THE EFFECTS OF ENRICHING ENVIRONMENTS ON THE DEVELOPMENT OF REPETITIVE MOTOR BEHAVIORS AND THEIR NEUROBIOLOGICAL CORRELATES

By

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To my Mom

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I would like to thank my family for their endless encouragement as I chase my dreams around the world. I must thank the members of the Lewis Lab for their immeasurable support and kindness. Our conversations have carried me, and I will miss them dearly. I would love to thank my little one, whose smile reminds me every day that life is beautiful.

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LIST OF ABBREVIATIONS

AD	Alzheimer’s disease
ASD	autism spectrum disorder
CNS	central nervous system
CO	cytochrome oxidase
DLS	dorsolateral striatum
EE	environmental enrichment
GP	globus pallidus
MRI	magnetic resonance imaging
SH	standard housed
SN	substantia nigra
SNR	substantia nigra pars reticulata
STN	subthalamic nucleus
STR	striatum

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Despite the prevalence of repetitive motor behaviors in neurodevelopmental disorders, neurobiological mechanisms mediating the development of such behaviors are, as yet, unknown. Not surprisingly, selective pharmacotherapies are not available. Environmental enrichment (EE) attenuates repetitive behavior development in animals, albeit by unknown mechanisms. Thus, we sought to identify environmentally mediated mechanisms of repetitive behavior development using the deer mouse model of repetitive behavior. Measures of neuronal activation and morphology were used to interrogate the structural and functional role of the indirect basal ganglia pathway in mediating the EE-induced attenuation of repetitive behavior. Findings provided support for the importance of indirect pathway nuclei in mediating this environmental outcome on behavior. In addition, we characterized the development of repetitive motor behaviors in deer mice reared in EE and investigated whether the attenuating effects of EE on the development of repetitive motor behavior would extend to non-enriched offspring of EE mice.

The beneficial effects of EE on repetitive behavior occurred by the second week of such housing, with significant differences from standard housed mice emerging by

three weeks. Levels of increasing neuronal activation in indirect pathway nuclei were associated with the beneficial effects of EE. We also showed a novel, beneficial transgenerational effect of EE on the development of repetitive behavior in mice never exposed to EE.

Finally, the molecular underpinnings of repetitive behavior development were investigated by proteomic profiling of one indirect pathway nucleus: the subthalamic nucleus (STN), from standard housed mice (high repetitive behaviors) and EE mice (low repetitive behaviors). Two mass-spectrometric based proteomic approaches were employed that implicated molecular pathways largely involved in cell growth, survival and death in the development of repetitive behavior. Global analyses from the two profiling methods also identified several common upstream regulators (e.g. amyloid precursor protein), as well as disease categories and functions (e.g. neurological disease, movement disorders). The current studies provide novel findings about how EE acts on repetitive behavior and how such effects are mediated in brain. These findings support the importance of the indirect basal ganglia pathway and point to novel potential intervention targets.

CHAPTER 1
GENERAL INTRODUCTION

Restricted Repetitive Behaviors in Autism Spectrum Disorder

Restricted repetitive behaviors (RRB), one of three diagnostic domains for autism spectrum disorder (ASD), refers to the broad range of responses that include stereotyped motor movements, self-injurious behavior, repetitive manipulation of objects, compulsions, rituals and routines, insistence on sameness, and narrow and circumscribed interests (Lewis and Bodfish, 1998). These forms of RRB have been categorized as either “lower-order” motor actions (stereotyped movements, self-injury, repetitive manipulation of objects) involving repetition of movement, or “higher-order” behaviors (compulsions, rituals, insistence on sameness, and circumscribed interests) involving more complex behaviors characterized by rigidity or inflexibility (Lewis and Bodfish, 1998; Turner, 1999, Rutter, 1978). This categorization has been empirically supported by factor analyses (Cuccaro et al., 2003; Szatmari et al., 2006) using relevant items from the Autism Diagnostic Interview-Revised (ADI-R). These two factors have been labeled repetitive sensory motor behavior and resistance to change/insistence on sameness. Other analyses have presented evidence for a third factor labeled circumscribed interests (Lam and Aman, 2007).

