD have demonstrated deficits in this task, specifically with action restraint resulting in increased instances of false alarms committed relative to controls[141]. It is important to note, however, that this seems dependent on subtype of diagnosis[142], highlighting the heterogeneity of behavioral inhibition even within a clinical group. By utilizing Go/NoGo and 5-CSRTT together one can differentiate between issues of waiting versus restraint.

The final type of impulsive action I will discuss is action cancellation or the ability to stop an already initiated action. Action cancellation can be measured with the Stop-Signal Reaction Time Task (SSRT task). This task requires a person or mouse to respond as quickly as possible to a go cue, however, a certain time after the presentation of the go cue a stop signal is presented[143, 144]. This requires that an action be stopped after initiation of the motor response. In this task the main measure is the stop signal reaction time, the time in which it takes for the stopping process to occur. From this task probability of stopping curves can also be generated by varying the delay between the go signal and presentation of the stop signal[143]. The SSRT task is a common tool used in the assessment of an ADHD diagnosis, as those with ADHD tend to have slower SSRTs relative to controls[145, 146]. Importantly this task was demonstrated to be a distinct form of impulsive action from both the waiting impulsivity that is tested with 5-CSRTT and action restraint that is examined with Go/NoGo, as its ultimate measure of behavioral inhibition is action cancellation[137, 141].

2.3 IMPULSIVE CHOICE

In addition to these measure of impulsive action, tasks have also been design to measure impulsive choice. As the name replies, this is no longer a measure of inhibition on prepotent motor responses, but choosing a less beneficial outcome because of either an inability for delayed reward gratification or heightened risk-taking behavior[135]. Several tasks have been developed to analyze impulsive choice, such as the Delay Discounting task. This task requires that a participant make a choice between a small immediate reward and a larger reward that is attainable after a delay. Delay Discounting can be done in mice such that an animal is presented with two levers and trained to press for reward. One lever is associated with either delivery of a larger reward, or increased time to access a reward after a set delay. Alternatively the other lever produces immediate presentation of the reward, but at either a reduced amount or reduced access time[147, 148]. An animal or person who has higher measures of delay discounting choose the smaller more frequent rewards over the higher yield option[135].

The Probability Discounting task is similar to Delay Discounting, but probes a different domain of impulsive choice. Probability discounting is a decision of high risk high reward. Normally for this task there is a range of several action-outcome options with the safest being a response or choice that has a 100% probability of resulting in a small reward, while the riskiest action-outcome pairing has a low probability of resulting in a large reward.

It is easy to imagine how deficits in both delay discounting and probability discounting could manifest in disruptive behaviors, deficits which are both present in ADHD. The ability to delay gratification is integral in optimal decision making. Some argue that the inability to delay gratification in the ADHD is an aversion response, because the act of waiting is an uncomfortable act[149]. Alternatively, others have proposed that it is a manifestation of dysregulated motivational

processes[150]. Regardless, the net result is that those with ADHD will make poor long time choices, such as financial choices that may appear as an immediate gain but result in long term losses.

2.4 COGNITIVE INFLEXIBILITY/COMPULSION

The final form of behavioral inhibition discussed herein is behavioral flexibility/compulsivity. This domain represents the ability to suppress previously learned responses when the outcome is no longer advantageous or the contingencies of the circumstance have changed. Several tests already mentioned here measure a subset of this type of behavioral inhibition in the form of perseverative responses. For example, in a task where a mouse is trained to nose-poke a stimulus a perseverative response would be additional nose-pokes to the stimulus after the initial rewarded response. These additional responses provide the animal with no additional benefit. This behavior is classically considered to align with compulsive behaviors,[151] however, in some contexts it is possible that these maladaptive behaviors may appear to be impulsive behaviors instead.

To more specifically target behavioral inflexibility, there are several types of operant tasks available. These tasks are generally measured in three different forms, extinction, set-shifting, and devaluation (contingency degradation) [152, 153]. With extinction assays an animal is trained for a specific action-outcome pairing. The reward component is then removed and the time it takes for the animal to cease responding to stimulus is recorded. Alternatively, the contingencies necessary to obtain a reward are switched from the parameters that the animal was originally trained on. For example, an animal could be trained that when it is presented two levers pressing the one lever elicits delivery of reward, then after a set amount of time the rewarded lever is switched to the second lever, and pressing the first lever no longer earns a reward. The time required for the animal to learn the new rules of the task reflects the ability to inhibit previously learned behavioral responses and shift to the new set of behavioral rules[154].

One can also use devaluation to assess behavioral inflexibility. Devaluation paradigms assess the shift between goal-directed behaviors to habitual responding by altering the motivational state in which the task is performed, i.e. responding continues even when it no longer produces on outcome of positive value. Oftentimes in animal models this is done with food satiation. A maintenance of response rates under the devalued condition indicates that habitual responding is occurring rather than goal-directed behavior. Strength of habitual responding and speed at which habitual responding is acquired are generally measures for these types of assays[152, 155]. A variation of this measure is contingency degradation. Instead of looking at perseverative responding in the face of reward devaluation with satiation in the home cage, the reward is now presented freely at set intervals within the operant chamber. Presentation of the reward becomes non-contingent on action-outcome pairings, degrading the value of lever pressing behavior. Thus maintain this behavior is taken as an indication that compulsive or habitual behavior has formed[152]. This point will be discussed more in later chapters, but cognitive flexibility and habitual responding are both present in ADHD and several additional disorders that are thought to have a dopaminergic component[156-158].

2.5 NEURAL SUBSTRATES OF IMPULSIVITY AND DOPAMINE ASSOCIATIONS

Importantly we know that the various nodes of behavioral inhibition mentioned above have distinct neural substrates, which depending on the region could be differentially affected by alterations to the DA system. Specifically many of these tasks involve frontostriatal circuitries but each are associated with specific cortical regions projecting to discrete portions of the striatum. For instance, stopping behavior as measured by the SSRT task is thought to involve the dorsal medial striatum but not the nucleus accumbens core[143]. The subthalamic nucleus, which receives dopaminergic neuromodulation, also plays an important role to stopping behavior such that lesions

to this area seem to cause a deficit in stopping behavior with increased limited holds and an increase in spontaneous activity[143]. Additionally, lesions to the orbitalfrontal cortex result in a slowing of SSRTs[143]. Activation differences in frontal cortical areas have been demonstrated between SSRT tasks and Go/NoGo tasks. For instance patients with lesions in the lateral prefrontal cortex are capable of action cancellation but display deficits in action restraint[159]. Alternatively, the prelimbic/infralimbic cortex seems to play an important role in the performance of 5-CSRTT. Specifically, lesions to these areas result in an increase in premature responding[160]. Of note, increased levels of DOPAC and DA release have been observed in the prefrontal cortex during this task, supporting its role in 5-CSRTT[161]. This is separate of the roles that norepinephrine and acetylcholine have in these regions as they appear to be more important for the attentional aspects of the task[138].

The orbitalfrontal cortex and its projections to the dorsal lateral and dorsal medial striatum have been demonstrated to contribute cognitive flexibility,[155] while also playing a role in perseverative responding in 5-CSRTT[160]. Lesions to the anterior cingulate cortex, however, produce a preference for small immediate rewards, demonstrating its role in impulsive choice[161]. DA modulation also influences impulsive choice. Several studies in rats have demonstrated that changes in the extracellular DA levels in the ventral striatum correlate with impulsive choice and risky decision making[162]. Specifically, DA efflux increases are seen in circumstances of choice and decreased reward probability. Additionally, preference for choosing large risky rewards can be enhanced with AMPH, but reduced with the D2 receptor antagonist flupentixol[163]. Further evidence for the direct role of DA in regulating this type of behavior also comes by manipulating D1 and D2 receptors both in the PFC and ventral striatum to alter impulsive choice[164]. For instance, blockade of D1 receptors in the ventral striatum increased preference for small and safe rewards,

whereas stimulating the D1 receptor optimized risk taking behavior to choose high reward options when the probability was favorable[165].

Importantly, these brain regions which have been linked to impulsivity both receive neuromodulation from DA and project to heavily dopaminergic innervated regions, we also see a confirmation of DA's role in impulsivity with human genetic studies. These include variants of the D2 and D4 receptors[166]. The DAT1 gene has also been implicated. Specifically, those with two copies of the 10-repeat allele, which has historically been associated with ADHD risk, showed a trend toward an increase in false alarms in the Go/NoGo task accompanied by differential activation of several brain regions, some already discussed, including increased caudate activation on nogo trials and several frontal cortex regions[167]. Interestingly, one study has demonstrated that variants associated with ADHD in the DRD4 gene and DAT1 produce an additive slowing effect on the SSRT task in humans.[168] Additionally, the COMT genotype in patients with Parkinson's disease on DA replacement therapy affects impulsive measures in the SSRT task.[169] COMT genotype has also been associated with both impulsive action and impulsive choice within the ADHD population[69, 169-171].

In summation, several lines of evidence indicate an important role for DA in the expression of different domains of impulsivity. However, as discussed, these domains have discreet and dissociable neural underpinnings that can be manipulated independently with various pharmacological agents and by targeting specific brain regions. Additionally, it is not unreasonable to assume that mutations within the DA pathway itself could result in differential enrichment for impulsive phenotypes based on the region of expression. Enrichment in the ADHD population of both impulsive behaviors and genetic mutations associated with the DA system (see Chapter 1) further support this assertion. Thus the DAT Val559 mouse provides an opportunity to understand

how alterations in DAT can affect expression of impulsivity and lead to improved therapeutic endpoints. Additionally, since the mutation is expressed in multiple neurodevelopmental disorders, it also provides an opportunity to understand the origins of impulsivity across distinct clinical phenotypes.

SPECIFIC AIMS

The work herein seeks to understand the effects of lifelong expression of the DAT Val559 variant on behaviors perturbed in ADHD as well as expand the understanding of the underlying molecular changes that contribute to the altered psychostimulant induced locomotor responses previously demonstrated in the DAT Val559 mouse model. To achieve these goals, I pursued the following aims:

1) Assess DAT Val559 mouse for alterations in cognitive, attentional, and impulsivity domains through utilization of the 5-choice serial reaction time task (5-CSRTT), with a specific focus on impulsivity.

2) Utilize additional operant tasks to provide in depth characterization of any behavioral phenotypes observed in 5-CSRTT.

3) Assess DAT Val559 mouse for alterations in cognitive flexibility, specifically focused on alterations that may be present between goal-directed and habitual behaviors.

4) Determine molecular correlates that underlie the altered psychostimulant-induced locomotor response in DAT Val559 animals. Specifically through assessment of post-synaptic proteins known to be activated by AMPH and contribute to the normal AMPH-induced locomotor response.

CHAPTER 3

EVALUATION OF COGNITIVE AND IMPULSIVITY TRAITS IN DAT Val559 MICE USING THE 5-CHOICE SERIAL REACTION TIME TASK

3.1 INTRODUCTION

Multiple rodent models have been advanced to gain insights into the mechanisms driving traits associated with ADHD[172, 173], including models featuring a disruption of DA signaling. Recently, we introduced the DAT Val559 mouse model as a construct valid model of disorders with DAergic dysfunction[134, 174]. Although ADHD was the pathophysiological focus that lead to our identification of the DAT Val559 variant, the mutation was found in a female with BPD[129], as well as two unrelated males with ASD[175]. Notably, ADHD is more prevalent in relatives of BPD subjects[176] and many individuals diagnosed with ASD meet clinical criteria for ADHD[177, 178], suggesting that the DAT Val559 mice may be most properly considered to model DAergic alterations that can lead to distinct clinical trajectories depending on interacting genetic and environmental factors.

Impulsivity is one of the core components of an ADHD diagnosis, and is present in both ASD and BPD, the source of that impulsivity and the type of impulsivity, however, can vary greatly[179]. Understanding the underlying driving force of impulsivity can guide future circuit-based evaluations of mechanism and possibly assist in the development of better treatments.
Previous work with this model has already assessed it for hyperactivity domains. The DAT Val559 mice have no overt hyperactivity behaviors, but rather a hyper-reactivity termed "darting[174]." This makes the DAT Val559 mouse a particularly useful model to use in more operant style test settings

as the results of which could otherwise be confounded by a hyperactive model. As such, I sought to define altered behavioral characteristics that were in line with the symptomatology of ADHD, keeping in mind the mutation's presence in ASD and BPD.

Based on the behavioral symptoms that were predominant in the boys and maternal grandmother with the DAT Val559 mutation[127], I expected to see behavioral perturbations associated with issues of impulsivity. However, I did not want to miss any opportunity that could reveal alterations in aspects of attention or cognition. As such, I focused my initial exploration into the altered behaviors of the DAT Val559 mouse model with the 5-Choice Serial Reaction Time Task (5-CSRTT). The choosing of this initial task was very deliberate as it produces a wide array of data that simultaneously allows for the assessment of attention, cognition, and impulsivity. Furthermore, the assay has a wide variety of flexible variables, that when individually manipulated allows for closer assessment of attention and impulsive behaviors[180]. Additionally, the results from 5-CSRTT are highly translatable to the human literature as the task and related ones are often used as investigative tools in the human population[136]. Utilization of this task allowed for the identification of several key behavioral changes that will be discussed herein and forthcoming chapters.

3.2 METHODS

Animals: All experiments were performed under a protocol approved by the Institutional Animal Care and Use Committees at Vanderbilt University and Florida Atlantic University. Homozygous DAT Val559 and WT littermate mice used in the study were bred from heterozygous breeders on the hybrid background used in our prior studies[134] (75% 129S6 and 25% C57BL/6J). Males were evaluated in the present study owing to the bias toward male subjects for ADHD diagnoses [4]. Animals were housed on a 12:12 (L: D) cycle. Mice were tested during their active cycle, achieved

by either raising animals on a reverse light cycle with lights on and off at 3 pm and 3 am, respectively, or by raising mice on a normal light cycle, with lights on and off at 7 am and 7 pm and then with transferal of mice to the reverse light cycle at 5 weeks of age, after weaning. Mice were approximately 6-7 weeks old when training for different behavioral assays commenced. For all operant conditioning tasks mice were placed on food restriction one week prior to the start of training. Mice were brought to approximately 85%-90% of their baseline weight (weighed every other day). On the fourth and fifth days (still under food restriction), mice were exposed to 33% Vanilla Ensure Original, the reward used for all the operant tasks, for one hour in the home cage. Animals were run under red light, with house lights off in the operant chambers.

5-CSRTT: I utilized 5-CSRTT as it allows for a variety of traits to be measured including gross attention impairments (omissions), impulsivity issues (premature responses and preservative responses), and sustained attention (accuracy). Additionally, acquisition time of the task can be used as an indication of learning deficits that could result from difficulties in the above measures. 5-CSRTT performance can also be aligned with measures gathered from the continuous performance test, a common task used to assess and diagnose children with ADHD [139, 181]. I implemented the 5-CSRTT using Bussey-Saksida Mouse Touch Screen Chambers (Lafayette, IN) with a mask forming five 4 cm x 4 cm square touch zones arranged horizontally. All testing occurred under red light. See Figure 3A for training schematic and 3B for paradigm diagram. During the Habituation phase of training, mice were exposed to the testing chamber for 30 min during which a 30 µl liquid reward was delivered every 10 sec coinciding with illumination of the reward receptacle. The 10 sec reward delivery timer was reset by head entry into the reward receptacle. Mice had to collect 30 rewards on two consecutive days to move to the Initial Touch phase of training. During the Initial Touch phase of training, one of the five touch zones was

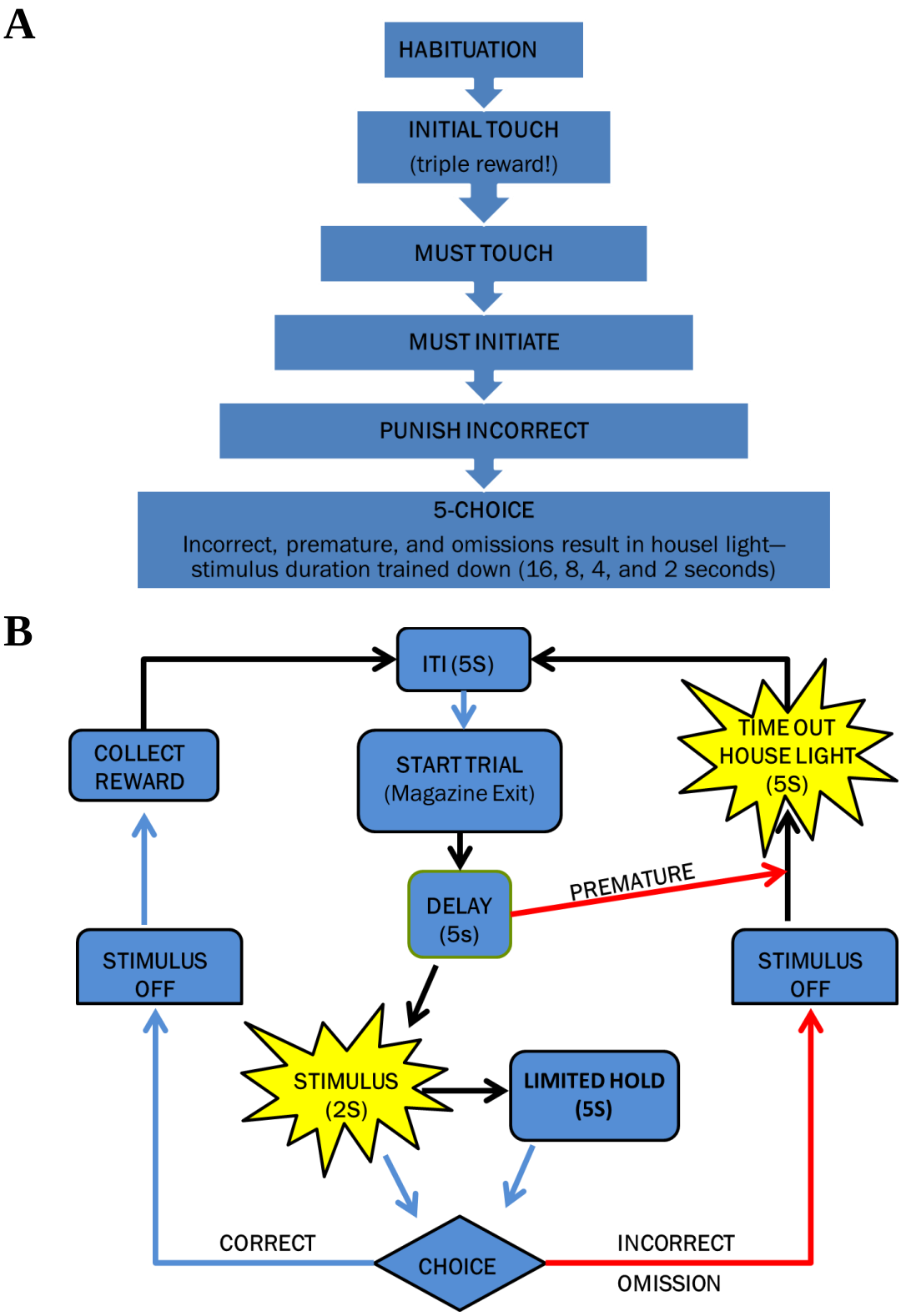


Figure 3. Training Schedule of 5-CSRTT (A) and paradigm schematic (B).

randomly illuminated for a 30 sec interval. Mice received reward regardless of touching the screen but received 3X reward if touch occurred during illumination, accompanied by a tone and illumination of reward receptacle. Mice had to collect 30 rewards in 30 min in order to move on to the Must Touch phase of training. During the Must Touch phase, single touch panels were illuminated pseudo-randomly (could not appear in the same location more than 3X consecutively) in 1 of the 5 locations. Illumination remained on until the correct location was touched. Blank touches (unlit locations) were not punished. Upon touching an illuminated panel, reward was delivered with concurrent tone and illumination of the reward receptacle. Upon head entry to receive reward, a five sec inter-trial interval (ITI) was initiated, followed by touch zone illumination to restart a trial. Mice had to collect 30 rewards in 60 min to move on to the Must Initiate phase of training. In the Must Initiate Phase, the session was initiated with delivery of a free reward. Once the reward was collected, a single touch zone was illuminated. Upon touch of this panel, reward was delivered concurrently with tone and reward receptacle illumination, followed by a 5 sec ITI. At this point, the reward receptacle was re-illuminated and mice were required to make a head entry into the reward receptacle to initiate a trial, at which time a single touch zone was illuminated to repeat the process. Mice had to collect 30 rewards in 60 min to move on to the Punish Incorrect phase.

In the Punish Incorrect phase, touch of an incorrect panel during stimulus presentation resulted in the stimulus being immediately turned off and the chamber being fully illuminated for a five sec time out. Subsequently, a five sec ITI was imposed before the receptacle light came on again for the initiation of a new trial. Mice had to complete 30 trials in 30 min at an accuracy of 80% correct before they could move on to the 5-choice evaluation phase. During the 5-choice phase, mice learned to respond to the illuminated zone with a progressively shorter stimulus presentation

duration (16s, 8s, 4s, and 2s). Mice had to initiate the trial and wait through a 5 sec delay before presentation of the stimulus. Responses had to occur either within the stimulus presentation or within a 5 sec window post-stimulus presentation (limited hold). Mice received a 5 sec timeout with the house light on if they responded during the 5 sec delay (before stimulus presentation but after trial initiation), made an incorrect response, or omitted a response (no response within stimulus duration plus 5 sec limited hold). If mice made a premature response the trial was reset. Mice were required to complete 80 trials in 60 min with at least 80% accuracy and less than 20% omissions for two days in a row before being moved to a shorter stimulus duration of the training series. All mice were brought to a baseline performance which consisted of trial initiation, 5 sec delay, 2 sec stimulus presentation, 5 sec limited hold (7 sec to make a response), reward delivery, and a 5 sec ITI. Measures collected for this assay included % correct, % omission, number of premature responses, number of perseverative responses, and session length. Once this was reached, the basic 5-CSRTT protocol as described above was further manipulated in a number of ways to more specifically probe attention and impulsivity. The individual manipulations done to this paradigm are listed below as well as what specific measures were being probed and the outcome measures that are potentially affected by said protocol changes.

Attentional Processes:

Short Stimulus Duration—In this version the stimulus duration was changed from 2 to 0.5 seconds for all 80 trials. The shorter stimulus increases the attentional load on the animal. Measures that are affected by this are accuracy and omissions.

Randomized Variable Short Stimulus Duration—In this version of the task the duration of the stimulus is further manipulated such that Instead of a 2 second stimulus duration the stimulus is a randomized set of 4 stimulus duration possibilities of 0.25, 0.5, 1, and 2 seconds. The shorter

stimulus durations combined with the randomized unpredictability of their presentation further increases the attentional load needed to perform the task. Again affecting measures of accuracy and omissions similarly to the short stimulus duration.

Impulsivity Measures:

Long Delay--A long delay session was performed in which the mice had to wait 15 sec between trial initiation and onset of stimulus. Measures that could be altered with an increase in delay times are number of premature responses.

Randomized Variable Delay--A separate set of trials was implemented where a 2, 5, 10, and 15 sec delays were intermixed randomly (20 trials of each). Variable delays have been indicated to increase measures of impulsivity as measured by premature responses.

Statistical Analyses: Statistical analyses was done using GraphPad Prism 7 software package. Statistical significance was set at $P < 0.05$ for all experimental results. The type of statistical analysis used was determined independently for each experiment and is listed in the relevant figure caption. Outlier analysis was performed using the ROUT method with the false discovery rate set at 2%.

3.3 RESULTS

DAT Val559 mice acquire 5-CSRTT faster than WT littermates. To explore multiple cognitive capacities, as well as impulsivity, of the DAT Val559 mice, I first evaluated their performance on the 5-CSRTT. During task acquisition (9 Phases), I observed no deficits in task acquisition compared to WT littermates (Figure 4A & 2B; WT n=18, VaL559, n=19). Instead, DAT Val559 mice progressed more quickly through both the early training phases (habituation-punish incorrect) and during the stimulus reduction phases than WT animals. Although accuracy assessed in the full 5-CSRTT was largely equivalent, I detected a difference on day 1 of testing (16 sec stimulus

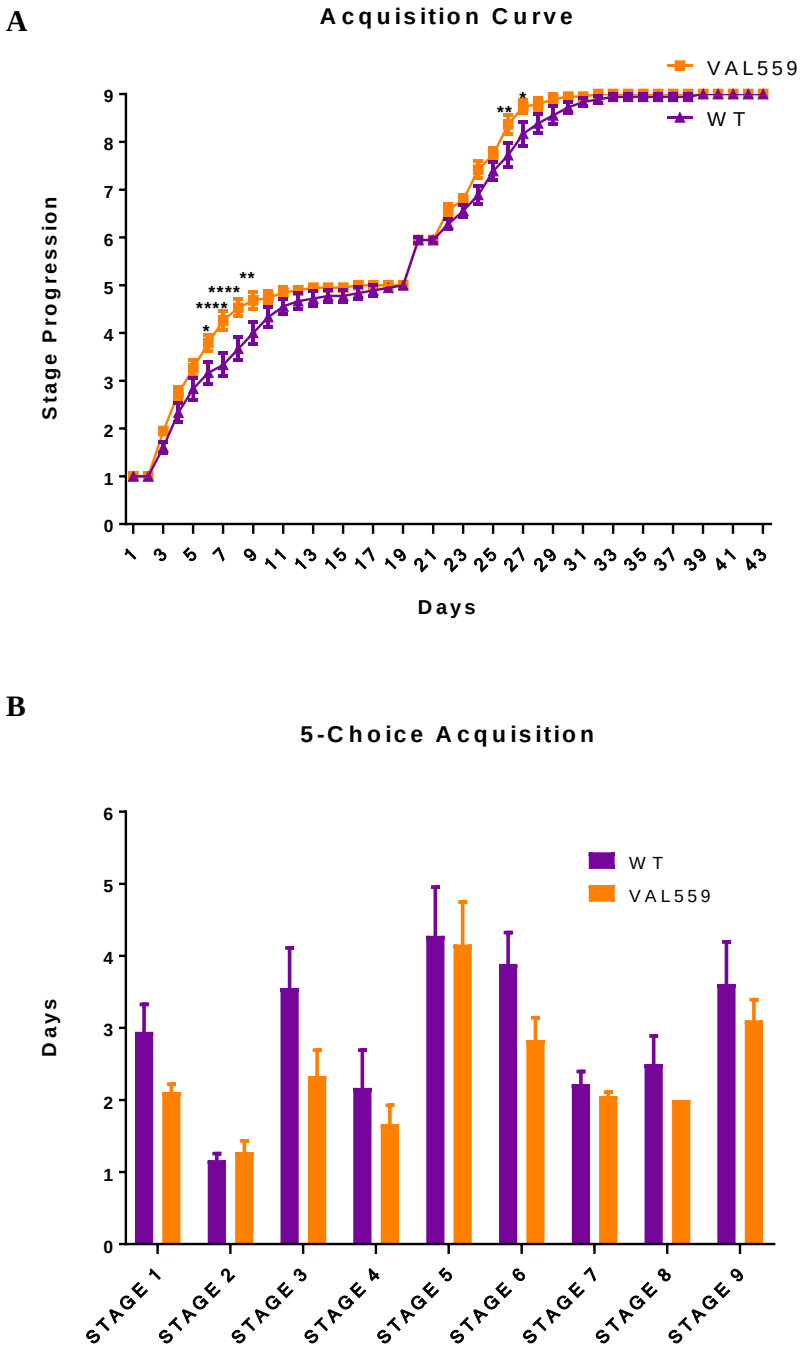


Figure 4. DAT VAL559 mice show alterations in 5-CSRTT acquisition. DAT Val559 mice learn 5-choice serial reaction time task (5-CSRTT) faster than their WT littermates. (WT=18; VaL559=19; Two-Way RM-ANOVA, time $P < 0.0001$, interaction $P < 0.0001$, genotype $P < 0.01$; post-hoc tests reveal $P < 0.05$ at day 6 and 27, $P < 0.01$ at day 9 and 26, and $P < 0.0001$ at Day 7 and 8) (A). Acquisition differences in 5-CSRTT (Two-Way ANOVA, main genotype effect $P < 0.001$ and main stage effect $P < 0.0001$ with no interaction) (B).

duration), which may also suggest that retention of the rules of the test were more effectively stored or retrieved in DAT Val559 mice (Figure 5A). In contrast to this, I saw no global differences in animal performance working down to the baseline protocol of 5-CSRTT in premature responses, perseverative responses, omissions, activity levels as measured by beam breaks, and experimental session length (Figure5B-F). There was a trend, however, for WT animals to have slightly higher omission rates (Figure 5D).