RRB at 2 years of age predicts autism diagnosis at age 9, is a major source of stress for parents, results in considerable accommodation by the family, and negatively impacts academic achievement (Lord and Jones, 2012). Despite this, treatment options for RRB are limited and there has been a dearth of adequately controlled

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studies examining such interventions (Boyd et al., 2011). Of particular relevance here, is that few, if any, pharmacological treatments for these behaviors have clearly demonstrated efficacy (Carrasco et al., 2012; King et al., 2009; Leekam et al., 2011). The lack of efficacious pharmacological treatments is, in large measure, due to the lack of understanding of the pathophysiological mechanisms that mediate the development and expression of repetitive behaviors in ASD. There are no post-mortem studies involving individuals with ASD that relate neuropathological findings to RRB (Amaral et al., 2008). Moreover, only a small number of MRI studies have related volumetric measurements to RRB and these results have been inconsistent (Hollander et al., 2005; Rojas et al., 2006; Sears et al., 1999).

Given this state of affairs, it would seem that animal models of RRB, given the requisite validity, could be particularly useful. Such models could identify various potential etiologies, characterize commonalities in pathophysiology, identify novel potential therapeutic targets, and guide the development and validation of novel treatments.

Modeling Restricted Repetitive Behavior in Animals

Repetitive sensory motor behaviors can take a number of forms in animals, depending on the species and context in which they are observed. These can include excessive grooming, stereotyped pacing, backward somersaulting, rhythmic body movements, head twirling, and excessive mouthing to name but several. These behaviors share important features with those observed in ASD in being not only repetitive, but having little variation in response form and no obvious purpose or function.

A clear challenge for animal studies is to model “higher order” RRB or resistance to change/insistence on sameness. Although stereotyped motor behaviors have

typically been the focus, some animal work has addressed the domain of cognitive rigidity or resistance to change. This domain can be assessed in animals using a variety of tasks including response extinction, reversal learning, and intra- and extra-dimensional set shifting (e.g., Colacicco et al., 2002). Specific examples of such tasks include extinction and reversal learning in a Morris water or T-maze task (Moy et al., 2007; Tanimura et al., 2008), and perseveration in a variation of a gambling or a two-choice guessing task (Garner et al, 2003; Dallaire et al., 2011; Gross et al., 2011). Other tasks such as marble burying behavior and restricted exploration in a hole-board task (Amodeo et al., 2012; Muehlmann et al., 2012; Pearson et al., 2011; Silverman et al., 2010) have been advanced to model perseveration or compulsion and restricted behavior or interest.

Models of restricted repetitive behavior in animals can be roughly organized into four different categories: repetitive behavior resulting from a specific CNS insult (e.g., gene mutation, lesion); repetitive behavior induced by specific pharmacological agents (e.g., amphetamine, cocaine); repetitive behavior consequent to confined or restricted housing (e.g., laboratory cage); and repetitive behavior associated with specific inbred mouse strains. In the following sections, we will update information from our previous review (Lewis et al., 2007), expand our treatment of animal models to specific inbred mouse strains, and provide a summary of our recent work on the neurobiology of repetitive behavior in mouse models.

Repetitive behavior following CNS insult

Genetic mutations

Mice carrying targeted genetic mutations as models of various clinical disorders have increased dramatically. Thus, it is not surprising to see more repetitive behavior

phenotypes associated with genetic alterations as a consequence. For example, Rett syndrome has been linked to mutations in the methylCpG binding protein 2 (MECP2), and mice with mutations in this protein demonstrate stereotypic forelimb behavior mimicking the characteristic hand stereotypies seen in patients (Moretti et al., 2005). Autism, along with Prader-Willi and Angelman syndromes has been linked to changes in a specific region (q11-13) of chromosome 15 carrying the GABRB3 gene. The creation of the *gabrb3* knockout mouse revealed a mouse model that displayed intense stereotyped circling behavior. RRB can also be modeled in mice with perturbations to the *Hoxb8* gene, which display excessive grooming that can lead to wound infliction (Greer and Capecchi, 2002).