DAT Val559 mice demonstrated no deficits in attentional processing in 5-CSRTT. Additional assessment of attentional measures, as probed by increasing the attentional load of the task with a shorter 0.5 sec stimulus duration and a randomized variable stimulus duration, demonstrated that there were no genotype differences between WT and DAT Val559 mice. Measures of accuracy (Figure 6A and 6D) and omissions (Figure 6B and 6E) were equivalent. Additionally, though not surprisingly, I saw no alterations in premature responses (Figure 6C and 6F).

DAT Val559 mice demonstrate waiting-dependent impulsivity in 5-CSRTT. Impulsivity can be assessed in the 5-CSRTT during training as well as when temporal variables are manipulated following training[96, 182]. DAT Val559 mice exhibited no differences in premature responses compared to WT during training (Figure 5C). However, when the delay between trial initiation and stimulus presentation was extended from the 5 sec delay used during training to a 15 sec delay, I detected a significant increase in premature responses in DAT Val559 mice (Figure 7A; WT n=15, VaL559, n=19), indicative of a waiting impulsivity[137]. Interestingly, when multiple delay times (2, 5, 10, and 15 secs) were randomized and delivered across a session, preventing a predictable use of time as a preparatory cue, DAT Val559 mice displayed fewer premature responses than WT animals (Figure 7B). When responses were analyzed for each of the delay values used, a trend was observed across all the tests, reaching statistical significance at the 15 sec

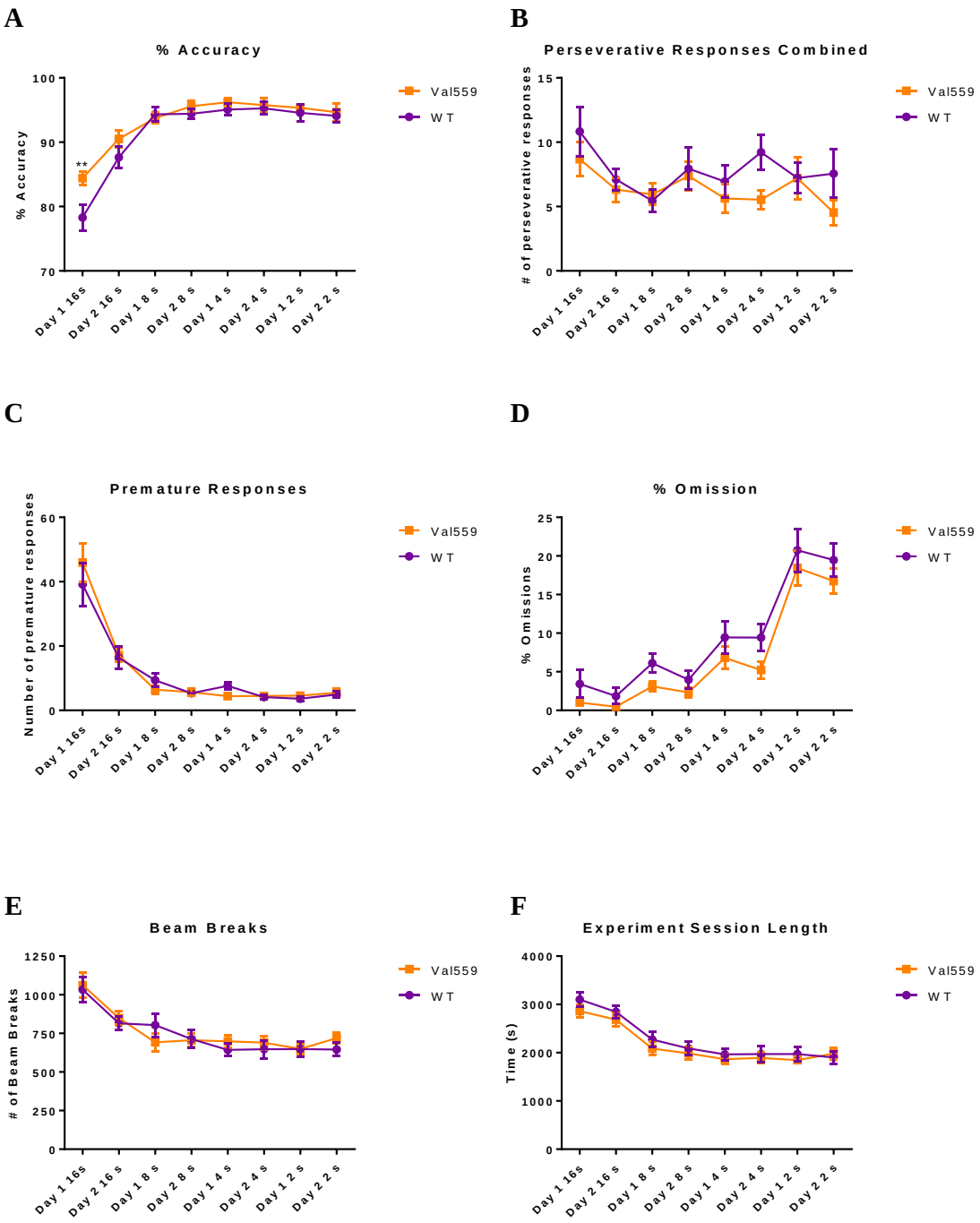
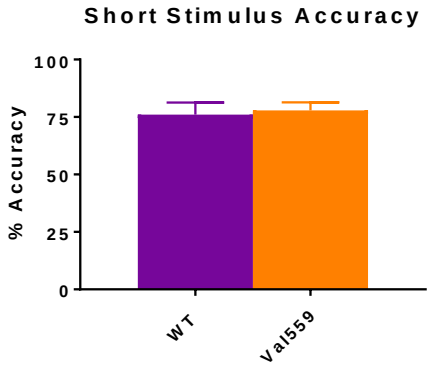
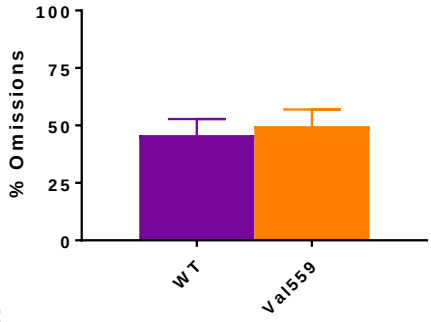


Figure 5. Baseline performance of the 5-choice task working from a 16s stimulus to a 2s stimulus duration. DAT Val559, similar to their faster acquisition of the task, also display better Accuracy on the 5-choice task on their first day experience of the full paradigm at a 16s stimulus duration (WT=18; VaL559=19; Two-Way RM-ANOVA, Time $P < 0.09$, Interaction $P < 0.0001$,Genotype $P < 0.05$; post hoc tests reveal $P < 0.01$ at Day 1 16s) (A). No genotype differences were observed for Perseverative Responses (B), Premature Responses (C), Omissions (D), Beam Breaks (E), and Experiment Session Length (F). There is a trend in % Omission, however, at genotype $P = 0.0568$.

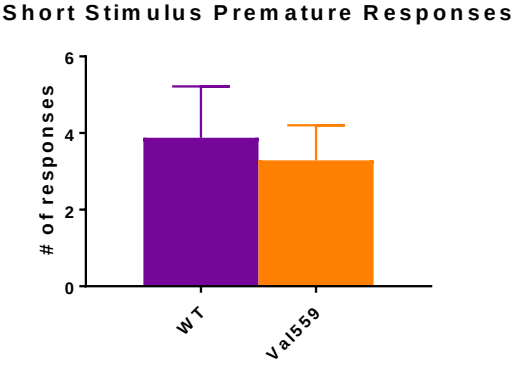
A


Short Stimulus Accuracy
% Accuracy
100
75
50
25
0
WT
Val559

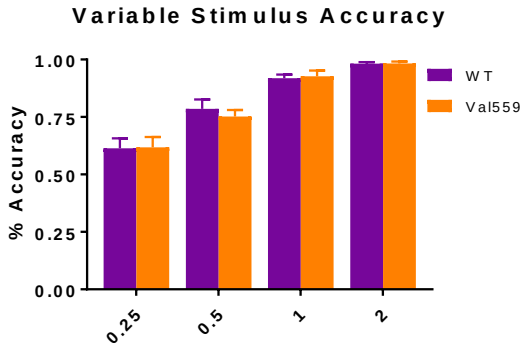
B


Short Stimulus % Omissions
% Omissions
100
75
50
25
0
WT
Val559

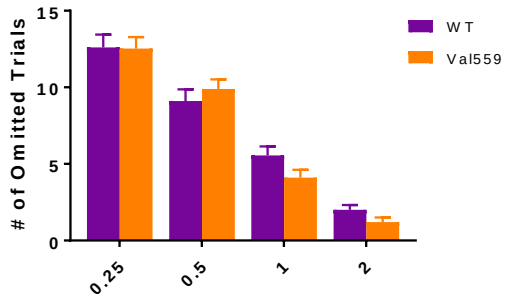
C


Short Stimulus Premature Responses
# of responses
6
4
2
0
WT
Val559

D


Variable Stimulus Accuracy
% Accuracy
1.00
0.75
0.50
0.25
0.00
0.25
0.5
1
2
WT
Val559

E


Variable Stimulus Omissions
# of Omitted Trials
15
10
5
0
0.25
0.5
1
2
WT
Val559

F

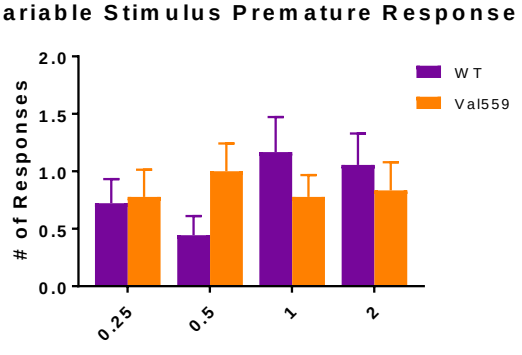

Variable Stimulus Premature Response
# of Responses
2.0
1.5
1.0
0.5
0.0
0.25
0.5
1
2
WT
Val559

Figure 6. DAT Val559 mice show no deficit in attentional processing as measured through 5-CSRTT. DAT Val559 mice show no difference in accuracy compared to WT mice when the stimulus was been shortened to 0.5 seconds (two-tailed Student's t-test, accuracy responses $P > 0.05$; WT = 8; Val559 = 7) (A). DAT Val559 and WT mice have equal measures of omissions under a short stimulus condition (two-tailed Student's t-test, % omissions $P > 0.05$) (B). Additionally, no differences in premature responses were seen between genotypes (two-tailed Student's t-test, premature responses $P > 0.05$) (C). No genotype difference was present for accuracy under a variable stimulus duration condition, though there was a significant main effect of stimulus duration on accuracy (Two-way ANOVA, genotype $P > 0.05$, interaction $P > 0.05$, stimulus duration $P < 0.0001$; WT = 18; Val559 = 19) (D). No genotype difference was seen in number of omitted trials with a variable stimulus duration, though there was a main effect of stimulus duration on number of omitted trials (Two-way ANOVA, genotype $P > 0.05$, interaction $P > 0.05$, stimulus duration $P < 0.0001$; WT = 18; Val559 = 19) (E). No genotype differences were seen in premature responses across stimulus durations (Two-way ANOVA, genotype $P > 0.05$, interaction $P > 0.05$, stimulus duration $P > 0.05$; WT = 18; Val559 = 18) (F).

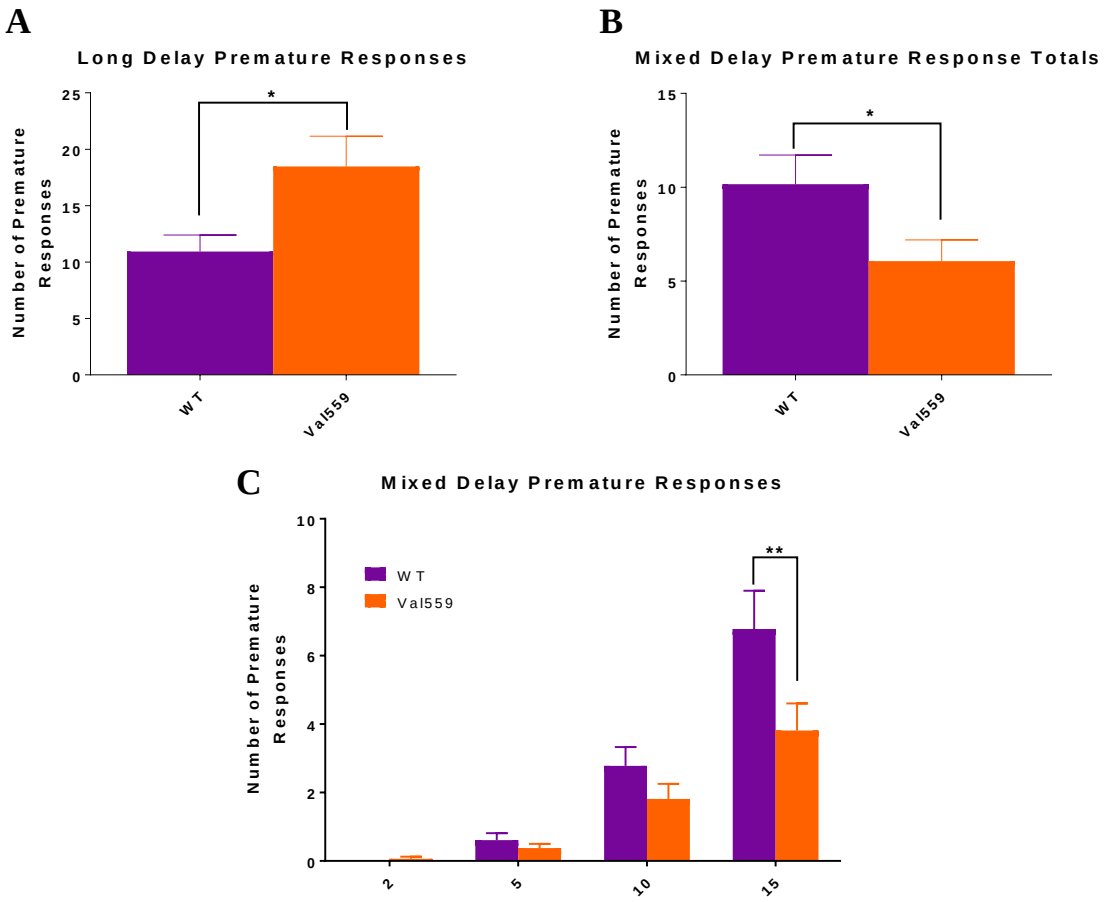


Figure 7. DAT Val559 mice show alterations in impulsivity on the 5-CSRTT. DAT Val559 mice display a higher impulsivity than WT mice when the delay between the start of a trial and stimulus presentation is increased from 5 sec to 15 sec (two-tailed Student's t-test, premature responses $P < 0.05$; WT=15; VaL559=19) (A). DAT Val559 mice display reduced impulsivity compared to WT mice when the delay between the start of a trial and stimulus presentation is variable/unpredictable (two-tailed Student's t-test, premature responses $P < 0.05$; WT = 15; Val559 = 19). (B) Additionally, when premature responses were separated out based on the delay duration, DAT Val559 mice specifically show a reduction at the 15 sec duration (delay $P < 0.0001$, genotype $P < 0.05$, interaction $P < 0.05$; post-hoc reveals a genotype difference at the 15 sec delay, $P < 0.01$) (C).

delay (Figure 7C).

3.4 DISCUSSION

Owing to elevated impulsivity traits in our two DAT Val559 expressing probands as well as the maternal transmitting grandmother[127], I initiated my testing efforts with the 5-CSRTT, a test that can reveal impairments in behavior ascribed to facets of attention, learning and impulsivity[180, 181, 183]. DAT Val559 mice demonstrated no deficiencies in learning the 5-CSRTT. Indeed, these animals reached task criteria during training more rapidly than their WT littermates. Although initially surprising, these findings may relate to studies in humans reporting a stronger association of DAT polymorphisms with impulsivity versus inattention[184], as well as brain activity linked to inhibitory response control versus attentional orienting[185]. This is particularly salient as when I specifically challenged the mice with both the short stimulus duration and the randomized variable stimulus duration to look at deficits in attention I observed no difference between genotypes.

Additionally, when DAT Val559 mice were challenged to withhold responses for three times the length of the fixed delay imposed during training (5s to 15s), they demonstrated significant premature actions, suggestive of impulsivity. Interestingly, when tested with a variable delay, DAT Val559 animals demonstrated reduced premature responses that became increasingly evident as the delay was extended, demonstrating a differential effect of timing context on impulsive responding. These results are interesting with respect to recent findings that performance of subjects with ADHD is context dependent, being diminished relative to controls when simple two choice tests are used, versus a more complex five choice option[186]. Additionally, studies done in AMPH-treated rats have shown a connection between training context and waiting, such that performance improves on a response inhibition timing task in a variable timing climate, compared to an increase in premature responses when treated rats are tested in a fixed timing climate[187]. As

such, this suggests that the chronic state of increased extracellular DA induced by the DAT Val559 results in the DAT Val559 mice using timing cues differently. This brings into question the idea that impulsivity could derive from a misuse of timing cues and that when timing is no longer a factor (as with a variable climate) the impulsive deficit is also no longer a factor.

CHAPTER 4

ASSESSING IMPULSIVITY DOMAINS AND CONTRIBUTIONS FROM INTERVAL TIMING AND MOTIVATION IN THE DAT Val559 MOUSE

4.1 INTRODUCTION

DAT Val559 mice have alterations in both learning and impulsivity compared to WT littermates. The lack of detrimental effects on learning in the DAT Val559 mice aligns with the idea that DAT variants play a bigger role in impulsivity than in attention.[168, 171, 184] Indeed, further testing in 5-CSRTT revealed no attentional differences in DAT Val559 mice. However, this does not explain the acceleration in task acquisition that was observed in the DAT Val559 mice. In fact, the increased acquisition speed could be indicative of changes in motivation/task salience. This is in line with previous work correlating extracellular DA levels with the motivation and performance of operant tasks[188, 189].

In addition to the learning curve alterations, I also demonstrated increases in impulsivity under long delay timing environments, with a rescue in impulsive measures in a variable delay timing environment (preventing delay time from being used as an indicator of when to expect stimulus presentation). As mentioned previously, a growing body of evidence indicates an important role for the DA system and specifically DAT in the ability to interval time[190-196] with disruptions also observed in the ADHD population[197, 198]. This brings into question the driving force behind the impulsivity seen in the DAT Val559 mice. As mentioned in Chapter 2, 5-CSRTT is often used as a measure of waiting impulsivity. Could it be that they are more impulsive under a long delay not

because they have trouble waiting but are perceiving the intervals in faster units relative to their WT littermates? Additionally, I have not determined if these animals have a global deficit in impulsive action or a specific problem with waiting impulsivity. To differentiate between these possibilities additional testing would be required with another paradigm that examines impulsive action.

To examine if the DAT Val559 mice are impulsive in 5-CSRTT due to an alteration in the perception of time I used the peak interval task, which allowed for the assessment of the timing capabilities of the DAT Val559 mice. Additionally, I used the Go/NoGo task to assess if the DAT Val559 mice expressed a global deficit in impulsive action. Finally, I utilized the progressive ratio paradigm to discern differences in motivational levels between the DAT Val559 and WT mice. Given the literature on DA de-regulation and impulsivity, I hypothesized that the increased levels of extracellular DA contribute to the increased learning speed of the DAT Val559 mice by enhancing their motivational levels while simultaneously altering the manner with which the DAT Val559 perceive time, manifesting as a specific waiting impulsivity rather than a global impulsive deficit.

4.2 METHODS

Animals & Statistical Analysis: All experiments were performed under a protocol approved by the Institutional Animal Care and Use Committees at Vanderbilt University and Florida Atlantic University. Homozygous DAT Val559 and WT littermate mice used in the study were bred from heterozygous breeders on the hybrid background used in our prior studies[134] (75% 129S6 and 25% C57BL/6J). Males were evaluated in the present study owing to the bias toward male subjects for ADHD diagnoses [4]. Animals were housed on a 12:12 (L: D) cycle. Mice were tested during their active cycle, achieved by either raising animals on a reverse light cycle with lights on and off at 3

pm and 3 am, respectively, or by raising mice on a normal light cycle, with lights on and off at 7 am and 7 pm and then with transferal of mice to the reverse light cycle at 5 weeks of age, after weaning. Mice were approximately 6-7 weeks old when training for different behavioral assays commenced. For all operant conditioning tasks mice were placed on food restriction one week prior to the start of training. Mice were brought to approximately 85%-90% of their baseline weight (weighed every other day). On the fourth and fifth days (still under food restriction), mice were exposed to 33% Vanilla Ensure Original, the reward used for all the operant tasks, for one hour in the home cage. Animals were run under red light, with house lights off in the operant chambers.

Peak Interval Task: To evaluate the ability of mice to estimate time as one possible explanation for impulsivity, I implemented a Peak Interval Task**.** Animals were tested in Med-Associates (St. Albans, VT) operant chambers housed in sound attenuation boxes. For the first 2 days of training, mice were presented with a free reward and given 5 sec to consume the reward upon head entry. Reward was then presented every 10 sec after reward consumption, unless a nose-poke was made in any of the 3 nose-poke holes (backlit by LEDs), which elicited reward delivery (a Fixed Ratio 1-Fixed Time 10 secs schedule; FR1-FT10). Sessions lasted 30 min and mice could earn up to 30 rewards. Days 3 and 4 utilized an FR1-FT30 schedule. On day 5, mice were moved to continuous reinforcement. Under this protocol only the center back nose-poke hole was lit and mice had to nose-poke to get a reward. Mice were given 60 min to obtain 30 rewards. Once all mice could collect 30 rewards within 60 min, they were moved to either a fixed interval schedule of 5 sec or 15 sec. The center nose-poke would illuminate, but it was only the first nose-poke after 5 or 15 sec of illumination that was rewarded. A 10 sec limited hold from the fixed interval mark was used. If mice made a nose-poke within that time, the nose-poke light went off, a click sounded, and the dipper was brought up. If no response was made within the limited hold, the nose-poke light went

off and an omission was counted. Mice had 5 sec to consume the reward upon head entry into dipper. Trials were separated by a variable ITI that averaged 11 sec between reward collection or response omission and center nose-poke re-illumination. Mice were trained on FI 5 sec or FI 15 sec until their response latencies stabilized. Mice were then moved to Peak Interval testing. This task functioned very similarly to Fixed Interval except with the addition of probe trials. During the probe trial, the nose-poke hole was lit for 3X the length of the fixed interval time, however, nose-poke responses produced no reward, allowing for a response curve to be recorded. Gaussian curves generated from the responses in the probe trials were used to provide information on interval timing variability (peak spread), maximum response rate (peak height), and timing accuracy (x-axis location of peak).

Go/NoGo Task: As a secondary measure to look at a different aspect of impulsivity, I used the Go/No-Go task. This assay is distinct from the impulsivity measures obtained in the 5-CSRTT in that Go/No-Go requires the mouse to learn to distinguish different signals associated with operant response and behavioral restraint in order to obtain a reward, making it a measure of motor inhibition/suppression versus a measure of waiting capabilities[135, 137, 199]. Mice were initially trained in the same manner as they were for 5-CSRTT up through the Punish Incorrect phase (see Chapter 3 methods), except mice had two larger (7.6 cm x 7 cm) touchscreen windows to interact with instead of five. Once mice completed the Punish Incorrect phase they were initially moved directly into Go/NoGo (60 trials, 20% No-Go trials). However, this was changed to Go-only trials upon failure of mice to complete the number of required trials and inability to acquire the No-Go behavior. Go-only trials consisted of trial initiation, 5 sec delay, and then the presentation of the stimulus in one of the two touchscreen windows for 3 sec with a 5 sec limited hold. The No-Go signal involved having both touchscreen windows lighting up together (Figure 8). Sessions

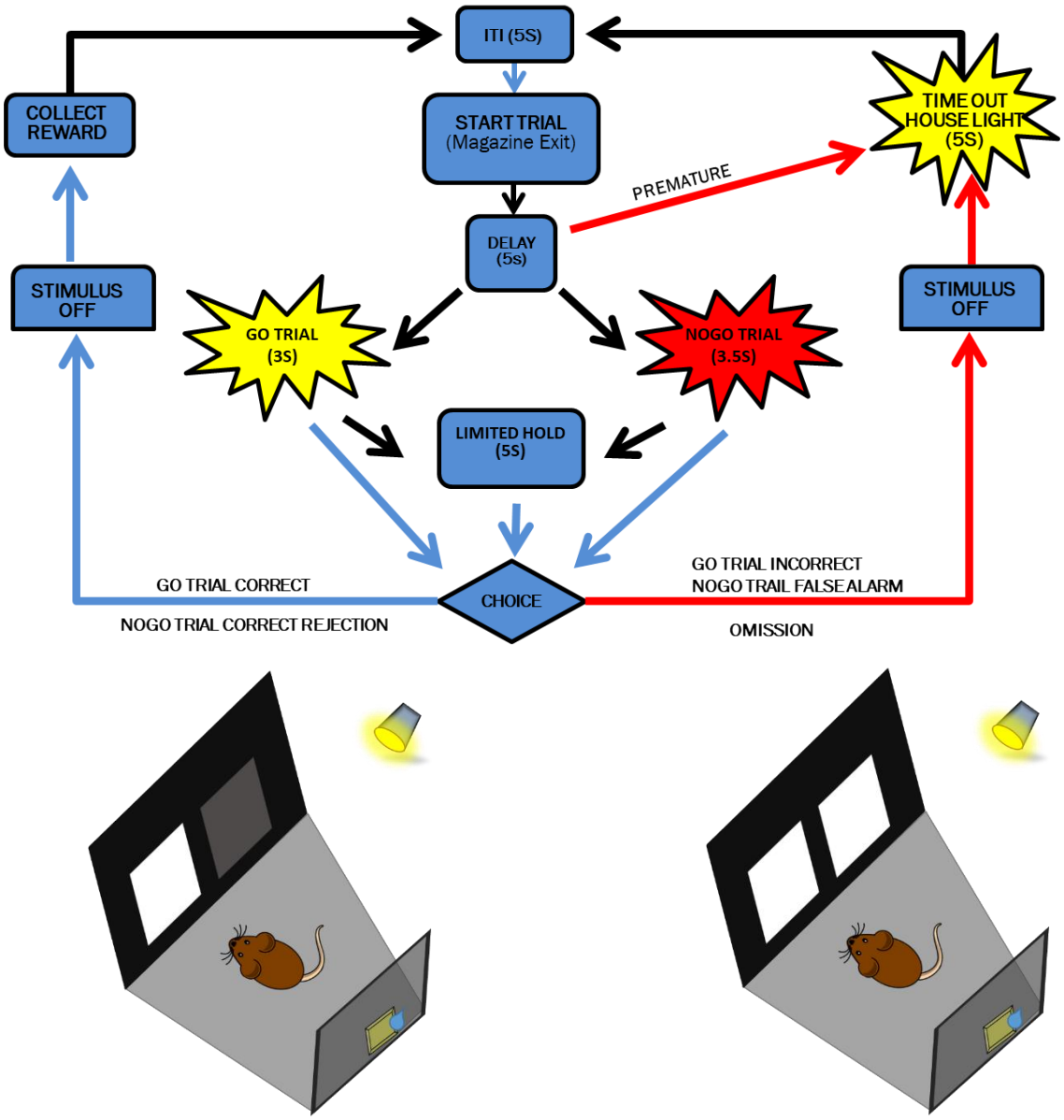


Figure 8. Go/NoGo paradigm schematic with cartoon representation of touchscreen chamber.

consisted of 30 trials within 30 min. Mice received 8 training sessions on go-only and were then moved to Go/NoGo, where mice were required to complete 30 trials within 30 min with 50% of the trials being No-Go trials. Mice were trained on Go/NoGo until stable performance rates were achieved (13 days). Mice were then tested in a single session where the delay between trial initiation and stimulus presentation was extended from 5 to 15 secs. The main dependent measures were omissions, premature responses, and correct rejections during No-Go trials (mice withheld responding).

Progressive Ratio: To evaluate motivation for reward, mice underwent 2 days of habituation to operant chambers with dipper training. Every 3 min of a 30 min session, mice received a free reward, but were able to obtain reward sooner with a nose-poke into the center hole opposite the dipper. The dipper remained raised until mice made a head entry into the food receptacle, and then animals had 10 sec to consume the reward. Mice were then moved to a continuous reinforcement (CRF) paradigm, where a nose-poke to the center hole on the opposite wall of the food receptacle was required for a reward. Nose-poke holes were also located to the left and right of the food receptacle and were used to measure non-specific nose-poke activity. Mice were required to make 50 corrects responses in 45 min, and given 10 sec to drink their reward. After 2 consecutive days of successful completion, mice were trained for an additional week (6-7 training sessions) at 50 correct responses in 45 minutes, with 5 sec to drink their rewards. Mice were then run through a progressive ratio schedule, requiring an increasing number of nose-pokes to obtain a reward. Sessions were ended after 60 min had elapsed or if the mouse was inactive for 5 minutes, whichever came first. Several measurements related to motivation are recorded, including the break point (the nose-poke requirement at which the mouse gives up), value met (last successful round of nose-pokes met before the mouse gives up), response rate, number of correct responses

made, and number of rewards earned. Animals were tested in Med-Associates (St. Albans, VT) operant chambers housed in sound attenuation boxes.

Sucrose Preference Test: To assess reward salience, I subjected mice to the Sucrose Preference test, singly housing mice with access to two bottles filled with water for a 2 day habituation, followed by substitution of water in 1 bottle with a 1% sucrose solution. On day 5, the sucrose solution was increased to 3%. Bottles were weighed daily and switched in placement. Sucrose consumption was calculated as the amount of sucrose solution consumed divided by total liquid consumption.

Statistical Analyses: Statistical analyses was done using GraphPad Prism 7 software package. Statistical significance was set at $P < 0.05$ for all experimental results. The type of statistical analysis used was determined independently for each experiment and is listed in the relevant figure caption. Outlier analysis was performed using the ROUT method with the false discovery rate set at 2%.

4.3 RESULTS

DAT Val559 mice display normal interval timing capacity with an increased response rate with increased waiting load. I hypothesized that the waiting impulsivity of DAT Val559 in the long delay condition in 5-CSRTT could be driven by a faster internal clock for interval timing. Agents and mutations that impact DA signaling, and DAT specifically[187, 192, 193, 200], have been demonstrated to impact interval timing, the ability of animals to accurately report time between cue onset and an imposed response delay. To look at the ability to time intervals we implemented the Peak Interval paradigm (Figure 9A). I observed no significant difference between the estimated peak for delay timing whether at 5 sec or 15 sec between the DAT Val559 and WT littermates (Figure 9B and 9C; WT, n=10 and 9 respectively, VAL559, n=10 and 10 respectively). However,

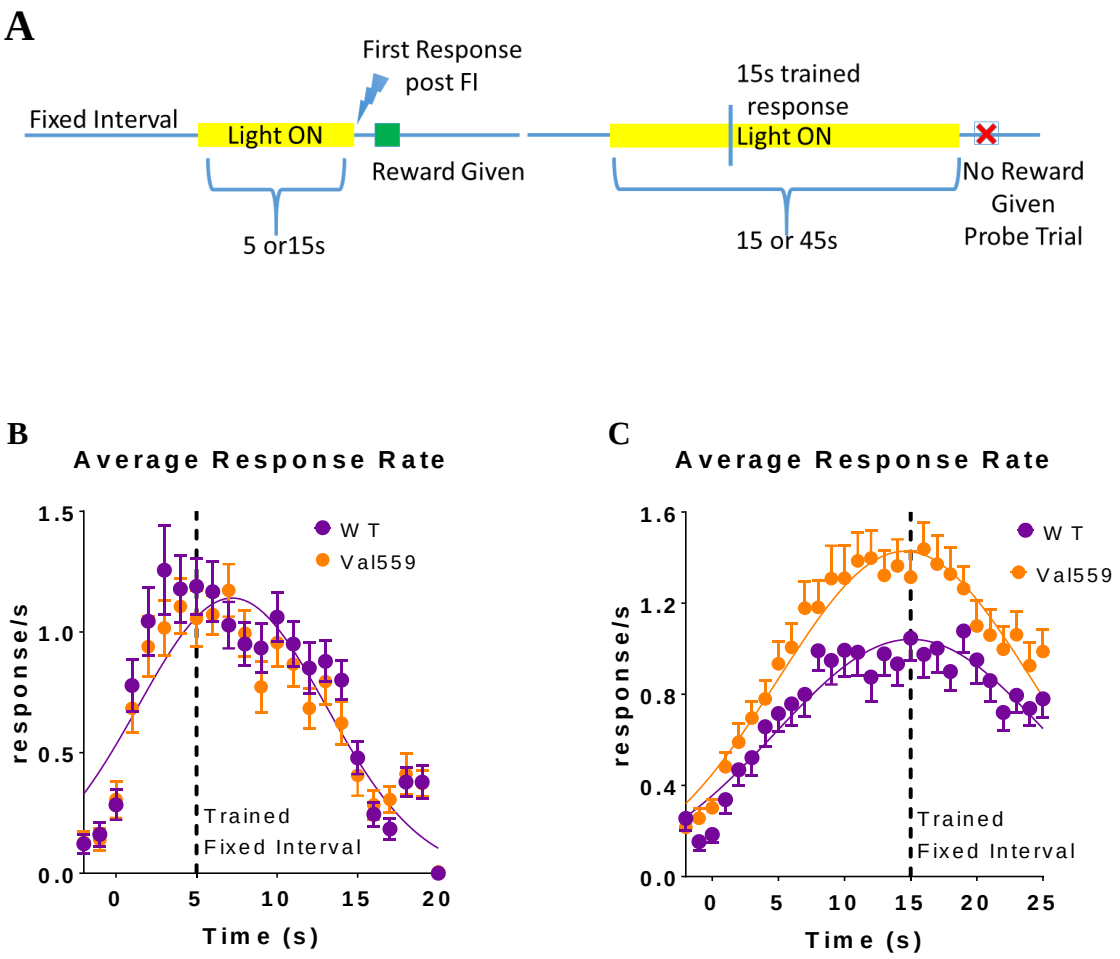


Figure 9. DAT Val559 mice have normal interval timing, but heightened response rates with longer wait times. Training schematic for Peak Interval task. Mice were trained on a fixed interval of 5 sec or 15 sec (WT=10 and 9 per training group, Val559=10 per training group) (A). Mice were then tested on a probe trial for accuracy of response timing. Both WT and Val559 mice display the same response curves at the 5 sec timing (Two-Way RM-ANOVA; interaction $P > 0.05$, time $P > 0.05$, genotype $P > 0.05$) (B). However when the interval is increased to 15 sec, DAT Val559 display an increase in response rate amplitude (Two-Way RM ANOVA; interaction $P < 0.0001$, time $P < 0.0001$, genotype $P = 0.0557$) (C).

at the 15 sec interval DAT VAL559 demonstrated a significantly elevated response rate (Figure 9C).

Alterations in Go/NoGo reveal that DAT Val559 mice can withhold responding when a withheld response has a probability of being rewarded. Given our findings related to impulsivity in the 5-CSRTT, I decided to utilize another test commonly implemented to assess this trait, the Go/NoGo paradigm. In this test, I detected a difference in acquisition of the task with DAT Val559 progressing through habituation to the punish incorrect phase faster (Figure 10A; WT, n=8, Val559, n=12), though for this task this effect predominantly seems to be driven by differences in task performance on stage 1 (Figure 10B). During the Go/NoGo sessions, when examined across sessions, DAT Val559 and WT mice initially demonstrated an equal percentage of omissions. By session 8, however, DAT Val559 mice demonstrated significantly more omissions than WT animals (Figure 11A). Increased omissions did not reflect an inferior recognition of the rules of the test as DAT Val559 were able to withhold responding during NoGo trials just as well as WT animals, if not better, as reflected by session correct rejections (Figure 11B). Moreover, no significant differences were detected across days for premature responding (Figure 11C). Next, in a single session, I extended the delay between trial initiation to stimulus presentation from 5 sec to 15 sec. This alteration produced no significant differences in percent omissions (Figure 11D). However, as with their superior performance during training, DAT Val559 mice made significantly more correct rejections than WT animals (Figure 11E) in the absence of significant premature responses (Figure 11F).

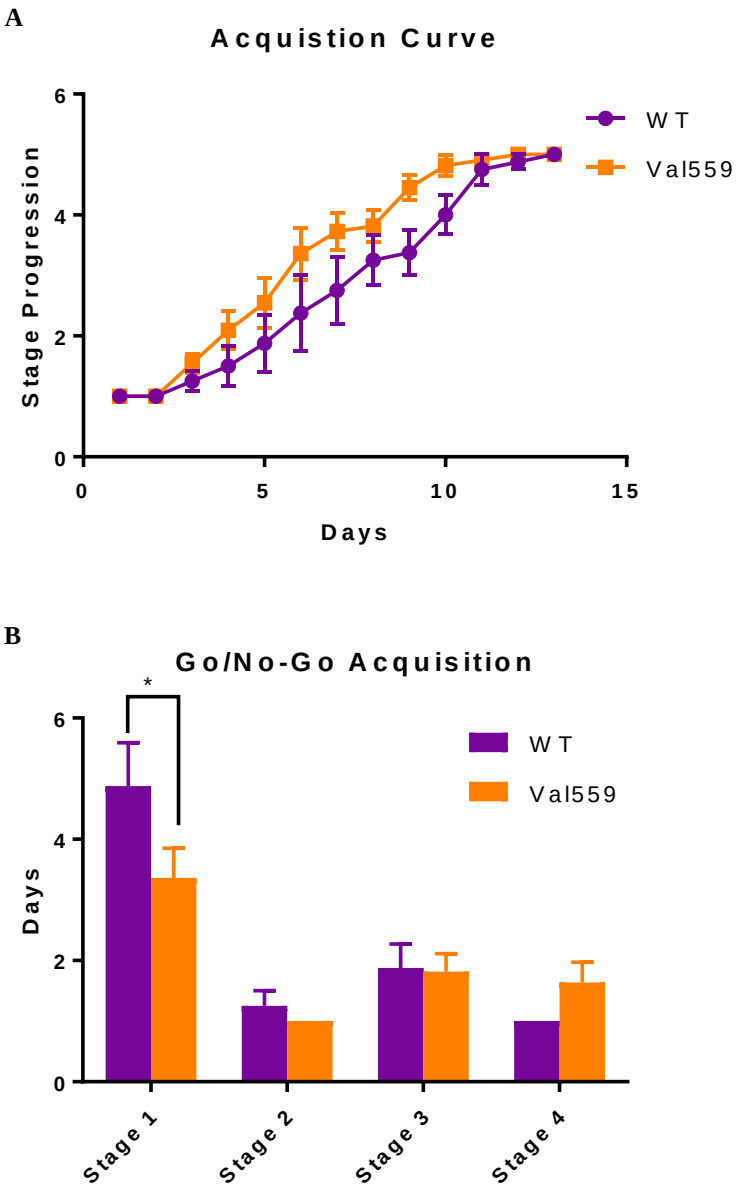


Figure 10. DAT Val559 mice acquire the Go/NoGo task differently than WT (Two-Way RM-ANOVA, genotype $P < 0.05$, day $P < 0.0001$, interaction $P < 0.05$; WT=8, Val559=12) (A). Acquisition difference in Go/NoGo are driven by WT taking longer to complete stage 1 (Two-Way ANOVA, stage effect $P < 0.0001$, genotype $P > 0.05$, interaction $P < 0.05$; Sidak's multiple comparison's test shows a significant difference between WT and Val559 at stage 1, $P < 0.05$) (B).

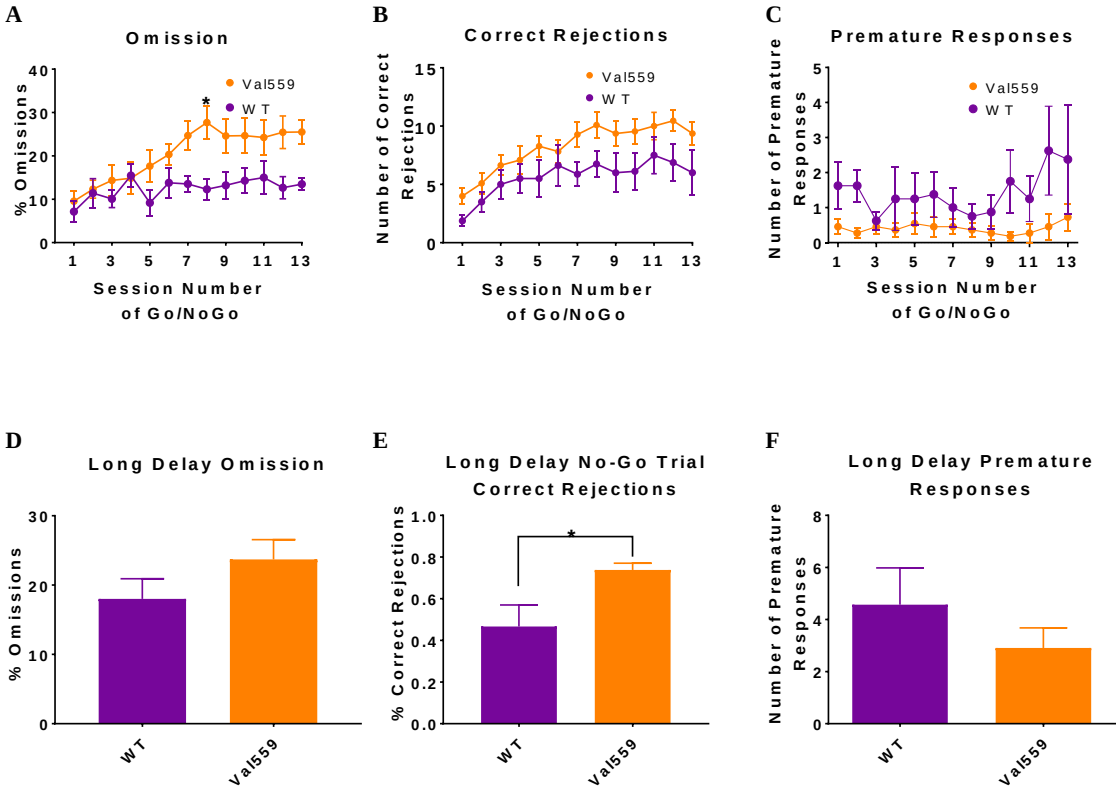
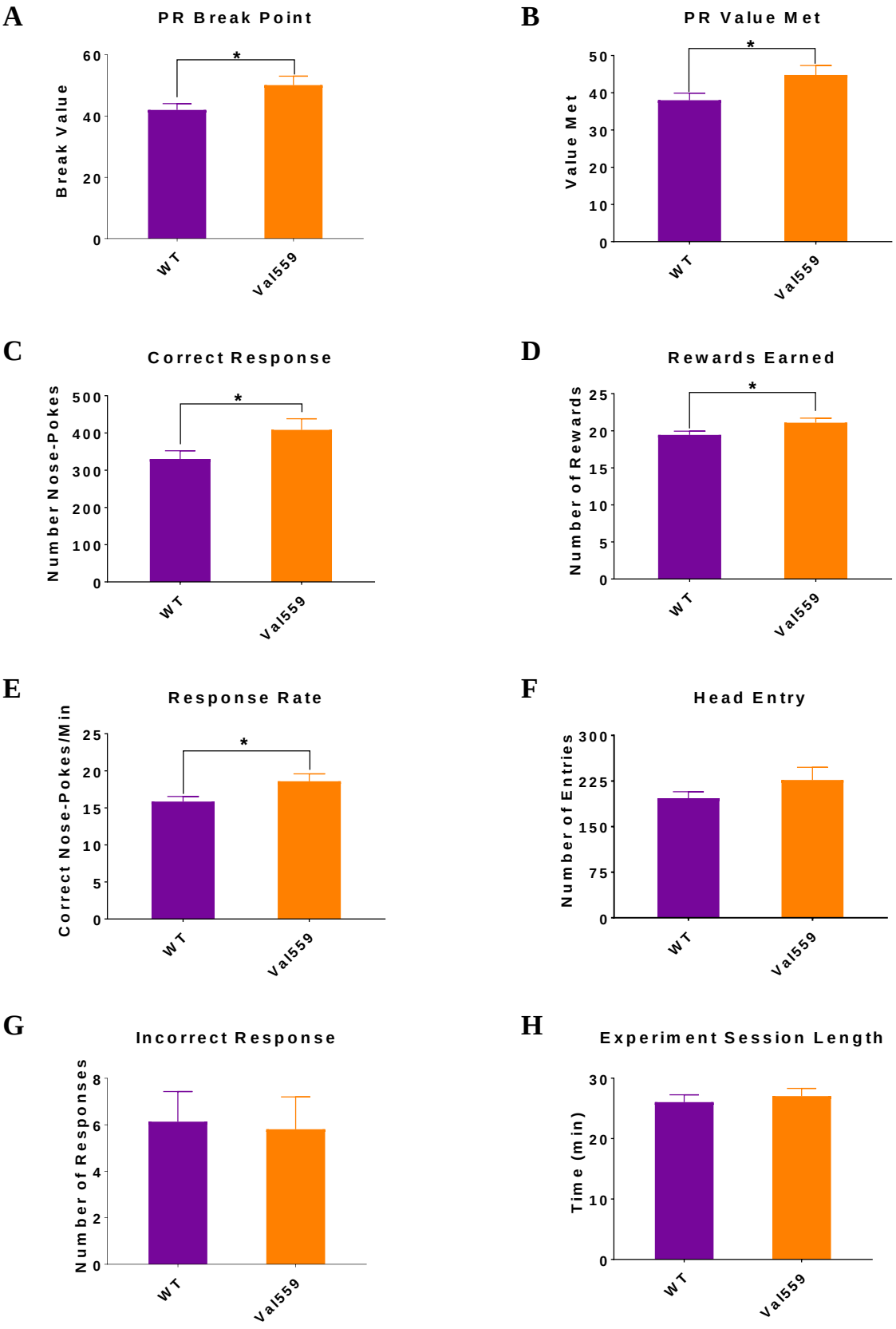


Figure 11. DAT Val559 mice show alterations in the Go/NoGo task. DAT Val559 mice progressively make more omissions (Two-Way RM-ANOVA, genotype $P < 0.05$, day $P < 0.0001$, interaction $P < 0.05$; post-hoc tests reveal $P < 0.05$ at day 8; WT=8, Val559=12) (A). DAT Val559 mice trend towards making more correct rejections than WT (Two-Way RM-ANOVA, genotype $P = 0.07$, day $P < 0.0001$**,** interaction $P > 0.05$) (B). No difference was seen in premature responses between genotypes (Two-Way RM-ANOVA, Two-Way RM-ANOVA; interaction $P > 0.05$, time $P > 0.05$, genotype $P > 0.05$) (C). No genotype difference was present in percent omissions during long delay (two-tailed Student's t-test, $P > 0.05$) (D). DAT Val559 mice made more correct rejections during long delay (two-tailed Student's t-test, $P < 0.05$) (E). No genotype difference was seen in premature responses during long delay (two-tailed Student's t-test, $P > 0.05$) (F).

DAT Val559 mice display enhanced motivation for rewards. In prior studies, an increased response rate in the interval timing paradigm was associated with increased motivation[190, 191, 196, 201]. To assess motivation more directly, I tested DAT Val559 and WT littermates for their willingness to sustain responses despite increasing demand, as assessed in the Progressive Ratio (PR) Test. Here, DAT Val559 mice exhibited clear evidence of enhanced motivation for reward, demonstrating a significantly increased average PR break point (Figure 12A; WT = 22, VaL559 = 21), greater PR value met (Figure 12B), increased correct responses (Figure 12C), greater number of rewards collected (Figure 12D), and overall a significantly higher response rate (Figure 12E). No differences were detected in total number of head entries made, number of incorrect responses made, or total experimental session time (Figure 12F-H). Importantly, there was no difference in sucrose preference (Figure 13, n = 8 per genotype), indicating that the changes detected in motivational measures in the PR test are likely not driven by a change in reward preference.


A
PR Break Point
Break Value
60
40
20
0
*
WT
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B
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Value Met
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C
Correct Response
Number Nose-Pokes
500
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300
200
100
0
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Val559
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Rewards Earned
Number of Rewards
25
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Val559
E
Response Rate
Correct Nose-Pokes/Min
25
20
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Val559
F
Head Entry
Number of Entries
300
225
150
75
0
WT
Val559
G
Incorrect Response
Number of Responses
8
6
4
2
0
WT
Val559
H
Experiment Session Length
Time (min)
30
20
10
0
WT
Val559

Figure 12. DAT Val559 mice have enhanced motivation as seen in progressive ratio. DAT Val559 mice display a higher break point in the Progressive Ratio (PR) task compared to WT (two-tailed Student's t-test, PR Break $P < 0.04$; WT = 22; VaL559 = 21) (A) and PR met (two-tailed Student's t-test; $P < 0.05$) (B). DAT Val559 made more correct responses (two-tailed Student's t-test, $P < 0.05$) (C) and received more rewards (two-tailed Student's t-test; $P < 0.05$) (D). Higher response rates were also seen (two-tailed Student's t-test; $P < 0.05$) (E). No significant genotype difference in number of head entries (two-tailed Student's t-test; $P > .05$) (F), incorrect responses (two-tailed Student's t-test; $P > .05$) (G), and session time (two-tailed Student's t-test; $P > .05$) (H) during the progressive ratio task.