More recently, alterations in molecular regulators of excitatory synaptic structure and function have emerged as mediators of aberrant repetitive behavior. For example, a targeted deletion of a postsynaptic scaffolding protein at excitatory synapses, SAPAP3, which is highly expressed in the striatum, produced a mouse model of reduced cortico-striatal synaptic transmission and glutamate receptor function, and excessive self-grooming behavior (Welch et al., 2007). SHANK genes encode another postsynaptic scaffolding protein family enriched at excitatory synapses, and mutations in ProSAPs/SHANK genes have been associated with autism. SHANK1 deletion has been identified in a small number of males with higher-functioning autism (Sato et al., 2012). SHANK2 and 3 mutations have been found in some, but not all cases of autism and intellectual disability (Berkel et al., 2010; Qin et al., 2009). Disruption of the *Shank3* gene in mice results in functional deficits to glutamatergic synapses and autistic-like behaviors, which include repetitive behavior in the form of increased grooming, sniffing

and object manipulation (e.g. Wang et al., 2011). Follow-up work found phenotypic specificity as a result of the precise location of the mutation within the SHANK3 gene (Yang et al., 2012). Comparison of *Shank2* and *Shank3* mutant mouse data similarly demonstrates that phenotypic differences can result from the different synaptic glutamate receptor expression abnormalities (Schmeisser et al., 2012). *Shank2* knockout mice display a range of autistic-like behaviors, including hyperactivity and repetitive jumping, although, decreased digging behavior (Schmeisser et al., 2012; Won et al., 2012). Some (Schmeisser et al., 2012), but not all (Won et al., 2012), investigators have reported increased grooming behavior in *Shank2* knockout mice. Other candidate genes for autism that are related to excitatory synapses include the neuroligin and neurexin genes. Neuroligins are a family of postsynaptic cell-adhesion molecules that associate with presynaptic neurexins to influence synaptic maturation. Blundell et al. (2010) characterized neuroligin 1 (NL1) deficient mice in tests relevant to autism. Compared to controls, NL1 KO mice groomed for twice the amount of time, and the behavior was associated with a ~30% reduction of the NMDA/AMPA ratio in the dorsal striatum. Systemic administration of a NMDA receptor partial co-agonist (d-cycloserine) rescued the abnormal grooming phenotype, suggesting a mechanism for decreased NMDA receptor-mediated synaptic transmission (Blundell et al., 2010). Deficits in spatial learning and memory that correlated with impaired hippocampal long-term potentiation and minimal social impairments were also noted. Although NL1 is ubiquitously expressed, KO mice were normal in a different task of repetitive behavior (marble burying), learning and memory (fear conditioning), and several other tasks (e.g. tests of anxiety, activity, motor function, sensory) (Blundell et al., 2010). Generation of

neurexin1α deficient mice revealed behavioral changes including increased grooming and impaired nest-building behavior, although no obvious deficits in social behavior or learning (Etherton et al., 2009).

Sala et al. (2011) have demonstrated deficits in reversal learning in oxytocin receptor knockout mice. Daily intracerebroventricular (ICV) injections of vehicle or oxytocin showed that oxytocin normalized reversal learning deficits in these mice. These mice also demonstrated deficits in social and communicative behavior (Takayanagi et al., 2005). A study by Hollander et al., (2005) evaluated the effect of oxytocin on repetitive behavior in adults. ASD subjects received both oxytocin and placebos challenges, each serving as their own control, and then were observed for repetitive behavior (repetitive behavior categories: need to know, repeating, ordering, need to tell/ask, self-injury, and touching). Repetitive behavior decreased following oxytocin infusion (Hollander et al., 2005).

Non-genomic factors

Other animal models take advantage of the strong influence the prenatal environment has on risk for offspring development of autistic-like behaviors. For example, in utero injections of valproic acid (VPA) during sensitive periods of embryonic development produce rodent offspring that show developmental delays, impairments in social behavior, and increased lifetime stereotypic behavior (Schneider and Przewlocki, 2005; Schneider et al., 2006, 2008). Environmental enrichment has been shown to attenuate the repetitive behavior associated with *in utero* exposure to VPA (Schneider et al., 2006).