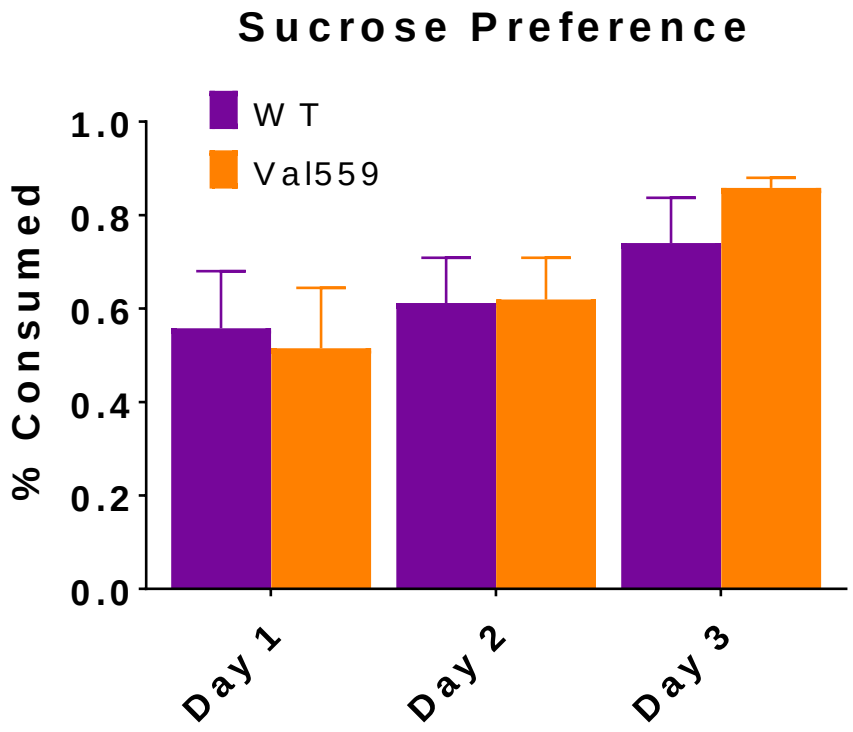


Figure 13. DAT Val559 mice show no difference in sucrose preference. Sucrose preference was calculated showing no statistical difference between genotype though there was a main effect of day (Two-Way RM ANOVA, genotype $P > 0.05$, day $P < 0.05$, interaction $P > 0.05$; WT = 8, Val559 = 8).

4.4 DISCUSSION

In this chapter, I demonstrate that DAT Val559 have a waiting impulsivity, driven by a heightened motivational state. Given the 5-CSRTT randomized delay paradigm may prevent mice from using interval time as a cue of when to expect the stimulus, I hypothesized, that increased premature responses by the DAT Val559 mice in the long delay paradigm may reflect perturbed time-estimation, underlying the appearance of impulsivity versus deficits in response inhibition *per se*. In support of this idea, significant evidence supports a contribution of DA signaling in interval timing[192, 194, 195, 202-205]. For example, normal human subjects given AMPH exhibit an increase in perceived elapsed time[200]. These and other findings have led to suggestions that disruptions in the ability to perceive time intervals (e.g. sped up internal clocks and/or increased clock variability) may represent an ADHD endophenotype[197, 198, 206-208]. However, DAT knockdown mice demonstrate an ability to estimate elapsed time correctly in the peak interval task, though they initiate responding sooner than WT animals and display a general increase in overall response rate[191]. To investigate this issue, I examined DAT Val559 mice and their WT littermates in the peak interval task, training mice to report the elapse of 5 or 15 secs, recapitulating the times used in 5-CSRTT (Chapter 3). If time estimation was contributing to the differences observed in the 5-CSRTT, I hypothesized that I would observe genotype differences with the 15, but not the 5 sec interval, manifested as a leftward shift in the peak interval response curve of DAT Val559 mice. Instead, I failed to observe a genotype effect on time estimation at either time point, but instead detected an increase in response amplitude for the 15 sec interval by the DAT Val559 mice versus WT animals.

Increased response amplitude in the peak interval task is suggested to reflect increased motivation[191, 195]. Additionally, DA signaling is directly implicated in response rate in the peak

interval task, as overexpression of the DA D2 receptors (D2Rs) selectively in striatal medium spiny neurons delays the onset of response initiation and reduces response rates in the peak interval task[196, 209]. Interestingly, the early start of response and the overall heightened responses seen in the peak interval task with DAT knockdown mice can be normalized by low doses of raclopride, a D2R antagonist, in a range thought to predominantly impact D2ARs[191]. This is an intriguing with respect to the DAT Val559 model, as D2ARs are both constitutively activated by tonic DAT Val559-mediated DA efflux[133] and drive efflux[210]. Our ongoing studies seek to determine whether all, or subsets of DAergic projections are equivalently influenced in the DATVal559 model by functional and/or structural D2R/DAT interactions, with previously published data indicating changes in the substantia nigra[134] and preliminary data indicating potentially interesting alterations in the dorsal striatum in particular.

My peak interval studies indicate that DAT Val559 mice exhibit impulsive action when required to wait if trained on a fixed, predictable timing delay, independent of the ability of the animals to estimate elapsed time. This waiting impulsivity does not reflect blanket deficits in pre-potent motor inhibition as assessed in the Go/NoGo test. Thus, DAT Val559 mice had no trouble withholding behavioral responses on NoGo trials. In fact, as mice progressed through sequential sessions, I found that DAT Val559 mice actually trend towards being better at correct rejections than their WT littermates. Intriguingly, this performance appears to come at the cost of an increase in percentage of omitted responses, in contrast to the lack of a genotype difference in omissions in 5-CSRTT. Moreover, unlike my findings with the 5-CSRTT, I observed no increase in measures of impulsivity in Go/NoGo test when the delay before stimulus presentation was increased from 5 to 15 secs. In fact, the DAT Val559 mice made significantly more correct rejections than WT, arguing for better inhibitory control. These findings can be reconciled if the DAT Val559 variant

instills an increased motivation/anticipatory drive rather than a frank deficit in motor inhibition. It may be that different motivational and/or response inhibition demands of the two tasks contribute to the distinct phenotypes of DAT Val559 mice on each task. My studies of DAT Val559 mice in the progressive ratio task supports this idea, where increased motivation is suggested by a significant increase in break point, an increased number of values met before reaching a break point, an increased number of correct responses made and rewards earned, and a significant overall increase in response rate, relative to WT littermates.

I do not believe that this enhanced motivation is driven by a general increased hedonic value of rewards as no genotype differences were observed in sucrose consumption in the two-bottle choice test. Instead, I suspect that the increased willingness of DAT Val559 mice to expend effort for reward indicates an intrinsically elevated drive for reward. In contrast, DAT KO mice show a very strong increased sucrose preference that increases with exposure[211], but do not show the same increase in motivational indices of progressive ratio tests[212]. These findings presumably reflect the differences in the genetic insult driving increased extracellular DA in the DAT Val559 model vs the DAT KO (e.g. DA leak versus constitutive loss of DA uptake) and the degree to which different DAergic pathways rely on the transporter to maintain extracellular and intracellular DA homeostasis.

Enhanced motivation for rewards in the DAT Val559 mice is at first blush difficult to align with reports of reduced reward motivation in ADHD subjects[150, 213], or reports of a lack of perturbed motivation altogether[214]. Certainly, ADHD is not a homogeneous disorder and subjects with and without motivational changes likely arise from the distinct etiological mechanisms. Studies with construct-valid models such as the DAT Val559 mouse may thus assist in the dissection of subgroups defined not by overt behavioral categories but by underlying

pathophysiology. In this regard, prior studies have demonstrated that increased levels of extracellular DA increase both motivation and risky/impulsive behaviors[162, 215]. Motivational changes may also be reward-specific, accommodating reduced, normal or even elevated motivation for some rewards but not others[216]. Some studies have argued that although intrinsic motivation may be low in ADHD subjects, they are more positively affected by the promise of an immediate extrinsic reward, leading to greater improvements in task performance compared to non-ADHD children[217-219]. Thus, children with ADHD can improve their performance in measures such as error monitoring relative to their age-matched controls, in the context of specific extrinsic motivational conditions (i.e. performing a task for money or gifts rather than praise or a letter grade)[217]. Additionally, receipt of rewards appears able to normalize impulsivity tendencies, suggesting a significant intersection between motivational and impulse control circuits[220, 221]. An enhanced motivational drive for reward could also explain the increased performance of DAT Val559 mice in the Go/NoGo test where the mutants may be more motivated by the 50% chance of receiving a reward through behavioral inhibition, as compared to WT animals, yielding higher instances of correct rejections and low premature responses.

It seems reasonable to propose that the increased speed in task acquisition in both 5-CSRTT and the Go/NoGo task is driven by the increased motivation of the DAT Val559 mice. This idea nicely dovetails with the Reinforcement Sensitivity Theory from the human literature in ADHD, specifically the Behavioral Approach System (BAS), which hypothesizes that high BAS traits and resultant impulsivity are driven by an increased motivation for reward[222, 223]. A high BAS trait index would be expected to allow for increased sensitivity to response-based reward and faster establishment between instrumental learning and reward, similar to the increased acquisition seen in the DAT Val559 with 5-CSRTT. Imaging studies involving BAS-related tasks have implicated

several brain regions heavily involved in DA signaling including the dorsal and ventral striatum and orbital frontal cortices[222]. Furthermore, children and adults with ADHD, specifically of the hyperactive/impulsive subtype, score higher on traits of BAS and display increased behavioral approach tendencies to reward associated tasks[224, 225], bringing to mind the premature responses seen with the increased delay in 5-CRTT and the increased response rate in the peak interval task in DAT Val559 mice.

The idea that impulsivity in the clinical context may be for some subjects, at its core, a disorder of dysregulated motivation is important for the development of both behavioral and pharmacological interventions for disorders involving DA dysfunction including ADHD. Finally, given that the DAT Val559 variant was identified in BPD and ASD, studies with the mouse model affords a unique opportunity to examine how various genetic and environmental factors shape the different clinical presentations that arise with changes arising from singular insults in DAergic signaling.

In conclusion, my studies reveal that DAT Val559 mice display several behavioral phenotypes with direct relevance to ADHD symptomology. As such, my work offers potential insights into the contribution that heritable alterations in DAT expression and function make to the pathophysiology of neurodevelopmental disorders. My work predicts that molecular changes that impact tonic extracellular DA levels can enhance motivation for reward, which can emerge phenotypically as elevated impulsivity, effects separate from perturbations of intrinsic time estimation or deficits in attention. DAT Val559 mice may therefore prove useful as a construct valid model with which to elaborate the circuitry and signaling disruptions that drive clinical syndromes characterized by impulsivity as well as their treatment.

CHAPTER 5

PERSISTENT CHECKING BEHAVIOR FORMATION IN DEVALUED GOAL-DIRECTED AND HABITUAL SCHEDULES IN THE DAT Val559 MOUSE MODEL

5.1 INTRODUCTION

Previously I demonstrated the effects of DAT Val559 expression on waiting impulsivity and how my data supports that the DAT Val559 mice resting in a state of enhanced motivation which may drive this inhibitory deficit[226]. Based on this increased motivation and the enhanced learning curve of the DAT Val559 mouse in the 5-CSRTT paradigm, one possible interpretation is that expression of the DAT Val559 variant places the mice in a neurochemical state that pre-disposes them to be better at learning goal-directed behaviors. This is in contrast to what I initially expected, as cue-directed behaviors rely on phasic DA release [41, 42]. As such, increased extracellular DA tone, as seen in the DAT Val559 mice, could alter the signal to noise ratio (phasic DA: tonic DA) necessary to establish cue salience required for goal-directed behavior. Additionally, subjects with ADHD, ASD, Tourette Syndrome, and OCD display functional deficits that may bias them to rely excessively on striatal-dependent habit memory/learning[227]. Given the discrepancies between what was expected of the DAT Val559 mice and was demonstrated, I wondered instead whether the mice were more readily able to shift or more prone to form habitual behavior, rather than having an immediate deficit in goal-directed behaviors, more reflective of behavioral flexibility issues seen in a variety of neuropsychiatric diseases thought to have a dopaminergic component[154, 228-231].

The dorsal medial striatum has been demonstrated to be critical for goal-directed behavior

however the dorsal lateral striatum has been heavily implicated in habit formation and behaviors. Importantly, it was demonstrated that these two regions balance each other and when one region becomes disrupted the other becomes dominant, resulting in a shift of behavioral strategies[155]. Additionally, unpublished structural imaging data on the DAT Val559 mice from Dr. Ellegood from the Mouse Imaging Centre at the Hospital for Sick Children in Toronto Canada revealed alterations in brain regions important to goal-directed behavior (Figure 14). Specifically, we see a volume reduction in the dorsal medial striatum of the DAT Val559 mice, whereas the dorsal lateral striatum has no volumetric changes relative to WT (manuscript in preparation).

The enhanced motivational state of the DAT Val559 mice could also contribute to a predisposition for more rapid formation of habitual responding. Such that, initially the DAT Val559 mice form stronger or faster stimulus-response connections compared to WT which then shifts more quickly to habitual responding as a result of an imbalance in circuitry caused by the reduced dorsal medial striatum. As such, it seemed possible to expect to see differences in habit formation in the DAT Val559 mice only when they were directly challenged for a distinction between goal-directed and habitual responding. By utilizing both a devalued progressive ratio paradigm and a novel within subject lever pressing paradigm, I assessed within subject goal-directed versus habit formation with the hypothesis was that the DAT Val559 animals would be pre-disposed for habit formation.

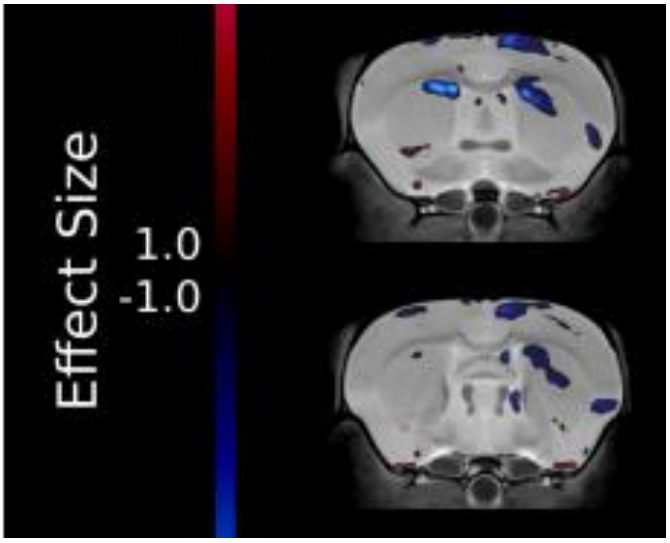


Figure 14. DAT Val559 mice display a reduction in the dorsal medial striatum relative to age matched WT. Data provided with the permission of Dr. Ellegood from the Mouse Imaging Centre at the Hospital for Sick Children in Toronto Canada. Cooler colors indicate a relative reduction in volume with warmer colors indicating a volume increase relative to matched WT controls.

5.2 METHODS

Animals: All experiments were performed under a protocol approved by the Institutional Animal Care and Use Committees at Vanderbilt University and Florida Atlantic University. Homozygous DAT Val559 and WT littermate mice used in the study were bred from heterozygous breeders on the hybrid background used in our prior studies[134] (75% 129S6 and 25% C57BL/6J). Males were evaluated in the present study owing to the bias toward male subjects for ADHD diagnoses [4]. Animals were housed on a 12:12 (L: D) cycle. Mice were tested during their active cycle, achieved by either raising animals on a reverse light cycle with lights on and off at 3 pm and 3 am, respectively, or by raising mice on a normal light cycle, with lights on and off at 7 am and 7 pm and then with transferal of mice to the reverse light cycle at 5 weeks of age, after weaning. Mice were approximately 6-7 weeks old when training for different behavioral assays commenced. For all operant conditioning tasks mice were placed on food restriction one week prior to the start of training. Mice were brought to approximately 85%-90% of their baseline weight (weighed every other day). On the fourth and fifth days (still under food restriction), mice were exposed to 33% Vanilla Ensure Original, the reward used for all the operant tasks, for one hour in the home cage. Animals were run under red light, with house lights off in the operant chambers.

Goal vs Habit Lever Press Paradigm: Modified from the methods in Gremel et al 2013, this assay was utilized to look at goal-directed and habit behavior[155]. This assay relies on the ability of different lever-press schedules to predispose an animal to utilize a goal-directed behavioral strategy (random ratio) or support habit formation (random interval). Mice underwent two training sessions each day in two distinct contexts (clear plastic walls vs black and white stripped walls). Animals were tested in Med-Associates (St. Albans, VT) operant chambers housed in sound attenuation boxes. The training schedule consisted of two days of FR1-FT10 (fixed ratio 1-fixed

time 10) where the trial started with the delivery of a free reward that the mouse had 5 sec to consume upon head entry into the dipper delivery zone. The mouse could then earn a reward by making a nosepoke into a nosepoke hole backlit by LEDs, otherwise a reward was freely delivered every 10 seconds (FR1-FT10). Mice had three possible holes into which they could make a nosepoke into, but only one of the three holes was the active hole (indicated by a lit LED in the back of the hole) that would elicit delivery of a reward. Mice were run under the FR1-FT10 schedule for 15 minute sessions and 15 rewards/session for each environmental context. Mice were balanced across environmental context for which hole was the active hole (i.e. for mouse 1 the active nosepoke hole was the left hole in the clear plastic environmental context while the right hole was the active nosepoke hole in the striped environmental context, but this condition was reversed for mouse 2). In this paradigm, the back center nosepoke hole was never the active hole, only the holes to the left and right of where the reward delivery dipper appeared. This was followed by 2 days of FR1-FT30 (15 minute sessions, 15 rewards/session). Mice then underwent CRF training (60 minute sessions, 15 rewards/context). Under this protocol mice received a free reward at the beginning of the session, but then had to complete a nosepoke on the active for hole for that training context in order to earn additional rewards. All mice had to reach the criterion of earning 15 rewards within 60 minutes before moving to the next training stage as a group. To prevent overtraining, mice that obtained this criterion sooner were rested, and total training sessions at CRF 15 were kept equivalent across mice +/- 1 training session. Mice were then run three days on CRF with 30 rewards possible. This was followed by three days of training on random ratio 10 (RR10; reward was delivered on average every 10 nosepokes with a 0.1 probability that a given nosepoke would produce a reward) and random interval 30 seconds (RI30; reward was delivered upon nosepoke on average every 30 seconds regardless of nosepoke vigor). The random ratio and

random interval schedules were counterbalanced across context and active nosepoke hole. After the RR10 and RI30 training the intensity of the schedules were increased to random ratio 20 (RR20) and random interval 60 (RI60) where animals were given 60 minutes in each context to earn 30 rewards. Upon completion of this training schedule there were two days of five minute non-reinforced probe tests done under devalued (Ensure given in home cage for 1 hour) and valued states (mouse chow given in home cage for 1 hour),counterbalanced across days, context, and genotype (Figure 15). Prior to the start of the non-reinforced probe tests mice were separated into clean cages and allowed access to either mouse chow (valued probe test days) or Ensure (devalued probe test days) for an hour. Amount of chow and Ensure consumed in home cage was measured to confirm that there were no consumption differences across genotypes.

Devaluation of Progressive Ratio: The protocol and baseline data for this assay can be found in Davis et al[226]. Please refer to the methods section there for the training protocol prior to devaluation. In this experiment mice were tested on progressive ratio in a devalued state the day after they were tested on progressive ratio under normal conditions. For devaluation mice were given access to ensure in their home cage for one hour prior to testing. This deviates from the devaluation above as mice were not separated into single housing, and trials were reinforced, the mice could still earn ensure by completing the nose-poke requirements as determined by the progressive ratio program.

Statistical Analyses: Statistical analyses was performed using GraphPad Prism 7 software package. Statistical significance was set at $P < 0.05$ for all experimental results. The type of statistical analysis used was determined independently for each experiment and is listed in the relevant figure caption. Outlier analysis was performed using the ROUT method with the false discovery rate set at 2%.

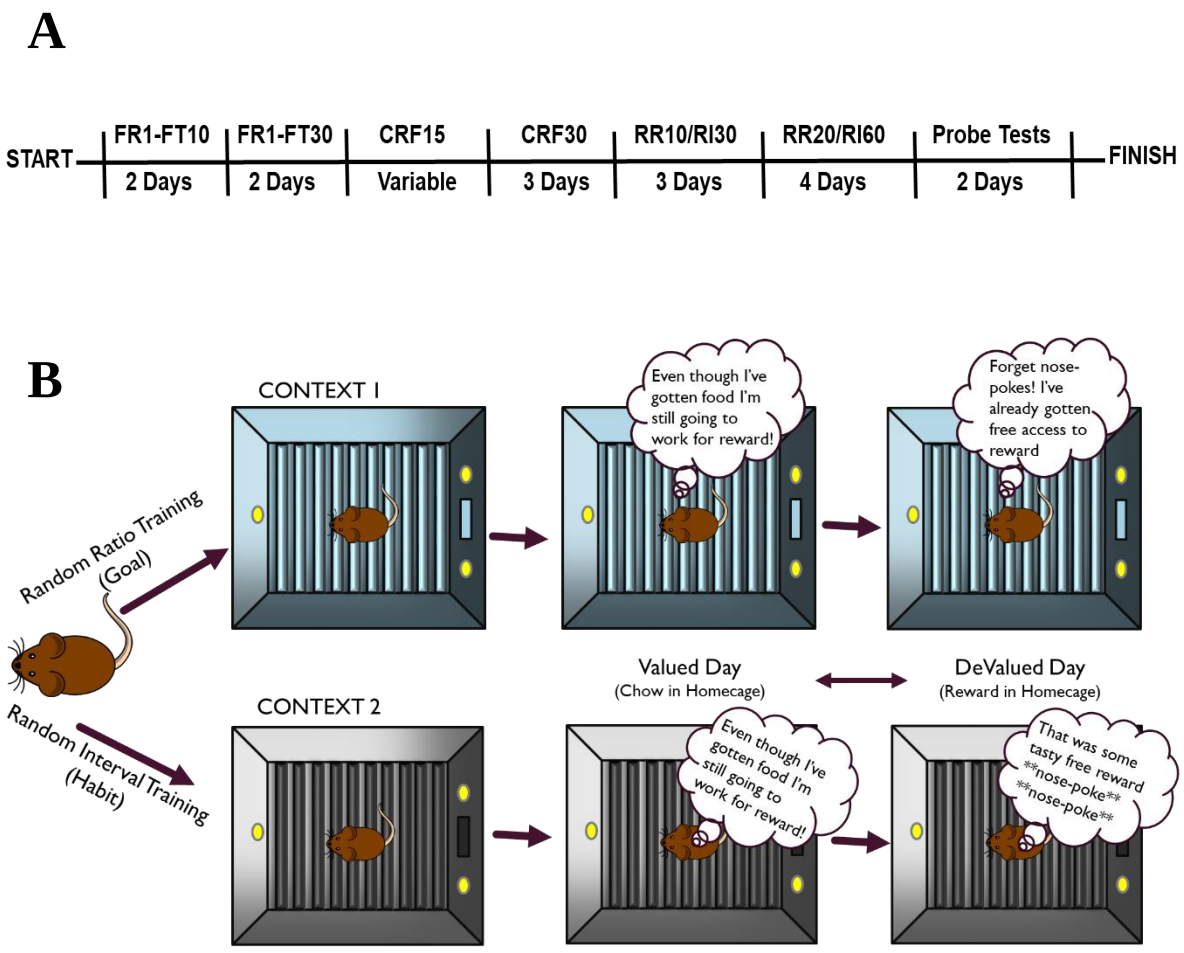


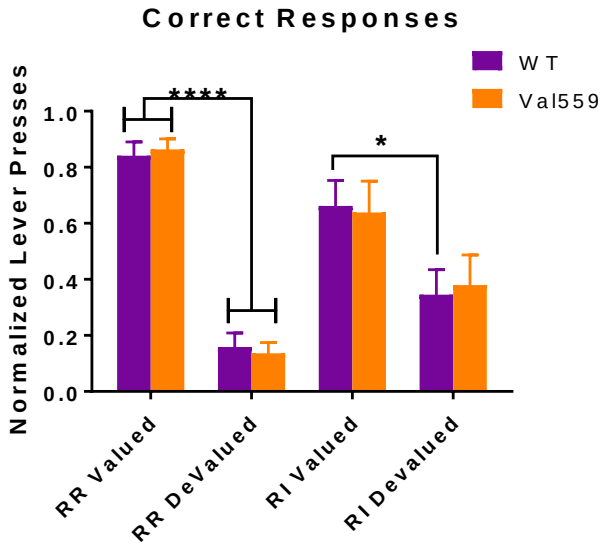
Figure 15. Training schedule of lever pressing paradigm. Mice are trained in two environmental contexts from start to finish of the paradigm. Mice start by learning where reward is delivered with the ability to acquire nose-poke behavior with two days of Fixed Ratio 1-Fixed Time 10s schedule, where one nose-poke will elicit delivery of reward, but reward is freely delivered every 10 seconds regardless of nose-poke behavior. This is then moved to free reward delivered every 30 seconds (FR1-FT30). Mice must then nose-poke to earn a total of 15 rewards followed by the ability to earn 30 rewards in each training environment. Mice were then trained on a Random Ratio10 (RR10) schedule in one environmental context and a Random Interval30 (RI30) in the other environmental context. They were then moved to RR20 and RI60. This was then followed by two days of probe tests under either valued or devalued conditions (A) Representation of outcome expectations based on training schedule (B)

5.3 RESULTS

DAT VAL559 Mice Display Seeking Compulsion in Devalued States for Both Goal-Directed and Habit Driven Behavioral Contexts. I hypothesized that DAT Val559 mice would show a bias for habit formation even in a behavioral context that promotes goal-oriented behavior [155]. To test this assertion, a within subject lever pressing paradigm was performed that allowed for evaluation of goal-directed and habit behavior[155]. WT littermates were expected to show sensitivity to devaluation in the goal-directed context, but not in the habit context, whereas DAT Val559 were expected to show enhanced nose-poking under devalued states in both the goal-directed and habit context. Contrary to my hypothesis, both genotypes were sensitive to devaluation in goal-directed context, while still displaying heightened nose-poke behavior in the habit context (Figure 16A). Interestingly, the number of head entries made under the devalued condition in both behavioral contexts was significantly elevated in the DAT Val559 mice relative to WT (Figure 16B). Importantly, no consumption differences in home cage were seen for either chow or ensure between genotypes (Figure 16C).

DAT Val559 Dysregulated Seeking is also Present in Progressive Ratio under Devalued Conditions. A similar phenotype was seen in progressive ratio when done in a devalued condition; DAT Val559 displayed significantly higher head entries (Figure 17A). Additionally, the DAT Val559 mice trend towards having higher break points (Figure 17B) and require significantly more experimental session time before they quit the experiment (Figure 17C). Interestingly, while the vigor in which both genotypes nose poke for reward is decreased (Figure 17D) relative to the task done in a non-devalued state[226], the DAT Val559 mice do not maintain their heightened level of response/sec, eliminating the genotype difference in response rate that was seen[226].

A


Correct Responses
WT
Val559
Normalized Lever Presses
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*
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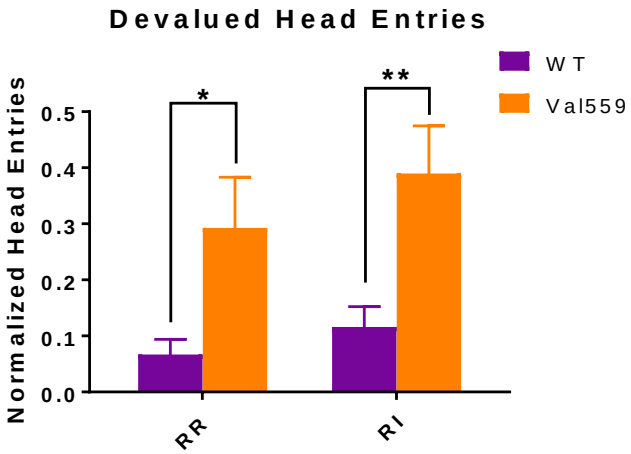
B


Devalued Head Entries
WT
Val559
Normalized Head Entries
0.5
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**
RR
RI

C

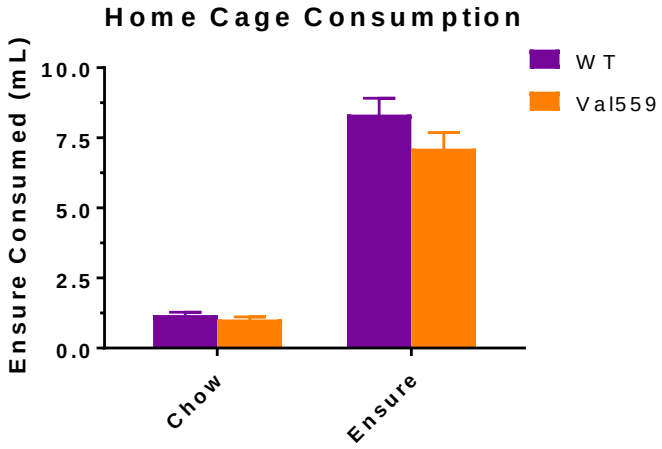

Home Cage Consumption
WT
Val559
Ensure Consumed (mL)
10.0
7.5
5.0
2.5
0.0
Chow
Ensure

Figure 16. DAT Val559 mice display seeking compulsion in devalued states for both goal-directed and habit driven behavioral contexts. WT and DAT Val559 mice have very significantly reduced nose-poke behavior in the goal-directed context under devaluation and WT have reduced nose-poke behavior in the habit context with no significant change for HOM in the habit context (2-Way ANOVA, condition $P < 0.0001$; Tukey's Multiple Comparisons Test, WT RR Valued vs. RR DeValued $P < 0.0001$, Val559 RR Valued vs. RR DeValued $p < 0.0001$, WT RI Valued vs. RI DeValued $P < 0.05$) (A). DAT Val559 mice have increased head entries compared to WT in RR DeValued and RI DeValued (two-tailed Student's t-test $P < 0.05$ and $P < 0.0$ respectively; WT = 12, Val559 = 10) (B). There is no statistical difference between the amount consumed in home cage prior to testing for both valued and devalued days (C).

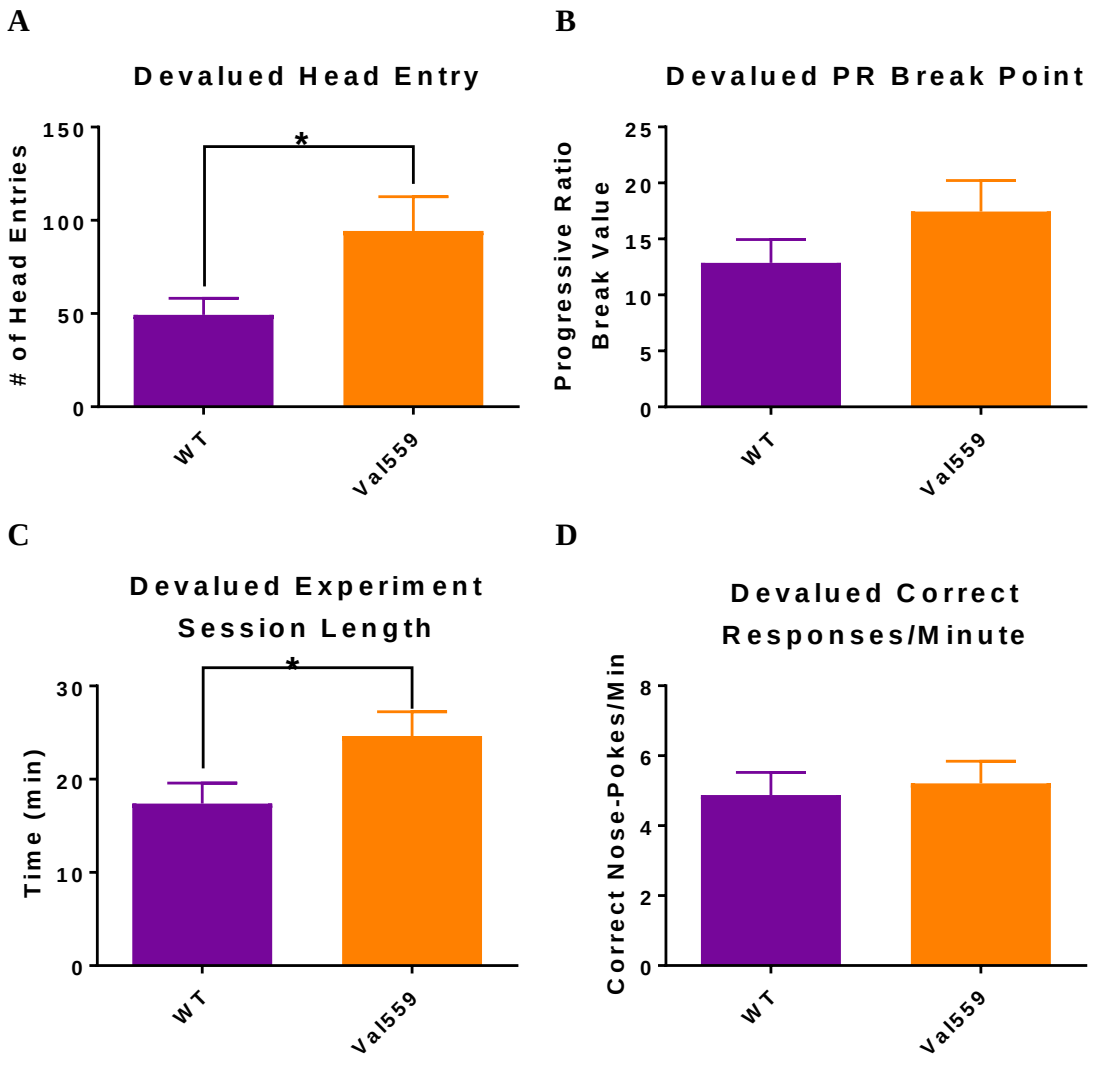


Figure 17. DAT Val559 mice show elevated seeking phenotypes during devalued progressive ratio. DAT Val559 mice had significantly increased number of head entries compared to WT (two-tailed Student's t-test $P < 0.05$; WT = 22, HOM = 21) (A). No significant genotype difference was seen for the break point (two-tailed Student's t-test) (B). DAT Val559 mice had a significant increase in the experiment session length (two-tailed Student's t-test $P < 0.05$) (C). There was no significant difference in response rate (two-tailed Student's t-test) (D).

5.4 DISCUSSION

Habit formation is a necessary component of healthy behavior. It allows certain stimulus responses to become automatic, freeing up cognitive processes to focus on other things. However, it is imperative that this process remain balanced with goal-directed behaviors and dynamic when necessary. Certain deficits in behavioral inhibition can alter one's abilities to suppress or change habitual responses, resulting in a cost or detriment in continuing to perform the behavior. This dysregulated habit formation expresses itself in the form of impulses or compulsions. Alterations in the circuitry associated with behavioral inhibition can place an individual in a state of increased susceptibility for the formation of these maladaptive behaviors, increasing the risk for the development of detrimental behaviors such as compulsive shopping, excessive gambling, and substance abuse. We know that DA plays an important role in these processes, both specific receptors and circuity heavily associated with DA rich brain regions[166, 232, 233]. Specifically, the orbital frontal cortex sends projections to both the dorsal medial and dorsal lateral striatum. These pathways work in concert to strike a balance between goal-directed and habit behaviors, providing a necessary mechanism to support behavioral flexibility[155]. Additionally, it is well documented that patients given L-DOPA, the precursor to DA, to treat Parkinson's disease have an increased risk of developing compulsive issues[234]. Of note, people diagnosed with ADHD are at higher risk for formation of these behaviors, specifically substance abuse. Compulsive behaviors are also prevalent in the ASD population. As the A559V mutation was identified in both disorders, it is perhaps not surprising that I was able to detect significant alterations in compulsive-like behaviors in animals harboring this mutation.

By using the DAT Val559 mouse model I was able to assess the role that expression of this disease relevant mutation has on the balance between goal-directed and habit behaviors. There are

several ways in which to assess behavioral flexibility, often utilizing devaluation paradigms or through reversal learning/extinction paradigms. However, I chose a devaluation based paradigm as it allowed for parallel, within subject assessment of the balance between goal and habit behaviors in the DAT Val559 mice.

I initially expected to see that the DAT Val559 mice would display heightened nose-poke behavior under devaluation in both the goal-directed and the habit associated contexts, as this would indicated that there is a misbalance in their circuitry allowing for stronger habit formation at the cost of goal-directed behavior. Instead both genotypes show a significant reduction in nose-poke behavior when the reward is devalued in the goal-directed context with both genotypes also demonstrating elevated nose-poke behavior under devaluation in the habit associated context, indicative of expected habitual nose-poke behavior. Contrary to this, however, I did observe an interesting phenotype with head entries. In both the goal-directed and habit associated contexts, the DAT Val559 mice show significantly elevated levels of head entries into the port where the food reward is normally delivered. This is likely not due to a difference in satiation levels as no difference in home cage consumption of chow or Ensure was observed. The presence of elevated head entries in both the habit-associated and goal-directed contexts begs the question as whether it is a measure that also represents habitual behavioral impulse or a checking compulsion, a phenotype that may precede habitual lever-pressing.

Interestingly, I observed a similar head entry phenotype when the progressive ratio schedule is run under devalued conditions. This paradigm is slightly different than the lever pressing paradigm as the mice still have the opportunity to earn reward during the probe trial when the reward is devalued. As such, DAT Val559 mice trend towards having a higher break point in the devalued state, similar to what was previously seen under normal conditions[226]. However, the DAT Val559

mice also now take longer before they time out of the progressive ratio experiment, but no longer maintain their more rapid response rate, unlike what was previously seen, where DAT Val559 mice displayed a significant elevation in response vigor. The normalization of response rate between genotypes in the devalued state is of particular interest, as it is an independent measure of motivation from break point. As such, the reduction in vigor could indicate a reduction in motivation for the reward. Again, this points to the elevated head entry response as being an indicator of altered circuitry that could result in a predisposition for the formation of a habitual checking/seeking behavior.

As discussed, the dorsal medial striatum in the DAT Val559 mice appears to exhibit volume reductions, potentially priming the animals to be more prone to habitual behavior. Lesion of the dorsal medial striatum maintains head entry under devalued conditions, but not independent of nose-poke responses[155]. This could indicate that the perseverative checking behavior is driven from a different circuit. Lesions to the nigrostriatal dopaminergic pathway have also been shown to prevent the switch from goal-directed responding to habitual responding when animals have been over trained, showing both elevated lever press behavior and head entries. Intrastriatal application of both D1 and D2 agonists does not rescue lever pressing, such that over trained animals with the nigrostriatal lesions are still susceptible to devaluation of the reward. However, with administration of the D1 agonist animals maintain elevated head entries despite devaluation of the reward, while administration of the D2 agonist did not[235]. This could indicate that DAergic innervation of the dorsal striatum from the substantia nigra could play a modulatory role through specific DA receptors that effects checking behavior independent of lever pressing behavior.

Additionally, the Blakely lab has previously demonstrated D2/DAT mediated alterations in synaptic function in DAT Val559 mice[174]. Several interesting studies have been conducted that

indicate a strong role for the D2 receptor in compulsive checking behavior, demonstrating that chronic administration of the D2/D3 agonist quinpirole can induce compulsive checking behaviors[236-238]. Of particular interest, an operant paradigm for rats was designed to look a checking behavior in a food motivated context (as opposed to a compulsion induced by aversion avoidance)[239]. In this task there were two active levers each with associated with a contingency for lever press behavior to earn reward. The lever that was the active lever would change across trials within a session. An observational lever, when pushed, would activate a light over the active lever within a set trial, providing no reward, but giving the animal necessary information regarding what lever press would lead to reward. Chronic treatment of quinpirole produced perseverative lever pushing of the observational lever, without providing further benefit to the animal. This compulsive pressing of the observational lever was mitigated with administration of sulpiride, a D2 antagonist. Our data supports the idea that the D2 autoreceptor is resting in a constitutively active state in the DAT Val559 animal as a result of DAT anomalous DA efflux, which could then contribute to the observed habitual checking behavior. Future work in the DAT Val559 mice may seek to confirm participation of D2R signaling in this aberrant phenotype.

It is unclear whether the elevated head entries in the DAT Val559 mouse represents a compulsion or an impulse to check for reward. Regardless, the elevated head entries arguably represent an alteration in the processing of habits. Although additional operant assays are under way to further assess cognitive flexibility in the DAT Val559 animal, we do have another measure that aligns with this idea of dysregulated habits. Unpublished data collected by Blakely lab post-doctoral researcher Adele Stewart demonstrated that male DAT Val559 mice display deficits in extinction. Specifically, male DAT Val559 mice take longer to extinguish cocaine-induced conditioned place preference. These data strongly align with the literature that those with

impulsive natures and deficits in cognitive flexibility are more prone substance abuse and addiction from a dysregulation in control of habit formation.

Understanding the exact changes to the circuitry in the DAT Val559 animals affected in the habit-associated regions could prove very insightful in understanding cognitive flexibility and the requirements to transition from healthy habits to impulse/compulsion formation. As already mentioned, we know the DAT Val559 have a localized reduction in the size of the dorsal medial striatum as well as alterations in presynaptic function of nigral DA neurons, both of which have been implicated in balancing habit formation. Dissection of the molecular and circuit level changes driving these unique behavioral alterations present in the DAT Val559 mice could provide insights into treatment options not just for the neurodevelopmental disorders associated with DAT Val559, but also potentially help with those suffering from stand-alone impulse control disorders.

CHAPTER 6

ALTERATIONS IN AMPHETAMINE-INDUCED ERK1/2 PHOSPHORYLATION IN DAT Val559 MOUSE

6.1 INTRODUCTION

In the previous chapters I focused on understanding how the lifelong expression of the DAT Val559 mutation altered ADHD-associated behavior. However, I also want to understand the underlying molecular and circuitry changes that drive these behavioral alterations. As such, I pursued a biochemical approach in parallel focused on demonstrated behavioral differences between the DAT Val559 mouse and WT controls: blunting of the locomotor response in the DAT Val559 mice to moderate dose of AMPH[174].

Previous research done on the DAT Val559 mouse strongly supports the idea that the blunted locomotor response results, at least in part, from presynaptic changes that impact vesicular release of DA. Specifically, Mergy et al demonstrated alterations in both AMPH and 4-AP induced DA release in striatal slices, mirroring the locomotor data. Application of the D2 antagonist sulpiride to DAT Val559 slices restored stimulated release, whereas quinpirole, the D2 receptor agonist, blunted release in slices collected from WT animals[174]. Together these data indicate that vesicular DA release in DAT Val559 mice is subject to constitutive D2R-mediated suppression.

Although there is a strong presynaptic component to explain the alteration in AMPH produced behavioral response, post-synaptic signaling in DAT Val559 animals has largely been left unexplored. Unlike with other hyperdopaminergic models[71], we know that post-synaptic receptor

density in the striatum is equivalent between genotypes. Since the total surface expression of the post-synaptic receptors is unchanged any likely post-synaptic component contributing to the altered locomotor response likely occurs at the signaling level. Additionally, no genotype differences were seen for locomotor behavior in response to a D1 agonist[174] indicating that, at least for the D1 receptor, intrinsic post-synaptic receptor sensitivity is equivalent[134].

Several excellent studies have extensively studied the downstream signaling cascades of the D1 receptor in response to AMPH, examining the proteins activated and the role they play in the behavioral response. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are of particular interest as they have been shown to be strongly activated by AMPH in the D1 MSNs and in the prefrontal cortex[240, 241]. Blockade of the D1 receptor through an antagonist or by knocking out the D1 receptor in mice prevents the activation of ERK1/2 in response to AMPH in the striatum[240]. Additionally, blockade of ERK1/2 activation by systemic injection or intrastriatal infusions of an ERK1/2 inhibitor produces a blunting effect on AMPH induced locomotor response[242, 243]. The DAT KO mouse displays elevated levels of activated ERK1/2 under basal conditions that are reduced in correlation with a reduction in locomotor behavior after administration of psychostimulants. Importantly, inhibition of activated ERK1/2 rescues the hyperactivity seen in DAT KO mice.[241]

As such, ERK1/2 served as promising initial targets to begin correlating behavioral alterations in the DAT Val559 mice with corresponding biochemical changes. I hypothesized that the blunted locomotor response in the DAT Val559 mice would be reflected in blunted ERK1/2 activation as well. Here I demonstrate that there are different patterns of ERK1/2 activation in the DAT Val559 model that are dependent on treatment, genotype, gender, and time of day.

6.2 METHODS

Animals: All experiments were performed under a protocol approved by the Institutional Animal Care and Use Committees at Vanderbilt University and Florida Atlantic University. Homozygous DAT Val559 and WT littermate mice used in the study were bred from a mix of heterozygous (het) breeders and homozygous (hom) breeders on the hybrid background used in our prior studies[134] (75% 129S6 and 25% C57BL/6J). Hom breeders were supplied through het x het breeding to prevent genetic drift. No difference in biochemical measures were seen between pups derived from het x het pairings versus hom x hom pairings. As such, animals were used as available. In this set of experiments both male and female mice were used. Animals were housed on a 12:12 (L: D) cycle. To be able to test mice in the active cycle animals were either raised on a reverse light cycle with lights on and off at 3 pm and 3 am, respectively, or by raising mice on a normal light cycle, with lights on and off at 7 am and 7 pm and then with transferal of mice to the reverse light cycle at 5 weeks of age, after weaning. Mice were tested in either their active (lights off) or inactive phase (lights on), as indicated. Mice were approximately 6-8 weeks of age when used for biochemical experiments.

Biochemistry: Animals were separated into singly housed cages (day 1). Mice received 100 µL saline injections to habituate to injection stress. This occurred during the same time of day (active or inactive) that the experiment would be completed. On day 4 mice were brought into the dissection room one at a time to be weighed and injected with either saline or 3 mg/kg of AMPH at a volume of 5ml/kg. Rapid decapitation occurred 15 minutes post-drug injection. Brains were rapidly removed from skull and placed onto a pre-chilled metal stage covered by a piece of filter paper (Whatman). The corpus callosum was severed and the brain was butterflied out to reveal each striatal hemi section and cortex. Prefrontal cortex (PFC) and striatum were rapidly dissected

from the brain and placed into separate microcentrifuge tubes filled with 500 µL of lysis buffer containing protease and phosphatase inhibitors (1% SDS, 800 µM EDTA, 0.5 M Tris Base) preheated to 95◦ and immediately homogenized with a hand held homogenizer. Samples were then placed in a 95◦ heat block for 10 minutes before being placed on ice. Samples were vortexed and then spun down for 10 minutes at 10.5 rpm. Clean supernatant was removed and placed into a clean microcentrifuge tube for protein concentration analysis by a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Laemmli buffer was then added to all of the samples. Samples were loaded onto 10% SDS-polyacrylamide gels at a volume containing 20 µg of protein and separated by electrophoresis and then analyzed through western blotting. Proteins were transferred onto Immobilon-FL PVDF membrane (Millipore, catalog #IPFL00010) and then blocked for one hour at room temperature in a phosphoprotein blocker (Millipore, catalog #WBAVDP001). Membranes were placed in a primary antibody mix containing both mouse anti-ERK1/2 (Cell Signaling, catalog# 9107) and rabbit anti-phosphoERK1/2 (Cell Signaling, catalog# 9101) at a 1:1000 dilution in the phosphoprotein blocker and allowed to gently shake overnight at 4◦. Washes were with TBST (0.1% Tween) and Li-cor Odyssey IRDye secondaries were used (IRDye800 goat anti mouse at a 1:10000 dilution, IRDye680 goat anti rabbit at a 1:15000 dilution) to allow for multiplexing imaging of the protein bands. Band images were obtained using either an Odyssey Clx Imager or an Odyssey Fc Imager and quantified using Li-cor's Image Studio software. Phospho ERK1/2 bands were normalized to their Total ERK1/2 bands to account for differences in loading.

Statistical Analysis: Samples were analyzed by normalizing to the WT saline control. Prism7 statistical software package was then used to perform statistical analyses. To see data that was normalized to each genotype's respective saline control please see Appendix 5.

6.3 RESULTS

Both male and female DAT Val559 mice have an altered ERK1/2 response in the inactive phase in response to an AMPH drug challenge. WT females trend towards the expected increase in phosphorylated ERK1/2 with AMPH treatment. DAT Val559 females display elevated but non-significant levels phosphorylated ERK1/2 under the saline condition with no change produced by injection of AMPH in the PFC (Figure 18A). In the striatum, WT females have significantly elevated levels of phosphorylated ERK1/2 after injection of AMPH compared to DAT Val559 females that received AMPH (Figure 18B). Analysis of males in the inactive phase revealed that the WT animals trend towards the expected increase in phosphorylation of ERK1/2 in the PFC (Figure 18C) whereas the dorsal striatum shows no change in phosphorylated ERK1/2 in response to an AMPH challenge in either genotypes (Figure 18D).

Female mice do not have an AMPH-induced ERK1/2 response during the active phase. Interestingly, neither WT nor DAT Val559 females had an increase in ERK1/2 activation in response to a 3mg/kg injections of AMPH in either the PFC (Figure 19A) or the striatum (Figure 179) during the active phase.

Male DAT Val559 mice have an altered AMPH-induced ERK1/2 response in the active phase that is different from the inactive phase response. In the PFC both DAT Val559 and WT mice have an increase in phospho ERK1/2 in response to AMPH injection (Figure 19C). The striatum, however, only shows a trend for increased phosphorylated in the WT animals, with no change present in the DAT Val559 mice between saline and AMPH conditions (Figure 19D). Additionally there is a nonsignificant trend of basal ERK1/2 phosphorylation levels being elevated in the male DAT Val559 mice in both regions during the active phase.