Repetitive behavior and other autistic-like behaviors have also been linked to perturbations during early development, such as lesion-induced damage. For example,

lesioning the amygdala and hippocampus in early postnatal development of rhesus monkeys caused delayed development of stereotypies, first apparent in post-weaning juveniles (Bauman et al., 2008). Further, lesion-specific topographies of repetitive behavior were documented, such that amygdala lesioned infants were more likely to develop self-directed stereotypies (body rocking, self-biting and self-clasping) compared to hippocampal lesioned infants that were more likely to develop repetitive head-twisting behavior (Bauman et al., 2008). However, similar lesions in adult animals failed to produce the same severity of repetitive behavior (Bauman et al., 2008). These recent findings complement previous studies in rats that found specific lesions of the hippocampus in early development (postnatal day 3) increased repetitive behavior, while the same lesion in later development (postnatal day 14) and adults attenuated repetitive behavior (Wood et al., 1997). These studies support a behavioral outcome that is dependent on the timing of lesions and a potential sensitive period for the development of stereotypic behavior.

Immune factors

A potential role for altered immune function in the genesis of autism is an area of considerable interest. Several recent reports have highlighted altered immune processes associated with the development of repetitive behavior in animals. The first of these was an intriguing study by Martin et al. (2008) examining the effect of maternal antibodies on non-human primate fetal brain. Here, pregnant rhesus macaques were exposed to purified IgG from sera of human mothers who had at least two children with ASD and whose sera was shown to be reactive to fetal brain protein. Offspring of the exposed macaques exhibited motor stereotypies not observed in control monkeys. Additional evidence for a link between anti-neuronal antibodies and repetitive behavior

comes from exposing Balb/c mice to IgM antibodies to streptococcus group A bacteria (Zhang et al., 2012). Mice so treated exhibited repetitive stereotyped movements including head bobbing, intense grooming, and sniffing and showed increased Fos-like immunoreactivity in regions included within cortico-striato-thalamic circuitry. These findings are consistent with previous reports that infusion of serum or purified IgG from Tourette syndrome patients into rat striatum induced motor stereotypies (Taylor et al., 2002). Immune responses are mediated, in part by various cytokines (e.g., interleukins, interferons) and their receptors. Soluble interleukin-6 receptor administration has been shown by Patel et al. (2012) to induce motor stereotypies in Balb/c mice. These behavioral effects were accompanied by evidence for localization of these IL-6 receptors in brain regions included in cortical basal ganglia circuitry. Finally, maternal infection, a risk factor for autism, was modeled in mice by administration of poly(I:C), to induce a proinflammatory antiviral response, starting at embryonic day 10.5 (Malkova et al., 2012). As adults, offspring of these maternally infected mice exhibited increased marble burying and elevated self-grooming. Interestingly, marble burying levels were normalized to controls following irradiation and bone-marrow transplantation of poly(I:C) exposed offspring (Hsiao et al., 2012).

Drug-induced repetitive behavior

For more than four decades, we have known that specific pharmacological agents (e.g., amphetamine, apomorphine) can induce repetitive motor behavior in humans and animals. Early experiments highlighted the importance of basal ganglia in mediating the induction of repetitive behavior by such drugs. For example, injection of dopamine or a dopamine agonist (e.g. apomorphine) into the striatum of rats induced repetitive behavior (Ernst and Smelik, 1966). Bi-directional models of selective

pharmacological agents further affirm the role of the cortical-basal ganglia circuitry in repetitive behavior. For example, modulation of dorsal striatal glutamate receptors by intrastriatal injection of NMDA, a glutamate receptor ligand, induced stereotypic behavior, whereas intrastriatal injection of CPP, an NMDA receptor antagonist, reduced stereotypic behavior (Karler et al., 1997). Amphetamine induced stereotypy can be enhanced by intracortical administration of D_2 or GABA antagonists, and attenuated by DA or GABAergic agonists (reviewed in Lewis et al., 2007). Evidence of the important role of the cortical-basal ganglia circuitry in repetitive behaviors is further demonstrated in studies that alter levels of drug-induced stereotypy by manipulations to the substantia nigra pars reticulata (SNpr) and subthalamic nucleus (STN). The SNpr sends GABAergic projections to the thalamus as part of the direct pathway of the basal ganglia (see following sections), whereas the STN sends glutamatergic projections to the SNpr as part of the indirect pathway. Increased stereotypy as a result of intranigral GABA agonist administration and reduced stereotypy by injection of serotonergic antagonists into the STN thus support the hypothesized role of these structures and respective pathways in repetitive behavior (reviewed in Lewis et al., 2007). Finally, Grabli et al. (2004) have reported induction of stereotyped behavior (e.g. licking and biting of fingers) in monkeys by the GABA antagonist bicuculline microinjected into the limbic aspect of the GPe (part of the indirect pathway). In a follow-up study (Baup et al., 2008), this group showed that deep brain stimulation (DBS) applied to the STN dramatically reduced these drug-induced repetitive behaviors.

Repetitive behavior and environment

Repetitive behavior following environmental restriction

Abnormal repetitive behaviors are commonly seen across species maintained in confined or restricted environments (e.g., zoos, farms, laboratories) (Mason and Rushen, 2006), or reared under conditions of early social deprivation (e.g., Harlow et al., 1965; Latham and Mason, 2008). Estimated numbers of stereotypic captive animals exceed 85 million (Mason and Latham, 2004), supporting repetitive behavior as the most common category of abnormal behavior observed in environmentally restricted animals (Würbel, 2001). Some examples of confinement-induced repetitive behavior include bar-biting in sows and laboratory mice; pacing in bears, monkeys, and birds; and head-twirling in mink (Mason and Rushen, 2006). Our own work shows that deer mice reared in standard laboratory caging display high levels of vertical jumping and backward somersaulting, behaviors that appear early in development and persist through adulthood (e.g. Powell et al., 2000; Turner et al., 2002).

Environmental restriction has also been shown to be associated with cognitive inflexibility as well as motor stereotypies. This has been demonstrated using an extinction task with bears as well as bank voles (Garner and Mason, 2002; Vickery and Mason, 2005). Orange wing Amazon parrots with higher motor stereotypy scores exhibited greater sequential dependency in a variation of a gambling task, which indexed the tendency to repeat responses or perseverate (Garner et al., 2003). In our own work, we tested deer mice in a procedural reversal learning task that involved learning to turn right or left in a T-maze for reinforcement. Following acquisition, the reinforced arm was reversed. Our results indicate that high levels of stereotypy in deer

mice were associated with deficits in reversal learning in the T-maze (Tanimura et al., 2008).

Repetitive behavior following environmental enrichment

Compelling evidence for the causative role of environmental restriction on the induction of repetitive behavior comes from studies of environmental enrichment. Enrichment has been shown to induce rapid, profound, and persistent effects on brain and behavioral development (Sale et al., 2009). Moreover, studies of rodent models of various brain disorders have highlighted the impact of environmental enrichment on attenuating disease onset, progression, and severity (Nithianantharajah and Hannan, 2006). Not surprisingly then, enrichment studies using multiple species have consistently shown that animals reared in complex environments show less stereotypic behavior than their environmentally restricted counterparts (Lewis et al., 2007; Mason et al., 2007). Moreover, we have shown that enrichment not only improved motor stereotypies but also increased cognitive flexibility in a reversal learning task (Tanimura et al., 2008).

Enrichment has been shown to impact a large number of measures of brain structure and function. For example, exposure to an enriched environment increased cortical thickness, dendritic length and spine density, and synaptic plasticity (Kolb and Whishaw, 1998; Nithianantharajah and Hannan, 2006). Despite decades of research on the neurobiological effects of enrichment, however, neurobiological mechanisms by which such experience alters repetitive behavior are still largely unidentified. Our own work using a deer mouse model demonstrated that environmental enrichment induced changes in cortical-basal ganglia circuitry (e.g. increased striatal dendritic spine density and BDNF) that were selectively associated with reduced stereotypic behavior (Turner

and Lewis, 2002; Turner et al., 2002, 2003). Moreover, we have shown that enrichment related changes in repetitive behavior were associated with increased indirect basal ganglia pathway activation (Tanimura et al., 2010).

Repetitive behavior in inbred mouse strains

Inbred strains of mice have become the most frequently employed model for studying human brain disorders. Thus, identifying an inbred strain that exhibits repetitive behavior not requiring a specific perturbation (lesion, drug, or genetic mutation) would be of significant importance to the field. Indeed, at least two inbred strains appear to be good candidate models. The BTBR mouse has been advanced as exhibiting a number of autistic-like traits (Pearson et al., 2011), including repetitive behavior in the form of elevated levels of self-grooming (Pearson et al., 2011; McFarlane et al., 2008). Interestingly, the mGluR5 antagonist, MPEP, was found to decrease repetitive self-grooming in these animals selectively (Silverman et al., 2010). To address the resistance to change/insistence on sameness behavioral domain, Amodeo et al. (2012) employed a spatial reversal learning task with BTBR mice. Compared to C57BL/6 mice, BTBR mice performed similarly to controls in acquiring the spatial discrimination but were impaired on reversal learning. Interestingly, this impairment was only observed when feedback for a correct choice was decreased to an 80% probability (i.e., occasional lack of reinforcement for a correct choice with occasional reinforcement for an incorrect choice). BTBR mice also display inflexibility in the exploration of a hole-board and more patterned sequences in sequential investigations of a novel object, suggesting this strain demonstrates both cognitive inflexibility and stereotypic motor behaviors (Pearson et al., 2011; Moy et al., 2008).