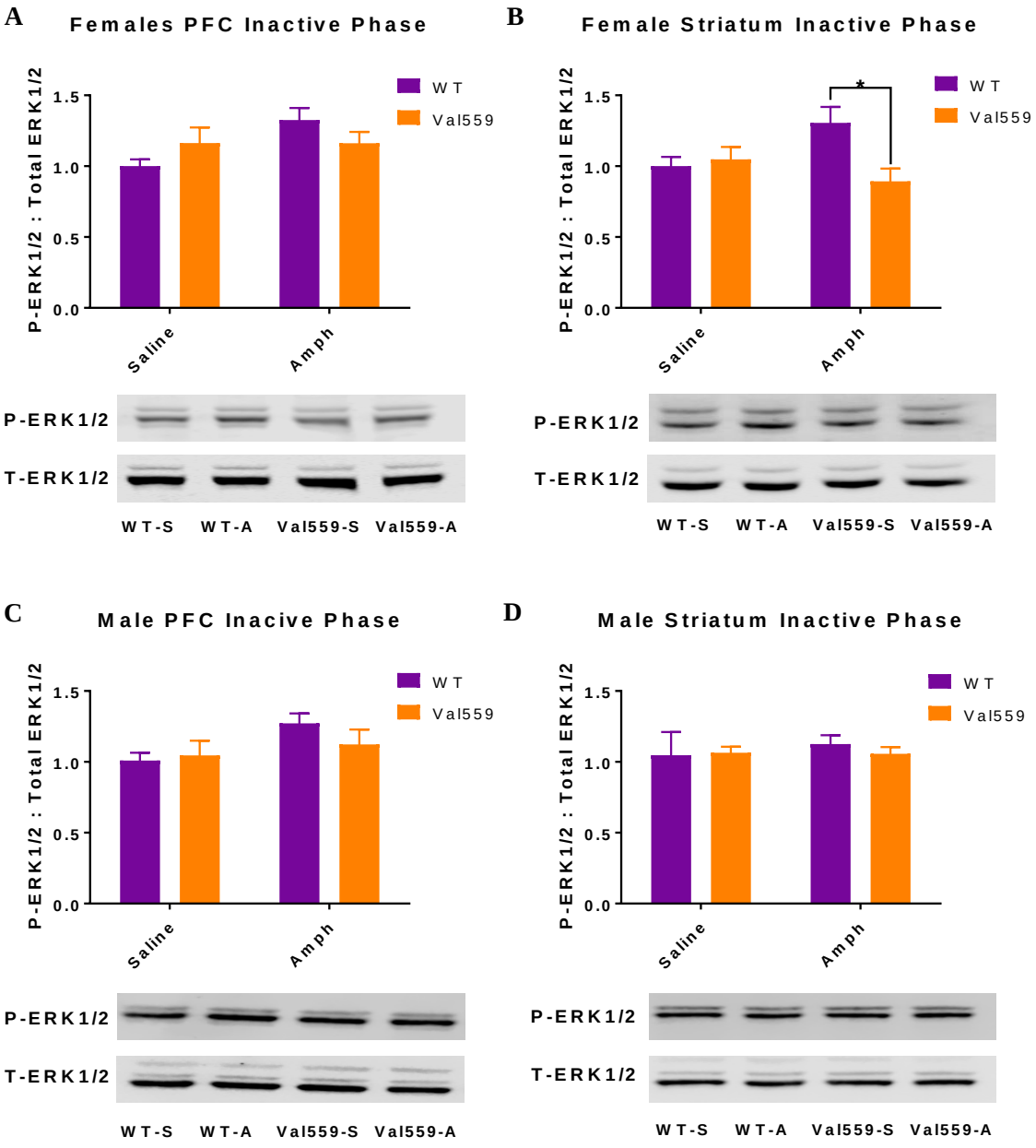

A
Females PFC Inactive Phase
P-ERK1/2 : Total ERK1/2
1.5
1.0
0.5
0.0
WT
Val559
Saline
Amph
P-ERK1/2
T-ERK1/2
WT-S
WT-A
Val559-S
Val559-A
B
Female Striatum Inactive Phase
*
C
Male PFC Inacive Phase
D
Male Striatum Inactive Phase

Figure 18. AMPH-induced ERK1/2 phosphorylation shows gender dependent differences in the inactive phase. WT female mice but not DAT Val559 female mice in the inactive phase show a trend towards increased phospho ERK1/2 in the PFC relative to WT saline controls (2-Way ANOVA genotype $P > 0.05$, treatment = 0.07, interaction = 0.06; WT = 6 per group, Val559 = 6 per group) (A). WT female mice have significantly elevated phospho ERK1/2 levels compared to DAT Val559 females challenged with AMPH in the striatum in the inactive phase (2-Way ANOVA, genotype $P = 0.06$, treatment $P > 0.05$, interaction $P < 0.05$; Tukey's multiple comparisons WT AMPH vs Val559 AMPH $P < 0.05$; WT = 6 per group, Val559 = 6 per group) (B). WT male mice have an elevated but nonsignificant increase in phosphorylated ERK1/2 in the PFC during the inactive phase (2-Way ANOVA genotype $P > 0.05$, treatment $P = 0.07$, interaction $P > 0.05$; WT = 5 saline and 6 AMPH, Val559 = 5 saline and 6 AMPH) (C). Both WT male mice and DAT Val559 male mice in the inactive phase do not show any increase in phospho ERK1/2 in the striatum relative to their saline controls (2-Way ANOVA, genotype $P > 0.05$, treatment $P > 0.05$, interaction $P > 0.05$; WT = 5 saline and 6 AMPH, Val559 = 6 saline and 5 AMPH)

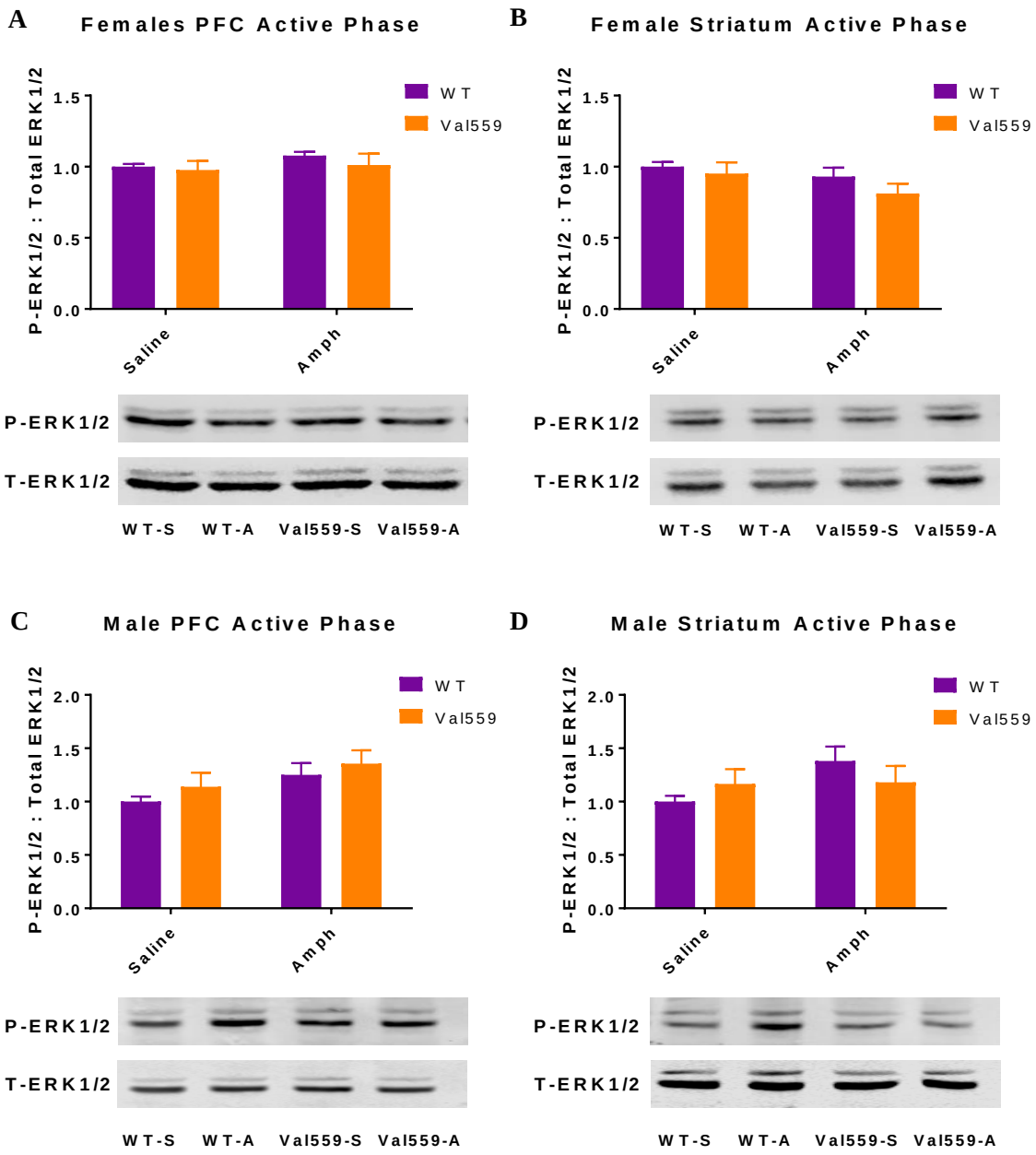

A
Females PFC Active Phase
P-ERK1/2 : Total ERK1/2
1.5
1.0
0.5
0.0
WT
Val559
Saline
Amph
P-ERK1/2
T-ERK1/2
WT-S
WT-A
Val559-S
Val559-A
B
Female Striatum Active Phase
C
Male PFC Active Phase
2.0
D
Male Striatum Active Phase

Figure 19. AMPH-induced ERK1/2 phosphorylation shows gender dependent differences in the active phase. Neither WT nor DAT Val559 females show differences in ERK1/2 phosphorylation in response to AMPH in both the PFC (A) and striatum (B) during the active phase (for both regions, 2-Way ANOVA, genotype $P > 0.05$, treatment $P > 0.05$, interaction $P > 0.05$; WT = 6 per group, Val559 = 7 per group). Both WT and DAT Val559 have an increase in phosphorylated ERK1/2 with an AMPH challenge in the PFC (2-way ANOVA, genotype $P > 0.05$, treatment $P < 0.05$, interaction $P > 0.05$; WT = 6 per group, Val559 = 6 per group) (C). WT male mice show a trend towards an elevated phosphorylated ERK1/2 response in the striatum with AMPH (2-Way ANOVA, genotype $P > 0.05$, treatment $P > 0.05$, interaction $P > 0.05$, WT = 6 per group, Val559 = 6 per group) (D).

6.4 DISCUSSION

I began assessment of molecular changes that may underlie the altered behaviors seen in the DAT Val559 mouse. I initiated this process by looking at the activation of ERK1/2 in response to AMPH, in hopes of understanding the post-synaptic component leading to the blunted AMPH induced locomotor response in the DAT Val559 mice.

In the PFC during the inactive phase, there was a trend towards an increase in phospho ERK1/2 after an AMPH challenge for both WT males and females. Whereas no change was observed in the DAT Val559 males and females in the PFC. In the striatum, WT females had significantly elevated level of phospho ERK1/2 after AMPH relative to DAT Val559 females, who trended toward a reduction of phospho ERK1/2 after AMPH administration. Interestingly, both the WT and DAT Val559 males failed to exhibit any drug-dependent alteration in phospho ERK1/2 in response to AMPH when tested in the inactive phase in the striatum.

We know that the DAT Val559 males respond to AMPH in the open field paradigm during the inactive cycle to a similar degree as male WTs (see Appendix 2) unlike the blunted response to AMPH exhibited by DAT Val559 in the active phase[134]. Further, previous work has shown that DAT activity changes in a circadian rhythm, such that, during the inactive phase both stimulated DA release and uptake rates are increased and extracellular DA levels are reduced relative to the active phase[244]. It has also been demonstrated that these alterations involve D2 autoreceptor (D2AR) dependent regulation of DAT as quinpirole has little to no effect on DA release during the inactive phase, but can significantly reduce DA-stimulated release in the active phase[244]. This supports the idea that WT and DAT Val559 mice have equivalent behavioral responses to AMPH in the inactive phase because a decrease in D2AR sensitivity may allow for an uncoupling of D2AR/DAT regulation, as we know that the DAT Val559 efflux is sustained by constitutively

active D2ARs[210]. I had hypothesized that I would see equivalent responses in ERK1/2 phosphorylation in response to AMPH during the inactive phase across genotypes. As such, it is surprising that, although behaviorally comparable, ERK1/2 activation levels differ in WT and DAT Val559 mice in the PFC when assayed during the inactive phase. In addition, I also failed to see the expected increase in AMPH-induced ERK1/2 phosphorylation in the striatum of the WT mice as reported in other strains. It is possible, thus, that the locomotive response in the inactive phase for the DAT Val559 mice is not being driven by activation of ERK1/2 and that the WT response in this strain is also different as ERK1/2 was demonstrated as a mediator of AMPH-induced locomotion in WT C57s animals[240-242]. It should also be mentioned that, even in the WT mice, the level of phospho ERK1/2 induced by AMPH in the PFC for males and both regions for females is inconsistent with other reports demonstrating a several fold increase in phospho ERK1/2 in response to AMPH. As these data were generated in another mouse strain (C57Bl/6), this is further evidence supporting the idea that strain may influence post-synaptic signaling mechanisms recruited following psychostimulant exposure.

Genotype-dependent alterations in the ERK1/2 response to AMPH were also present in the active cycle, and, interestingly, sex differences were also evident. For the females both WT and DAT Val559 animals exhibit the same lack of ERK1/2 phosphorylation in response to AMPH in either the PFC or striatum. Behaviorally DAT Val559 females and WTs both respond to AMPH in a locomotor chamber, failing to display the differences in AMPH-induced locomotion seen in males, it seems that phospho ERK1/2 may not play a role in this response. To our knowledge the ERK1/2 AMPH response has not been actively researched in females, with experiments occurring exclusively in males. The data would indicate that an alternate signaling mechanism must be at work to produce the locomotor effect of AMPH in females tested in the active phase.

The male response in the active phase also varied from the three previously discussed groups. Both WT and DAT Val559 males show an increase in phospho ERK1/2 in the PFC, while only WT males show an increase response in the striatum. Interestingly, work was done that indicates that the ERK1/2 response to AMPH in the PFC is not driven by DA but by norepinephrine[245]. Previously we had demonstrated that there are no differences in total tissue levels of norepinephrine or its metabolites in the DAT Val559 mice[174]. If ERK1/2 phosphorylation in the PFC is predominantly driven by AMPH-induced efflux of norepinephrine that would reconcile why there is still an AMPH-induced phospho ERK1/2 response in the PFC but not a drug response present in the striatum of the DAT Val559 male mice during the active cycle. This does not explain why there is no male or female DAT Val559 AMPH-induced phospho ERK1/2 response in the PFC of the inactive phase. However, the difference in AMPH-induced phospho ERK1/2 in the PFC of DAT Val559 males between the inactive and active phases provides support for changes in molecular response or even circuitry that could account for the time of day dependent differences of the AMPH-induced locomotor response in the DAT Val559 males.

In contrast to WT males in the inactive phase, AMPH-induced ERK1/2 phosphorylation increases in the striatum of WT males in the active phase.DAT Val559 males show no drug-induced changes in the striatum during the active phase. Phosphorylation of ERK1/2 in the striatum is dependent upon the convergence of both DA and glutamatergic signaling for the locomotor behavioral output[46, 246]. The maintained ERK1/2 response in the PFC of the DAT Val559 males provides support that glutamatergic inputs from the PFC to the striatum are appropriately activated by AMPH through a norepinephrine mechanism to support locomotive behavior and suggests that there are alterations in the DA afferents that produce both the lack of AMPH-induced ERK1/2 response and contributing to the blunted locomotor response. This is further supported by

previously reported data indicating reduced AMPH-induced DA release into the striatum[174]. As such, the regional differences in AMPH-induced ERK1/2 phosphorylation in the DAT Val559 males could indicate that there is an imbalance of DA and glutamatergic signaling within the striatum that ultimately plays a role in the AMPH-induced blunted locomotor response of DAT Val559 males in the active phase.

These data demonstrate that time of day, gender, region, and potentially strain, likely all affect the phosphorylation of ERK1/2 in response to AMPH. The data gathered in the male DAT Val559 mice in the active phase support the hypothesis that the blunted AMPH-induced locomotor response is produced from reduced AMPH-induced DA release in the striatum and is reflected by reduced phosphorylation of ERK. ERK activation, however, is not likely the sole post-synaptic driving force for this phenotype in the males or even necessarily a contributor to locomotor behavior at all, as demonstrated by the active phase females and inactive phase males. As such, other proteins associated with AMPH-induced locomotion should be investigated, such as β-arrestin signaling in D2 MSNs, as β-arrestin KOs have also been shown to have a blunted locomotor response to AMPH administration[247]. Regardless, I provide preliminary evidence arguing for post-synaptic changes in DAT Val559 mice that may be shaping behavior in addition to the presynaptic alterations induced by the expression of DAT Val559 already described[174]. Further, this work emphasizes the importance of circadian rhythms and gender in psychostimulant-induced intracellular signaling, which could profoundly impact drug response in humans.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

I utilized the first construct-valid model of ADHD to inform on behavioral perturbations driven by the alterations in the molecular and circuitry landscape produced by the expression of DAT Val559. Previous work has shown that the DAT Val559 produces alterations in neurochemistry and synaptic function. However, only very cursory behavioral analysis had been done to understand the consequences of an effluxing DAT. I sought to remedy this by pursuing behavioral assays that aligned with the clinical symptomatology of ADHD. The initial behavioral paradigm, 5-CSRTT, allowed me to assess multiple domains at once including attention, impulsivity, and cognition, though my main focus was geared toward impulsivity phenotypes as the initial identification of the mutation in the Blakely lab was strongly associated with an impulsivity phenotype both in the probands and their maternal grandmother.

Through this work I demonstrated that DAT Val559 mice display deficits in the ability to wait. However, this behavioral phenotype directly related to the delay schedule as a variable delay improved impulsive measures of the DAT Val559 relative to WT while long delays exacerbated impulsivity in DAT Val559 mice. This impulsivity was not a result of a generic deficit in prepotent motor responses nor a reflection of disordered interval timing as evaluated by Go/NoGo and peak interval, respectively. Instead, I demonstrated that the DAT Val559 mice rest in an enhanced motivational state that drives both enhanced task acquisition and context-dependent impulsivity.

Both a modified progressive ratio experiment and a within subject novel lever pressing experiment demonstrated that the DAT Val559 mouse are more resistant to the effects of reward devaluation, maintaining checking behavior of the food port. The increased motivational state of the DAT Val559 mouse may predispose it towards an increased susceptibility toward habit formation, potentially priming the animal to form maladaptive seeking behaviors. This could also be indicative that DAT Val559 will have issues with other measures of cognitive flexibility such as set-shifting.

Additionally, I used an AMPH challenge in an effort to begin to identify post-synaptic molecular changes that could contribute to altered psychostimulant responses resulting from the pre-synaptic expression of the DAT Val559. I demonstrated that the activation of ERK1/2 shows both gender and light cycle differences that could play a role in the blunted locomotor phenotype seen in the mole DAT Val559 mice in response to AMPH exclusively when the animals are tested during their active phase.

This work has revealed several interesting roles for the DAT Val559 both molecularly and behaviorally, but has also brought forth several important questions that still need to be assessed. The most obvious of these is assessing if AMPH has a therapeutic effect on any of the behavioral measures. Specifically, it would be informative to know if AMPH, a drug used clinically in the treatment of ADHD patients, normalizes the measures of enhanced motivation seen in the progressive ratio or peak interval task and, subsequently, the phenotype of waiting impulsivity observed in DAT Val559 mice. This could provide an additional layer of pharmacological evidence that the waiting impulsivity of this ADHD model is produced from their enhanced motivational state. It would also provide a level of predictive validity to the model that would then

validate it as a tool to be used for understanding how ADHD medications provide their therapeutic effect.

As mentioned, the devaluation studies could be indicative of broader issues with cognitive flexibility. A task such as Pairwise Discrimination with a reversal component could be used to further probe for such deficits. In this task mice learn to discriminate between two images, learning to associate that interaction with one image produces delivery of a reward while interacting with the other produces nothing or even a punishment. Once the animal has learned which image produces the reward, the contingencies are switched and the previously unrewarded stimulus is now the rewarded stimulus[180]. If DAT Val559 mice have broader deficits in cognitive flexibility produced by an inability to inhibit previously learned responses, then it is expected that they will take a longer number of sessions in order achieve response reversal. However, if the elevated head entries truly represent an increased susceptibility to form habitual seeking from a motivational nexus, then it is not unreasonable to also see the opposite occurring and that the DAT Val559 mice actually switch their behavior faster, going where the reward is.

Additionally, as of yet we have not done any assays to evaluate impulsive choice through tasks such as delay and probabilistic discounting. As previously discussed, the ADHD population is enriched for deficits in impulsive choice, preferring small immediate rewards to larger delayed ones as well as making riskier choices in choosing low probability, high reward options. It should be mentioned that this type of behavior is also present in BPD[248, 249] adding an additional layer of relevance for these tasks to be done as the variant was also identified in a BPD patient and share overlapping symptoms. Based on the waiting impulsivity seen in 5-CSRTT, I hypothesize that we will see higher levels of delay discounting in the DAT Val559 mice, resulting in the preference for the small but immediate rewards. Additionally, as elevated extracellular DA tone correlates with

preference for high reward, high risk options[162], I would hypothesize that the DAT Val599 mice would also prefer such choices.

Besides being of relevance for modeling of ADHD, this model also has cross-functionality potential for ASD and BPD. Thus, additional assays could be pursued to test for changes in additional ASD and BPD-related behaviors, as this model already has particular utility in the study of impulsivity domains shared across all three disorders. Indeed, pediatric BPD and ADHD can oftentimes be very difficult to differentiate from one another, showing similar cognitive and inhibition deficits[250, 251]. Cognitive flexibility issues are also present in all three disorders[231, 252, 253], underscoring the potential importance of the Pairwise Discrimination task. However, a line of inquiry that has been left largely unexplored are more social and emotional related tasks. As such, tasks related to social dominance such as the tube task, often used in models of ASD[254, 255], should be pursued. Additionally, using a chronic defeat model, we should analyze whether the DAT Val559 mice are more susceptible to bouts of mania or depressive phenotypes post-social stressors to look for more emotional disruptions associated with BPD[256].

Clarification on the molecular components should be investigated as well as more functional circuitry related pursuits, as the data discussed herein are limited in scope. Follow-up studies should focus on expanding the analysis to include other known proteins downstream of DA receptors in specific DAergic circuits that are activated or altered by administration of psychostimulants. Efforts are currently underway to broadly survey molecular changes in DA target regions using the transcription factor c-fos as a marker of neuronal activation. C-fos data could provide a gateway, in combination with existing literature, to the elucidation of pathways that could be manipulated to rescue aberrant behaviors in DAT Val559 mice utilizing novel neuromodulatory techniques such as optogenetics. Optogenetics provides the means to manipulate

DA circuits in a manner that is more specific than classical pharmacology. Based on the discussion I gave of the neural substrates of impulsivity in Chapter 2, I would expect that the DAT Val559 mice would show circuitry alterations in the prelimbic/infralimbic cortex as this region was linked to premature responding in the 5-CSRTT[161] as well as alterations in regions associated with motivation, such as the dorsal striatum. Although I did not directly test for the preference of immediate small rewards in a delay discounting task, I hypothesize that the motivation phenotype combined with the waiting impulsivity in the DAT Val559 mice might also produce an impulsive choice deficit. As such, it is possible that the DAT Val559 mice might have circuitry changes in the cingulate cortex as lesions to this area produce a preference for small immediate rewards. The end goal would be to pinpoint distinct pathways contributing to the waiting impulsivity and enhanced motivational state of DAT Val559 mice with the hope that this would assist in the identification of neural substrates amenable to therapeutic intervention.

In conclusion, I have demonstrated that the DAT Val559 model provides a unique opportunity to understand the role of a disease-relevant hyperdopaminergic state induced by the DAT Val559 variant. My studies have afforded an insight into a different mechanism by which impulsivity can arise as well as underscore the clear distinctions between subcategories of impulsive action. This effort has also further validated the DAT Val559 model as useful in understanding the clinical symptomatology present in ADHD, with strong crossover potential for ASD and BPD. The broad clinical relevance of this hyperdopaminergic model makes it ideal for future studies into development of improved therapies and understanding the mechanistic actions of current therapies. It is important not to over-inflate the importance of this model, but it does provide a promising next step for understanding the critical role of the DA system in neurodevelopmental disorders, providing insight upon which future work can be built.

APPENDIX 1: AGING EFFECTS ON PASSAGE OF DAT Val559 RISK ALLELE

INTRODUCTION

Epidemiological data has demonstrated a correlation between parental age and the likelihood of offspring presenting with certain neuropsychiatric disorders such as ASD and ADHD, with increased parental age tracking with an increased risk for affected offspring[257, 258]. Specifically, both maternal and paternal age have independently been correlated with risk for offspring with increasing age, with overall parental age having an additive risk correlation. Although striking, it still leaves the question as to causation.

De-regulation of dopaminergic neurotransmission is heavily implicated in ADHD, with drugs designed to interfere with activity of DAT used commonly as ADHD treatments. Catecholamines are present in human semen presumably coming from sympathetic nerve endings that innervate the testis[259]. The machinery necessary to transmit extracellular DA into intercellular signaling are also present, with DA D2 receptors detectable at the mRNA and protein level in the germ cells of various species[260, 261]. Recent work demonstrated the presence of functional DAT in equine sperm, where it appears to play a role in modulating acrosomal integrity and sperm motility[262]. Together these data point to a functional role for DA in sperm that could become dysregulated peripherally through mechanisms that have been described in the central nervous system. The DAT Val559 mouse (and perhaps other construct valid mouse models) could help test this hypothesis.

The DAT Val559 mouse is a construct valid mouse model of ADHD and dopaminergic dysfunction[134, 226]. This DAT variant was identified in probands with ADHD[127], ASD[128], and BPD[129], all of which can have overlapping symptoms associated with DA dysfunction. Earlier work demonstrated that this line followed a mostly traditional Mendelian distribution with a slight over-representation of WT at the cost of the homozygous allele [174]. However, here I demonstrate

that, as male breeders age, there is a distinctive shift, with an increase in the representation of the mutant allele and a decrease in the representation of the WT allele. This observation lead me to investigate the potential functional impact of the DAT Val559 mutation in sperm, comparing sperm of young (8-10 weeks) with aged (5-6 months) male WT and mutant animals. With the increased age at which people are conceiving offspring[263, 264] and the rise in diagnosis of conditions such as ADHD and ASD, the DAT Val559 mice could be a key tool to understanding the role of aging on the passage of risk alleles for psychiatric conditions.

METHODS

Animals: All experiments were performed under a protocol approved by the Institutional Animal Care and Use Committees at Vanderbilt University and Florida Atlantic University. DAT Val559 mice were maintained through heterozygous (Het) breeders on the hybrid background used in our prior studies[134] (75% 129S6 and 25% C57BL/6J). Animals were housed on a 12:12 (L: D) cycle.

Genotyping and Breeder Tracking: All animals from het x het breedings are tailed were tailed at approximately 21 days for genotyping. DNA was extracted from tissue samples using the Red Xtract-n-Amp Tissue PCR Kit (Sigma). DNA was then amplified in a thermocycler using KAPA2G Fast HotStart Genotyping PCR Mix (Kapa Biosystems) and the following forward and revers primers: 5'ctctctattcttggagacaata and 5'gggaccctattttcattgga respectively. Animals were genotyped based on the presence or absence of a leftover LoxP site (WT = 238 bp, HET = 238 bp and 309 bp, Val559 = 309 bp). Pup information including sire, dam, and date of birth, sex, and genotype was kept in an Excel database that was then used to determine genotype distribution in relation to sire age.