The second inbred mouse strain that would seem to hold considerable promise for furthering our understanding of the neurobiology of repetitive behavior is the C58 strain. The UNC group reported repetitive hindlimb jumping and persistent back-flipping in these mice (Moy et al., 2008; Ryan et al., 2010). Of note, the former topography was observed in some mice prior to weaning. Subsequently, we have confirmed these observations showing that compared to C57BL/6 mice, C58 mice exhibited high rates of spontaneous hindlimb jumping and backward somersaulting reaching asymptotic levels by 5 weeks post-weaning (Muehlmann et al., 2012). We also showed that six weeks of environmental enrichment following weaning substantially reduced repetitive behavior. In our hands, C58 mice did not exhibit increased marble burying nor did they display reduced exploratory behavior in the hole-board task. Further investigation of cognitive inflexibility in this strain will be important in determining the utility of this model for modeling resistance to change/insistence on sameness.

Cortical Basal Ganglia Circuitry and Repetitive Behavior

The models reviewed in the previous sections highlight the fact that repetitive behavior in animals, consistent with what we know in humans, can have multiple etiologies or inducing conditions. These include, but are not limited to, gene alterations, lesions, toxicants, ant-neuronal antibodies, and restricted environments. There is some, but limited, evidence that these etiologies share a common pathophysiology: alterations in cortical-basal ganglia circuitry. For example, some of the genetic mutations reviewed impact cortico-striatal glutamatergic synapses whereas some anti-neuronal antibodies associated with repetitive behavior are directed at basal ganglia. Selective pharmacological agents that induce repetitive behavior have molecular targets

expressed in basal ganglia and environmental restriction associated with repetitive behavior alters basal ganglia functioning.

As reviewed elsewhere, (e.g. Lewis and Kim, 2009), cortical basal ganglia circuitry involves pathways that project from select areas of cortex to striatum, then to other basal ganglia nuclei (globus pallidus, substantia nigra), then to thalamus and finally back to cortex. This cortico-striato-thalamo-cortical circuitry is thought to be comprised of multiple parallel loops that while interacting are functionally and anatomically distinct (Alexander et al., 1986; Langen et al., 2011). Five loops have been proposed based on their cortical targets: the motor, occulomotor, dorsolateral prefrontal, lateral orbitofrontal, and anterior cingulate loops. From a functional perspective, three loops are generally considered: the sensorimotor (motor and oculomotor), associative (dorsolateral prefrontal), and limbic (lateral orbitofrontal, and anterior cingulate) loops. These loops mediate motor, cognitive, and affective functions respectively. Of these, the motor circuit has been the most studied and emerges as the best candidate for mediation of repetitive motor movements. The limbic loop may be the best candidate for mediation of some “higher order” repetitive behaviors, particularly compulsions. This hypothesis is based largely on neuroimaging studies of individuals with obsessive compulsive disorder or OCD (Harrison et al., 2009; Reminjinse et al., 2009).

Each of these cortical basal ganglia loops makes use of two distinct basal ganglia pathways that originate from the striatum or caudate-putamen. The striatum is made up of medium spiny GABAergic projection neurons that receive input from sensory-motor and associative areas of cortex, and, in turn, give rise to the direct and indirect pathways. Approximately half of striatal neurons express the neuropeptide dynorphin as

well as D_1 dopamine receptors and A_1 adenosine receptors and constitute striatonigral or direct pathway neurons. These neurons send projections from the striatum to the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNpr). Striatal medium spiny neurons that express the neuropeptide enkephalin as well as D_2 dopamine receptors and A_{2A} adenosine receptors constitute striatopallidal or indirect pathway neurons. Indirect pathway neurons project to the external segment of globus pallidus (GPe) and then to subthalamic nucleus before projecting to GPi and SNpr. Output from the GPi/SNpr goes to thalamus and then on to cortex to complete the circuitry (Olanow et al., 2000). The classic view has been that the direct pathway facilitates movement via disinhibition of glutamatergic thalamo-cortical firing whereas the indirect pathway inhibits ongoing movement via inhibition of thalamo-cortical afferents (Gerfen et al., 1990).