Sperm Count and Density: Mice were anesthetized by carbon dioxide. Male reproductive tract was exposed and the caudate of the epididymis was removed bilaterally and wet tissue was

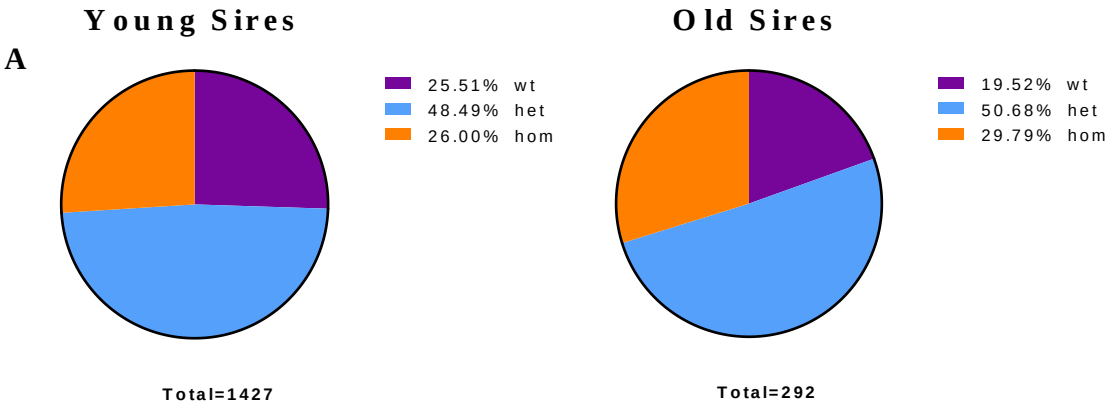
weighed. Tissue was then minced in a dish containing either one mL of warmed PBS. Dishes were placed in a 37 degree incubator for approximately 60 minutes to allow sperm time to swim out of the minced tissue. Sperm samples were then diluted by a factor 2,500,000 in saline. Sperm count was then obtained using a hemocytometer with samples counted in duplicate. If numbers obtained between duplicates were further than 10% apart from each other, the counting for that sample was done again. Persons quantifying were blind to the genotype associated with each sample. Based on sperm count and caudate weight, sperm density was calculated.

RESULTS

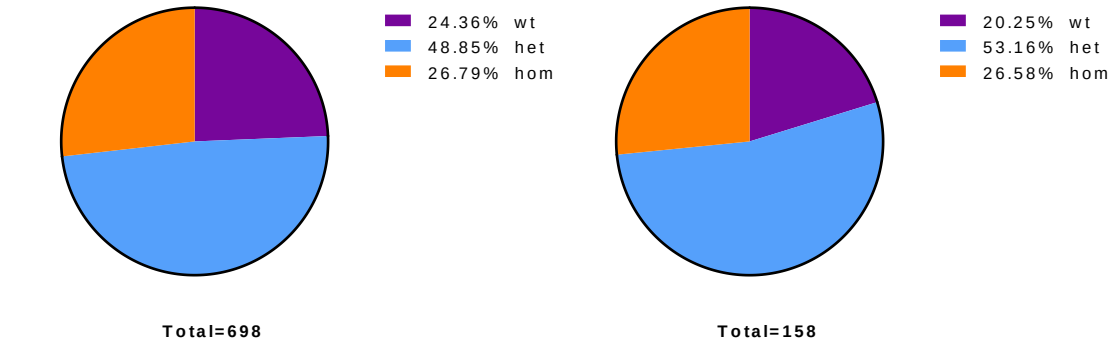
DAT Val559 representation increases in offspring with aging sire. The representation of the DAT Val559 allele was a significantly increased with sire age across all pups produced, resulting in a percentage increase in both homozygous and heterozygous pups (Figure 20A). Male pups from aged sires trended in an increase in percentage of heterozygous pups at the cost of WT (Figure 20B). Female pups displayed a large and significant percentage increase in homozygous pups (33.58%) at the cost of WT pups, with the amount of heterozygous pups remaining constant irrespective of sire age (Figure 20C).

DAT Val559 mice have increased sperm count with age. Given the allelic distribution shift in aged Het breeders, I sought to directly assess the impact of the DAT Val559 mutation on sperm. Sperm count and density were assessed in both young (8-10 weeks).

A


Young Sires
25.51% wt
48.49% het
26.00% hom
Total=1427
Old Sires
19.52% wt
50.68% het
29.79% hom
Total=292

B


24.36% wt
48.85% het
26.79% hom
Total=698
20.25% wt
53.16% het
26.58% hom
Total=158

C

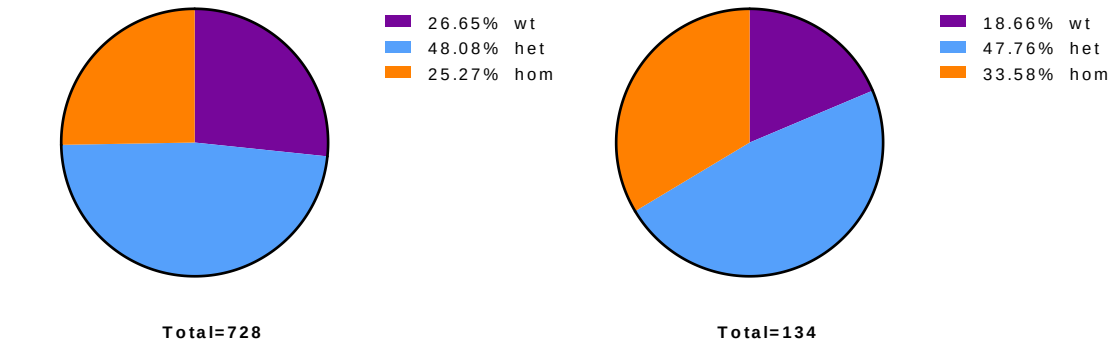

26.65% wt
48.08% het
25.27% hom
Total=728
18.66% wt
47.76% het
33.58% hom
Total=134

Figure 20. Genotype distributions shift with aged sires. Young het sires followed Mendelian inheritance looking at male and female distributions combined, while aged sires showed significant reductions in WT pup representation (Chi-Square test, $P < 0.05$) (A). Young het sire produce male pups as expected by Mendelian inheritance, while a 4% reduction in WT male pups was seen with aged het sires, though this did not reach significance (Chi-square test) (B). Young het sires produce female pups as expected by Mendelian inheritance, but older het sires produced WT female pups at a significant 6% reduction with an increase in DAT Val559 allele representation (Chi-square test, Old Sire allele distribution, $P < 0.05$) (C).

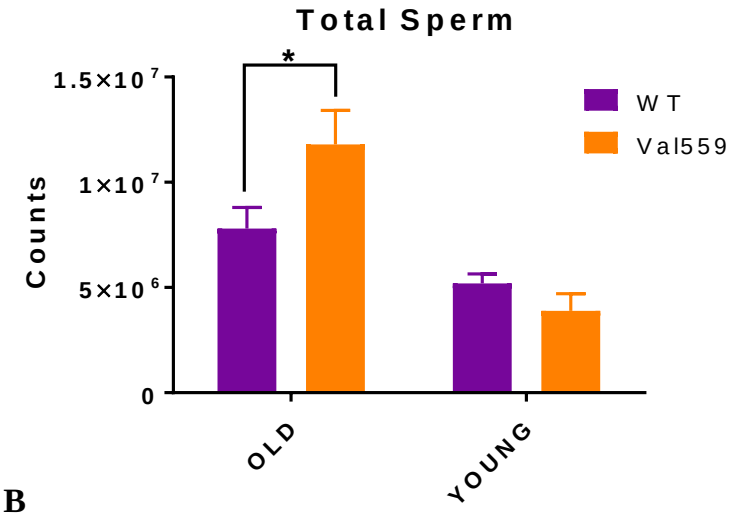
and old males (5-6 months) of both the WT and homozygous DAT Val559 genotypes. Interestingly, there is a trend to HOM mice having less sperm at the younger age, but as the mice age HOM have a significant increase in sperm count relative to age matched WT mice (Figure 21A). This is true for sperm density as well (Figure 21B). No difference was seen in weights of the caudate epididymis between genotypes (Figure 21C).

DISCUSSION

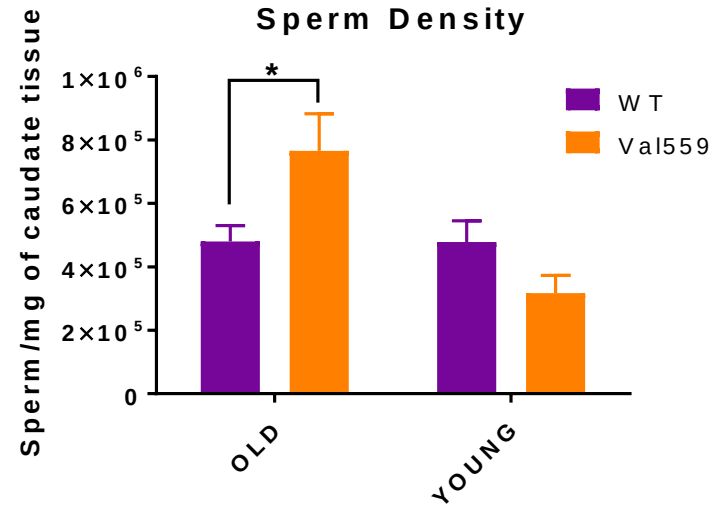
Anecdotal observations indicating overrepresentation of the homozygous DAT Val559 mutant allele in pups produced from aged sires led to a more directed analysis of allelic distribution with aging sire. Compilation of the mouse genotyping records revealed that there was a shift in DAT Val559 representation in both male and female pups. In male pups I observed a 4% increase in heterozygosity in litters sired by males over 6 months of age compared to litters sired by fathers younger than 6 months of age. This increase in heterozygous pups comes at the cost of the WT population. Interestingly, in females I observed a significantly large increase homozygous pups again, approximately 8%, at the cost of WT population. This correlates with a human study that shows an increased association between parental age and risk for ASD in female offspring[257].

Upon confirmation of this breeding shift with aged sires, I assessed young and aged male mice that were either WT or DAT Val559 homozygous for differences in sperm count and density. The increase DAT Val559 representation in litters of older sires seems to correlate with changes in the male reproductive system. Specifically, I found an age-specific shift in both total sperm count and sperm density in the DAT Val559 sires with increasing age. As such, DAT Val559 homozygous mice go from having a slightly lower sperm count and sperm density as young males to having significantly higher levels of sperm and sperm density compared to WT aged males.

A


Total Sperm
*
1.5×10 7
1×10 7
5×10 6
0
Counts
WT
Val559
OLD
YOUNG

B


Sperm Density
*
1×10 6
8×10 5
6×10 5
4×10 5
2×10 5
0
Sperm/mg of caudate tissue
WT
Val559
OLD
YOUNG

C

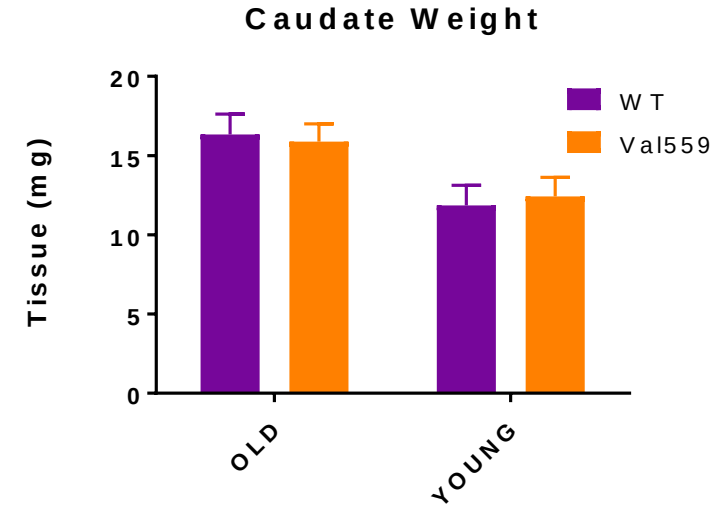

Caudate Weight
20
15
10
5
0
Tissue (mg)
WT
Val559
OLD
YOUNG

Figure 21. DAT Val559 male mice have age-related sperm alterations. Aged DAT Val559 mice have significantly more sperm than their WT counterparts, with a significant main effect of age between old and young male mice sperm counts (Two-way ANOVA, interaction $P < 0.05$, age $P < 0.0001$; Sidak's multiple comparisons comparing each cell mean with the other cell means in that row, Old WT vs Old DAT Val559 $P < 0.05$; Old WT = 9, Young WT = 7, Old DAT Val559 = 8, Young DAT Val559 = 7) (A). Aged DAT Val559 mice have a significantly higher sperm density than their WT counterparts, with a significant main effect of age between old and young male mice for sperm density (Two-way ANOVA, interaction $P < 0.01$, age $P < 0.01$; Sidak's multiple comparisons comparing each cell mean with the other cell means in that row, Old WT vs Old DAT Val559 $P < 0.05$) (B). No differences in the weight of the caudate epididymis were seen within an age group, but there was a main effect of age (Two-way ANOVA, age $P < 0.01$) (C).

The shift in sperm quantity with age could possibly explain the alterations in pup genotype distribution seen between young and aged heterozygous male breeders. If the balance in sperm quantity in the heterozygous breeders is shifting such that there is now a higher representation of sperm carrying the DAT Val559 allele, then with every breeding event the probability of a homozygous allele making it to a fertilization event is arguably increased. Studies are actively being pursued to determine if this is indeed what is occurring in the heterozygous male breeders.

The question now remains as to what mechanism(s) contribute to the increase sperm count in DAT Val559 mice that occur with age. One study argues that increased risk of disorders such as ASD are the result of hormonal changes that occur with increasing age[265]. Additionally, it was shown that DAT is expressed on sperm and plays a role in swimming speed, survival, and capacitation (a necessary phenomenon required just prior to fertilization). Specifically, it was demonstrated that increased extracellular DA levels reduces motility of stallion sperm and compromises acrosomal integrity in a DAT dependent manner[262]. Since DA is present in both the male and female reproductive tract, it could be that catecholamine levels change with age. As such, if DA uptake reduces motility and compromises integrity, then perhaps the DAT Val559 sperm are provided with a survival advantage in that sperm containing DAT Val559 would theoretically efflux out the DA, and so may be more resistant to the negative effects of environments with increased DA.

Understanding how sperm levels are affected by the interaction of age and the presence of DAT Val559 could be invaluable to understanding the rise in diagnoses of disorders such as ASD and ADHD, especially since both the maternal and paternal age at conception of first child is increasing[263, 264].

Several pivotal studies have made strong arguments for the role of parental age, both paternal

and maternal, have increasing both independent and combinatorial risk for disorder diagnosis in offspring. Studies have now begun to focus on the mechanism behind this age risk interaction with many pointing towards the accumulation of de novo SNP mutations accumulating in the germline[265, 266]. Given the strong heritability of ADHD, it is intriguing that an additional neurochemical mechanism(s) could be at work promoting the passage of these risk alleles. As such, the DAT Val559 mouse could provide powerful tools to investigate this question in an independent but complimentary manner to current work being done with both epigenetic factors and age-related independent mutation accumulation studies. This could allow for therapeutics to be developed specifically for aged couples who are partaking in intentional family planning to reduce the passage of risk alleles.

APPENDIX 2: OPEN FIELD EXPERIMENTS

Several open field experiments were conducted during the course of this theses looking at the pharmacologically effects of various agents on locomotor behavior. Locomotor behavior was measured in Med-Associate open field boxes. On day 1 mice were placed in chambers for a 30 minute habituation period. Day 2 was a day of rest followed by day 3 which consisted of a 30 minute habituation period followed by saline injection with a 60 minute recording period after that. This was done to form a baseline for any effects that injection stress could have on the measured behavior. Day 4 was a day of rest, but day 5 ran similarly to day 3, however, instead of a saline injection mice received a drug injection. Experiments were run in the active phase unless stated otherwise. DAT Val559 mice had a blunted locomotor response to 10mg/kg MPH (Figure 22). 3 mg/kg of AMPH delivered in the inactive phase did not reveal genotype differences in contrast to the behavioral response in the active phase (Figure 23A). 1 mg/kg AMPH produced a small but significant increase in WT locomotor behavior but not for DAT Val559 mice (Figure 23B, collected by Dr. Adele Stewart). Administration of 0.1 mg/kg raclopride has no effect on locomotor behavior in either genotypes, however, 0.1 mg/kg raclopride co-injected with 3 mg/kg AMPH appears to rescue the blunted locomotor phenotype in DAT Val559 animals (Figure 24A). Additionally, 5 mg/kg sulpiride co-injected with 3 mg/kg AMPH has opposite effects on locomotor behavior in WT and DAT Val559 mice while 5mg/kg of sulpiride produce no effects on its own (Figure 24B). It should be noted that experiments with sulpiride experienced some disruptions in experimental timeline due to unintentional light pulses during the active phase from which the mice had to recover and an interruption caused by hurricane Irma.

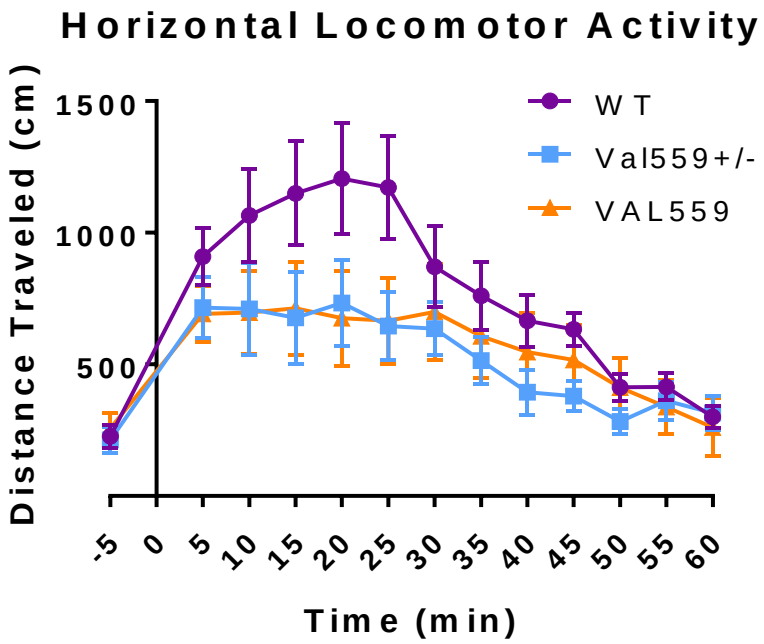
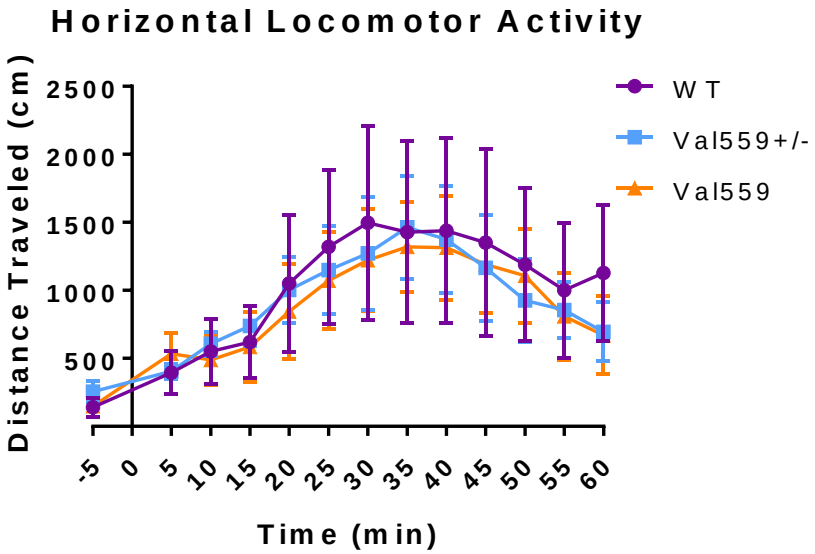


Figure 22. DAT Val559 and Val559+/- demonstrate a blunted horizontal locomotor response to a 10 mg/kg does of MPH. DAT Val559 and DAT Val559+/- mice show a strong trend for having a blunted locomotor response to 10 mg/kg of MPH given during the active phase (2-Way repeated measures ANOVA, genotype $P > 0.05$, time $P < 0.0001$, interaction $P = 0.06$; WT = 14, Val559+/- = 15, Val559 = 15).

A



B

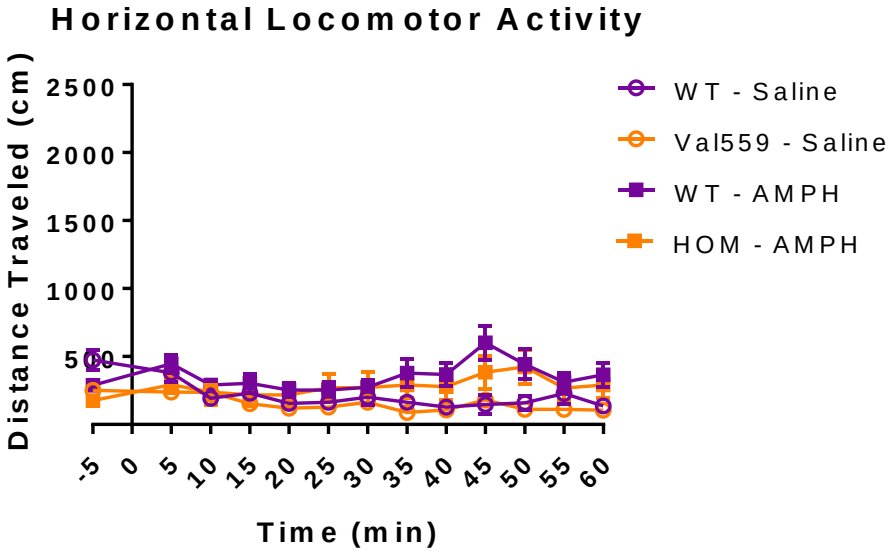
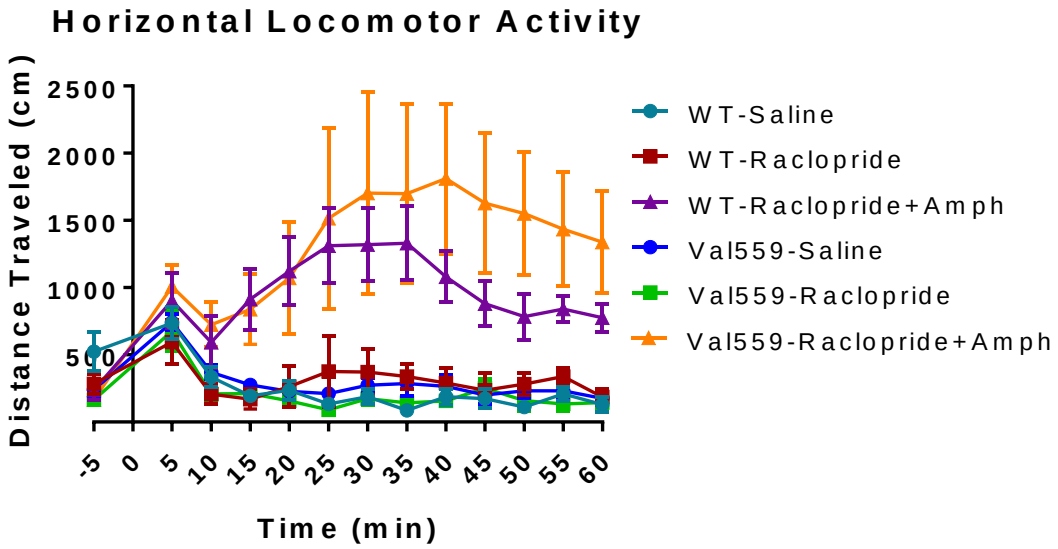


Figure 23. Locomotor responses to AMPH. DAT Val559 and Val559 +/- mice show no difference in locomotor behavior in response to 3 mg/kg of AMPH when given in the inactive phase (2-Way repeated measures ANOVA, genotype $P > 0.05$, time $P < 0.0001$, interaction $P > 0.05$; WT = 7, Val559 +/- = 7, Val559 =8) (A). A low 1 mg/kg dose of AMPH given in the active phase does not produce a locomotor response in DAT Val559 animals, but a mild one in WT animals (2-Way repeated measures ANOVA, genotype $P > 0.05$, drug $P < 0.0001$, interaction $P < 0.0001$; Tukey's multiple comparisons test, Val559 saline vs WT AMPH $P < 0.05$ at 35 min, $P < 0.01$ at 50 min, and $P < 0.001$ at 45 min; WT saline vs WT AMPH $P < 0.05$ at 50 min and $P < 0.001$ at 45 min; Val559 saline vs Val559 AMPH $P < 0.01$) (B).

A


Horizontal Locomotor Activity
Distance Traveled (cm)
2500
2000
1500
1000
500
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (min)
WT-Saline
WT-Raclopride
WT-Raclopride+Amph
Val559-Saline
Val559-Raclopride
Val559-Raclopride+Amph

B

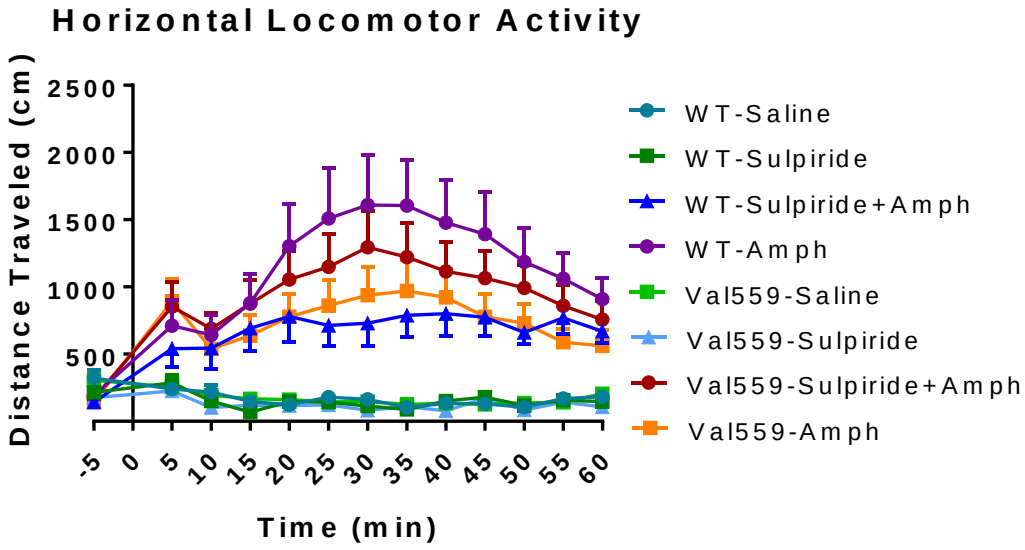
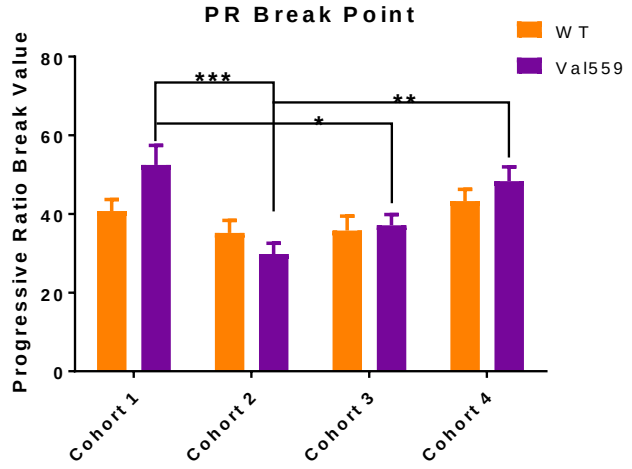

Horizontal Locomotor Activity
Distance Traveled (cm)
2500
2000
1500
1000
500
-5
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (min)
WT-Saline
WT-Sulpiride
WT-Sulpiride+Amph
WT-Amph
Val559-Saline
Val559-Sulpiride
Val559-Sulpiride+Amph
Val559-Amph

Figure 24. Rescue attempts for DAT Val559 blunted locomotor response phenotype in response to AMPH. A blunted locomotor phenotype is not seen in the DAT Val-559 mice with co-injection of 0.1 mg/kg of raclopride and 3 mg/kg of AMPH in the active phase (2-Way repeated measures ANOVA, genotype $P > 0.05$, time $P < 0.0001$, interaction $P > 0.05$; WT = 6, Val559 = 7). No genotype differences were produced by 0.1 mg/kg of raclopride compared to saline locomotor activity (2-Way repeated measures ANOVA, genotype $P > 0.05$, time $P < 0.0001$, interaction $P > 0.05$; WT = 6, Val559 = 7) (A). Co-injection of 5 mg/kg of sulpiride with 3 mg/kg AMPH in the active phase blunts WT locomotor response with a trend towards rescuing the blunted DAT Val559 AMPH response (2-Way repeated measures ANOVA, genotype $P > 0.05$, time $P < 0.0001$, interaction $P < 0.05$; Tukey's multiple comparisons WT sul/AMPH vs WT AMPH $P < 0.05$ at 25 min and 35 min, $P < 0.01$ at 30 min; WT sul/AMPH = 12, WT AMPH = 14, Val559 sul/AMPH = 14, Val559 AMPH = 12). The 5 mg/kg dose of sulpiride has no locomotor effect on its own as compared to saline controls (2-Way repeated measures ANOVA, genotype $P > 0.05$, time $P < 0.0001$, interaction $P > 0.05$; WT sulpiride = 17, WT saline = 17, Val559 sulpiride = 18, Val559 saline = 18) (B).

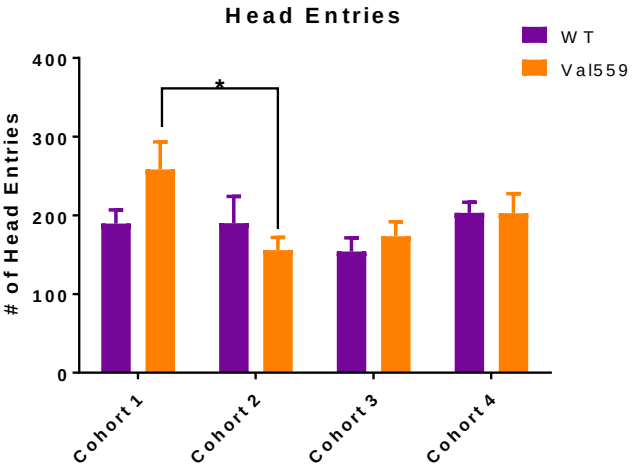
APPENDIX 3: MOTIVATION AND EARLY LIFE STRESS

Below data are presented on the potential effects of early life stress on the motivational measure of the DAT Val559 mice. As shown in chapter 4, the DAT Val559 mice display increased measures of motivation as determine by the progressive ratio task. Two cohorts of mice experienced early life stress such that the colony housing room flooded and mice had to be moved to an emergency space. This involved the racks being moved to a completely different floor with sub-optimal conditions for approximately two weeks and then moved back to their original housing room. During this period two behavioral cohorts were being produced, one group consisting of pups that were less than a week old during the start of the disturbance and one group still in the gestational stage of development. Data below shows that these two groups have alterations in measures obtained from progressive ratio. Importantly, once mice were allowed to reacclimatize after the event, progressive ratio measures returned to similar levels as data collected pre-flooding, i.e. DAT Val 559 having heightened measures of motivation. For groups that experienced early life stress the WT mice showed a slight but non-significant reduction in break points. The DAT Val559 mice showed a significant reduction in break point values (Figure 25A), with mice who were newly born during the disturbance showing the biggest deficit and mice who were in the gestational stages showing a milder but still present reduction (Figure 25A). Significant alterations were also present in measures of head entry (Figure 25B) as well as number of correct nose-pokes made (Figure 25C). This could have serious implications for how this mutation interacts with early life stress and warrants further more structured investigation with early life stress protocols such as maternal separation.

A


PR Break Point
WT
Val559
Progressive Ratio Break Value
80
60
40
20
0
***
**
*
Cohort 1
Cohort 2
Cohort 3
Cohort 4

B


Head Entries
WT
Val559
# of Head Entries
400
300
200
100
0
*
Cohort 1
Cohort 2
Cohort 3
Cohort 4

C

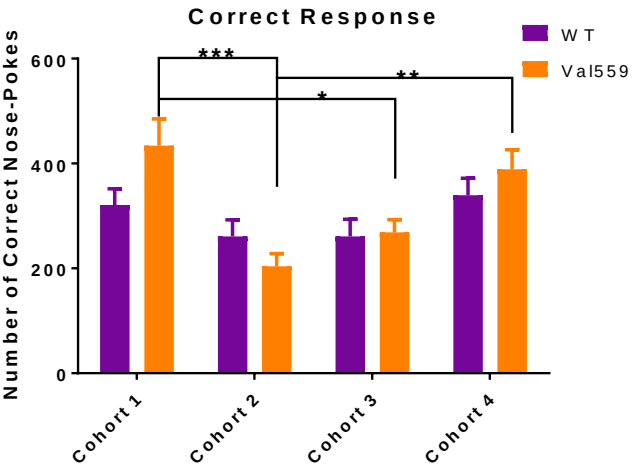

Correct Response
WT
Val559
Number of Correct Nose-Pokes
600
400
200
0
***
**
*
Cohort 1
Cohort 2
Cohort 3
Cohort 4

Figure 25. DAT Val559 mice show deficits in motivation produced by early life stress events. DAT Val559 mice show significant reductions in progressive ratio break point values achieved when exposed to early life stress, relative to DAT Val559 mice not exposed to early life stress (Two-way ANOVA, trending interaction $P = 0.09$, cohort $P < 0.0001$; Sidak's multiple comparisons comparing each cell mean with the other cell means in that row, cohort 1 vs cohort 2 DAT Val559 $P < 0.001$, cohort 1 vs cohort 2 DAT Val559 $P < 0.05$, cohort 2 vs cohort 4 $P < 0.01$; cohort 1 WT = 11, cohort 1 Val559 = 9, cohort 2 WT = 10, cohort 2 Val559 = 11, cohort 3 WT = 9, cohort 3 Val559 = 9, cohort 4 WT = 11, cohort 4 Val559 = 12) (A). DAT Val559 mice show a trend toward reduced head entries after early life stress compared to DAT Val559 animals that were not exposed to early life stress (Two-way ANOVA, cohort $P = 0.05$; Sidak's multiple comparisons comparing each cell mean with the other cell means in that row, cohort 1 vs cohort 2 DAT Val559 $P < 0.05$) (B). DAT Val559 mice show significant reductions in number of correct nose-pokes made when exposed to early life stress, relative to DAT Val559 mice not exposed to early life stress (Two-way ANOVA, trending interaction $P = 0.09$, cohort $P < 0.0001$; Sidak's multiple comparisons comparing each cell mean with the other cell means in that row, cohort 1 vs cohort 2 DAT Val559 $P < 0.001$, cohort 1 vs cohort 2 DAT Val559 $P < 0.05$, cohort 2 vs cohort 4 $P < 0.01$) (C).

APPENDIX 4: PERSEVERATIVE CHECKING BEHAVIOR RESCUE EXPERIMENTS

Below are results for preliminary investigation into the increased head entry phenotype discussed in chapter 5. In this experiment, I focused on rescuing the elevated head entry phenotype specifically with the random ratio training schedule (goal-directed schedule). Two cohorts were done where animals were treated with 1 mg/kg AMPH (see appendix 3 for locomotor data). 25 minutes post-injection mice were placed in the 5 minute non-rewarded probe trial. As can be seen below there is a cohort to cohort variability, where cohort one nicely replicates what was seen in the baseline data collected for the saline condition, with a trend of reduction of head entries seen in the DAT Val559 animals treated with 1 mg/kg AMPH (Figure 26A). I also see the expected nose-poke behaviors for valued versus devalued states, with a significant reduction of nosepokes made in the devalued context. Importantly, no difference in home cage consumption of chow or ensure was seen between genotypes (Figure 26B). For cohort two, however, DAT Val559 animals treated with saline have a reduced number of head entries compared to WT animals, contrary to previous experiments. Additionally, the application of AMPH seems to have a reducing effect on WT animals with no effect seen in DAT Val559 (Figure 26C). However, home cage consumption of Ensure is different for this cohort of animals. DAT Val559 mice have a significant increase in Ensure consumption relative to WT animals (Figure 26D) as well as increased consumption compared to the initial paradigm discussed in chapter 5 and the first cohort where a drug was rescue was attempted (Table 1). This could reasonably be the cause of the altered head entry behavior seen, making it incompatible for comparison to the previous cohorts. The increase in Ensure consumption for the second rescue cohort could also be an indicator of broader issues within that group of animals. It should be noted that there were some colony disruptions during the pre-weaning stage of the animals in rescue cohort two. Specifically, large fluctuations over

several days in temperature and humidity occurred due to a facility issue. The altered behavior seen in cohort 2 in comparison to cohort 1 and the cohorts from chapter 5 could be another indication of the effects of environmental stressors on behavioral phenotypes. A third cohort would be invaluable for determining the efficacy of the rescue experiment.

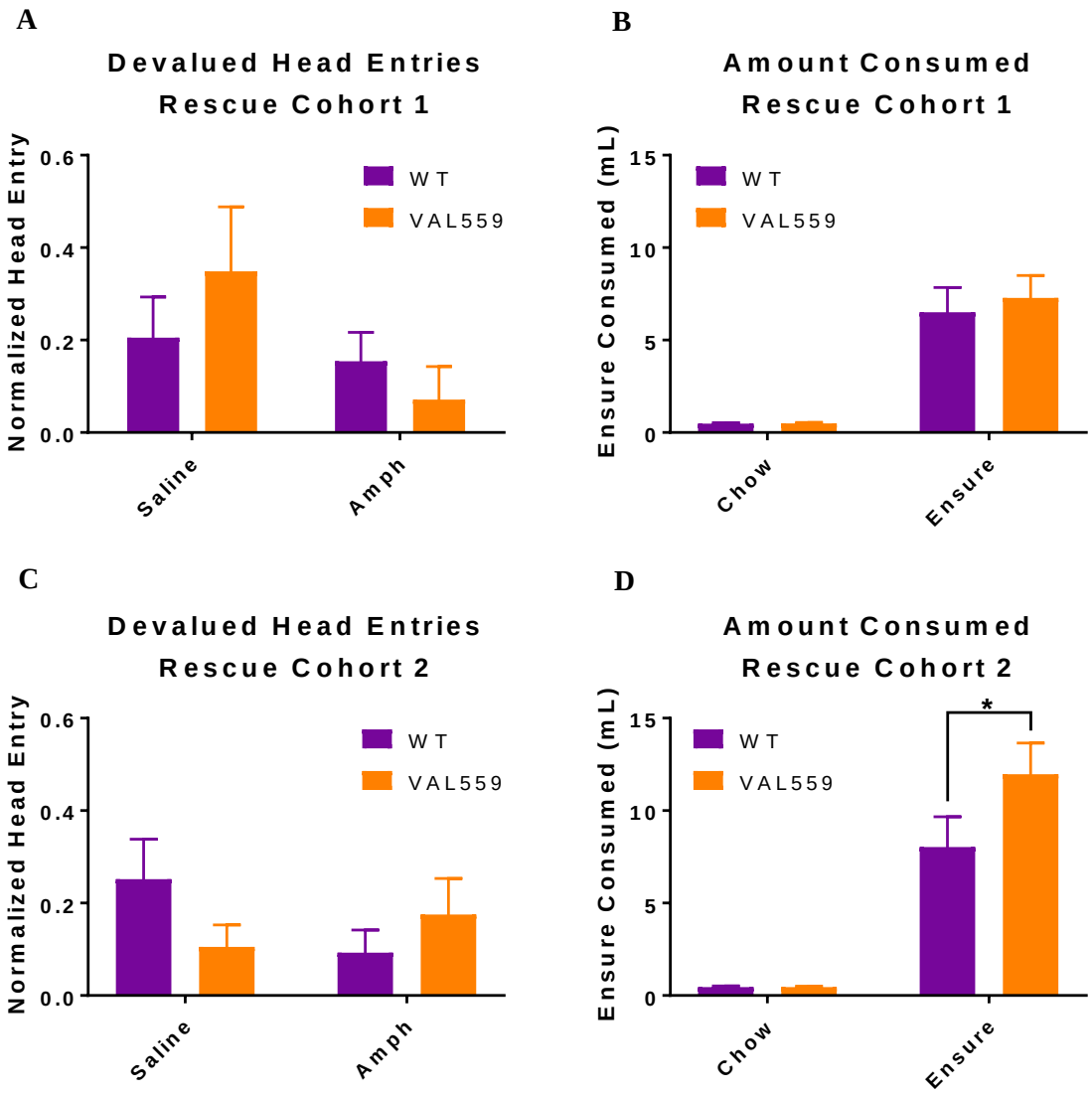

A
Devalued Head Entries
Rescue Cohort 1
Normalized Head Entry
0.6
0.4
0.2
0.0
WT
VAL559
Saline
Amph
B
Amount Consumed
Rescue Cohort 1
Ensure Consumed (mL)
15
10
5
0
WT
VAL559
Chow
Ensure
C
Devalued Head Entries
Rescue Cohort 2
Normalized Head Entry
0.6
0.4
0.2
0.0
WT
VAL559
Saline
Amph
D
Amount Consumed
Rescue Cohort 2
Ensure Consumed (mL)
15
10
5
0
*
WT
VAL559
Chow
Ensure

Figure 26. Trans-cohort variability obscure potential rescue effects of AMPH on perseverative checking phenotype. DAT Val559 mice trend toward having increased number of head entries under the saline condition relative to WT animals that appears to be reduced with administration of 1mg/kg AMPH (Two-way ANOVA, genotype $P > 0.05$, interaction $P > 0.05$, treatment $P > 0.05$; WT saline = 7, WT AMPH = 6, Val559 saline = 6, Val559 AMPH = 5) (A). No genotype differences were seen for amount consumed of chow or ensure (multiple t-tests with correction for multiple comparisons by Holm-Sidak method, WT vs Val559 chow $P > 0.05$, WT vs Val559 ensure $P > 0.05$) (B). DAT Val559 mice trend toward having a reduced number of head entries under the saline condition relative to WT animals with no effect of AMPH, while WT animals trend toward a reduced number of head entries with AMPH (Two-way ANOVA, genotype $P > 0.05$, interaction $P > 0.05$, treatment $P > 0.05$; WT saline = 6, WT AMPH = 5, Val559 saline = 6, Val559 AMPH = 5) (C). DAT Val559 mice show a significant increase in the amount of ensure consumed relative to WT animals (multiple t-tests with correction for multiple comparisons by Holm-Sidak method, WT vs Val559 chow $P > 0.05$, WT vs Val559 ensure $P < 0.05$) (D).

Table 1	Baseline		Rescue Experiment 1		Rescue Experiment 2	
	average	s.e.m	average	s.e.m	average	s.e.m
WT	8.33	0.59	6.51	1.34	8.04	1.63
Val559	7.11	0.57	7.28	1.22	11.97	1.69

Table 1. Ensure consumption comparisons across experiments. DAT Val559 mice consume significantly more Ensure during devaluation during rescue experiment 2 compared to baseline and rescue experiment 1 DAT Val559 mice (2-Way ANOVA, genotype $P < 0.05$, experiment $P > 0.05$, interaction $P > 0.05$; Tukey's multiple comparisons test within genotype, baseline vs rescue experiment 2 $P < 0.05$, rescue experiment 1 vs rescue experiment 2 $P < 0.05$; WT baseline = 12, WT rescue 1 = 13, WT rescue 2 = 11; Val 559 baseline = 10, Val 559 rescue 1 = 11, Val 559 rescue 2 = 11).

APPENDIX 5: ALTERNATIVE NORMALIZATION OF ERK1/2 DATA

The biochemistry data presented in Chapter 6 was presented so that all data sets were normalized to the WT saline control of each experimental condition (gender and activity phase). Presented here are the same data sets but normalized to genotype saline controls (e.g. Val599 AMPH treated normalized to Val559 saline treated samples). All data sets below were analyzed using 2-way ANOVA with Sidak's multiple comparisons. Post hoc tests compared means across drug conditions within the genotype.

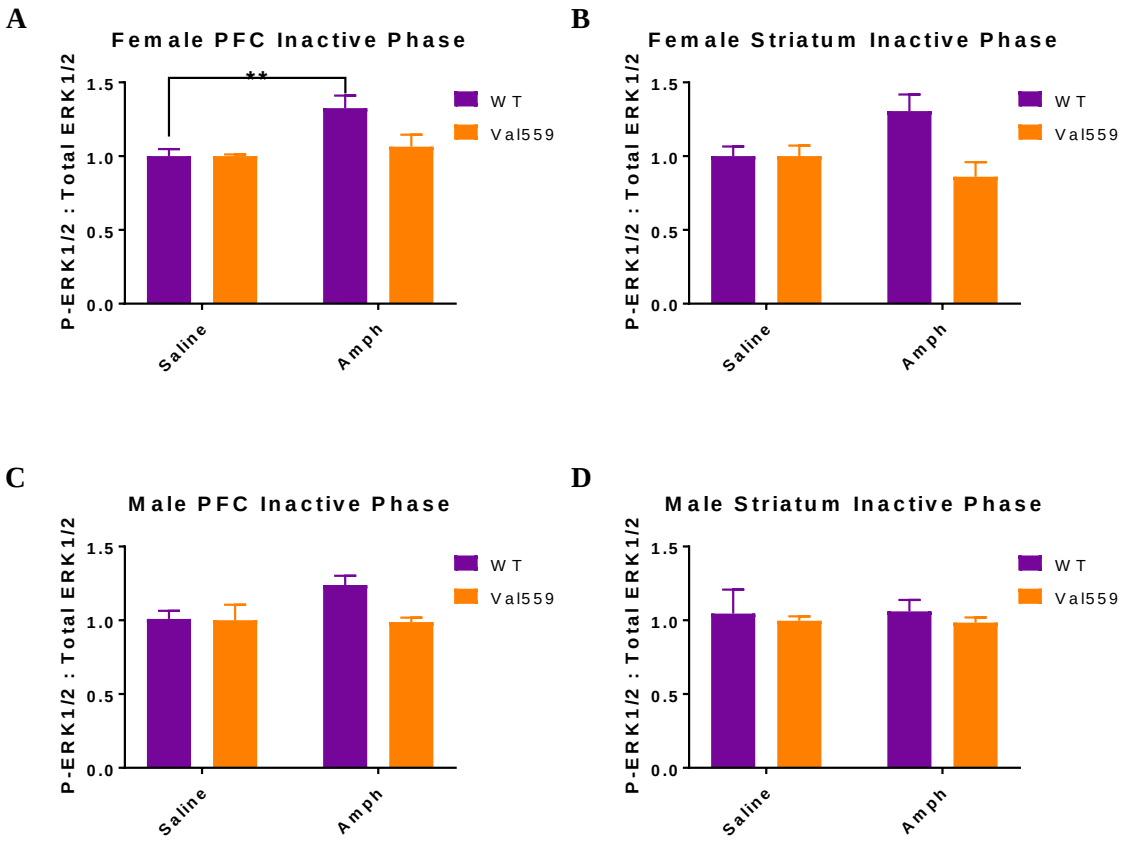


Figure 27. AMPH-induced ERK1/2 phosphorylation shows gender dependent differences in the inactive phase. WT female mice but not DAT Val559 female mice in the inactive phase show an increase in phospho ERK1/2 in the PFC relative to their saline controls (2-Way ANOVA, genotype P > 0.05, drug $P < 0.01$, interaction $P = 0.0526$; multiple comparisons WT saline vs WT AMPH $P < 0.01$ WT; WT = 6 per group, Val559 = 6 per group) (A). A similar difference in ERK1/2 activation patterns was also present in the striatum (2-Way ANOVA, genotype $P < 0.05$, interaction $P < 0.05$, drug $P > 0.05$) (B). WT male mice but not DAT Val5599 males in the inactive phase show a trend in increased phospho ERK1/2 in the PFC relative to their saline controls (2-Way ANOVA, genotype $P = 0.0955$, drug $P > 0.05$, interaction $P > 0.05$; WT = 5 saline and 6 AMPH, Val559 = 6 saline and 5 AMPH) (C). Both WT male mice and DAT Val559 male mice in the inactive phase do not show any increase in phospho ERK1/2 in the striatum relative to their saline controls (2-Way ANOVA, genotype $P > 0.05$, drug $P > 0.05$, interaction $P > 0.05$) (D).

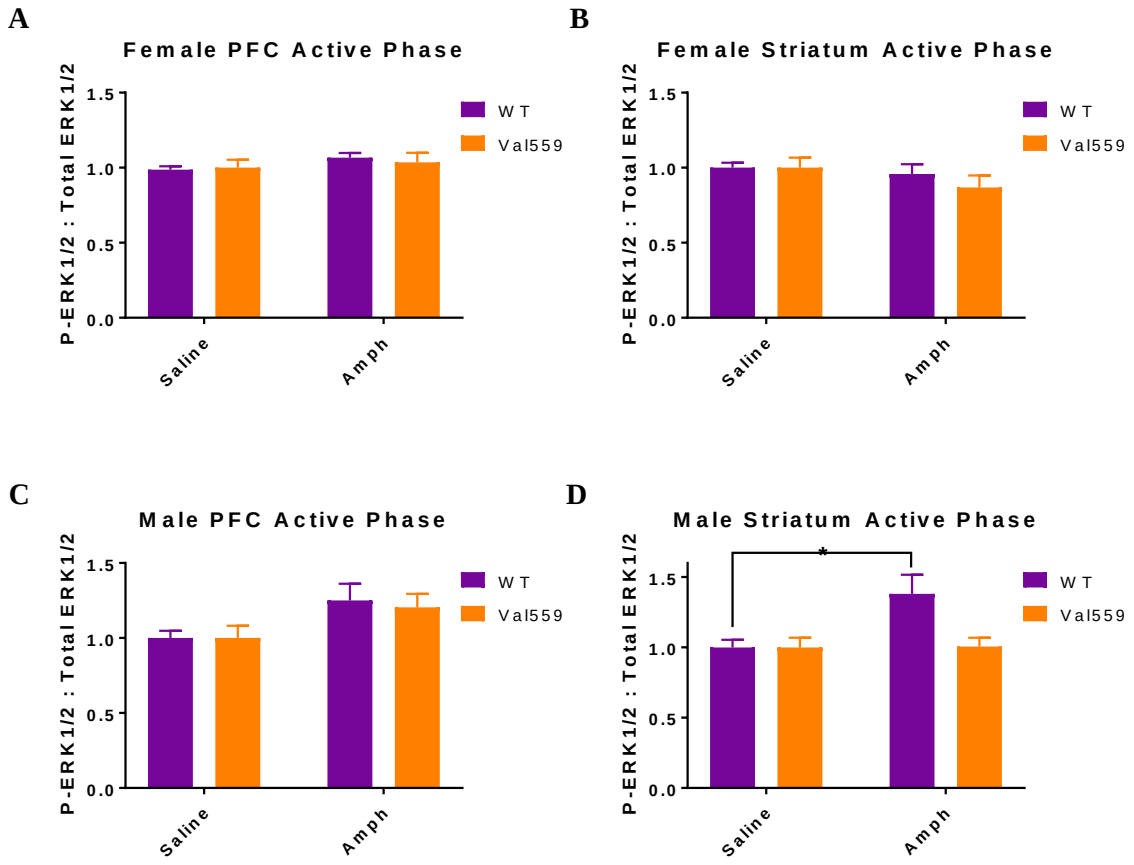


Figure 28. AMPH-induced gender and genotype specific ERK1/2 activation in the active phase. Both WT and DAT Val559 female mice in the active phase show no increase in ERK1/2 phosphorylation in response to AMPH in the PFC (2-Way ANOVA, genotype $P > 0.05$, drug $P > 0.05$, interaction $P > 0.05$; WT = 6 per group, Val559 = 7 per group) (A). Both WT and DAT Val559 female mice in the active phase show no increase in ERK1/2 phosphorylation in response to AMPH in the striatum (2-Way ANOVA, genotype $P > 0.05$, drug $P > 0.05$, interaction $P > 0.05$; WT = 6 per group, Val559 = 7 per group) (B). Both WT and DAT Val559 males show an increase in phospho ERK1/2 in response to AMPH in the PFC (2-Way ANOVA, genotype $P > 0.05$, drug $P < 0.05$, interaction, $P > 0.05$; WT = 6 per group, Val559 = 6 per group) (C). WT male mice show an increase in phospho ERK1/2 in the striatum (2-Way ANOVA, genotype $P < 0.05$, drug $P < 0.05$, interaction $P < 0.05$; Sidak's multiple comparisons WT saline versus WT AMPH $P < 0.05$; WT = 6 per group, Val559 = 6 per group) (D).

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