Indirect basal ganglia pathway and repetitive behavior. Work from our lab using the deer mouse model of spontaneous repetitive behavior (e.g. Powell et al., 2000; Presti and Lewis, 2005; Presti et al., 2003, 2004) has indicated that reduced indirect basal ganglia pathway activation mediates the expression of high levels of repetitive behavior. For example, as dynorphin and enkephalin serve as markers for direct and indirect pathway neurons, respectively, we measured the concentrations of these striatal neuropeptides in animals exhibiting high or low levels of repetitive behavior (Presti and Lewis, 2005). Results indicated significantly decreased enkephalin content in high-stereotypy mice relative to low-stereotypy mice. Moreover, a significant negative correlation was found for enkephalin content and frequency of stereotypy. To extend these findings, we assessed indirect pathway activation relative to stereotypy by

measuring neuronal metabolic activation of the subthalamic nucleus (STN), a key brain region in the indirect pathway [85]. Using cytochrome oxidase (CO) histochemistry to index long-term neuronal activation, we found that CO staining in the STN was significantly reduced in high-stereotypy mice. Further, CO staining was significantly negatively correlated with the frequency of stereotypy. Consistent with reduced glutamatergic innervation from STN, high stereotypy was also strongly associated with decreased CO staining in SNpr (Tanimura et al., 2011). Thus, higher rates of spontaneous stereotypy were associated with reduced neuronal activation of the indirect pathway.

In order to confirm the role of the indirect pathway in our model, we have used selective pharmacological agents to alter the activity of this pathway. Results from these experiments show that drug combinations designed to increase the activity of the indirect pathway markedly and selectively reduce repetitive behavior in deer mice [85]. Moreover, unpublished results indicate that drug combinations designed to suppress the activity of the indirect pathway significantly increased repetitive behavior. Beyond providing compelling evidence for the role of the indirect pathway in repetitive behavior, these findings point to specific potential therapeutic targets for drug development.

Summary

There are a number of animal models that have a robust repetitive behavior phenotype. Moreover, these models represent a variety of etiologies or inducing conditions, consistent with the clinical literature. A critical question to be pursued is to what extent these various etiologies share a common or overlapping pathophysiology. A number of models have not yet been systematically pursued to determine how a particular insult (genetic mutation, lesion, toxicant), rearing condition, or genetic

background alters neuronal signaling and neural circuitry to induce a complex behavior. The inbred mouse strains reviewed that exhibit a robust repetitive behavior phenotype provide a particularly promising vehicle for identifying important neurobiological mechanisms (e.g., differential gene expression) and altered neural circuitry mediating repetitive behavior. The link between altered immune function and repetitive behavior is an intriguing one and should be pursued using animal models. Identifying the role of maternal infection or maternal antibodies in the genesis of RRB using animal models would have substantial translational value.

A great deal more effort needs to be directed toward using animal models to understand the pathophysiology of repetitive behavior, as this is key to developing new effective treatments. To date, work directed at identifying specific potential therapeutic targets for drug development to treat RRB using animal models has been very limited. This is a critical need in the field as there are few, if any, pharmacological interventions for the treatment of restricted, repetitive behavior in ASD with established efficacy (Leekam et al., 2011). In that regard, very little of the work we have reviewed generally has been treatment focused including testing novel behavioral or biological treatments. Environmental enrichment has been examined by us and others as an experiential intervention (Mason and Latham, 2007; Tanimura et al., 2010). Novel psychopharmacological treatments have been largely limited to testing a mGluR5 antagonist (Mehta et al., 2012; Silverman et al., 2012) and our work examining drug combinations targeting receptor complexes expressed on indirect pathway neurons (Tanimura et al., 2009; 2010). Greater use of animal models of RRB to test potential treatments would increase the translational value of such models substantially.

Aims

To build on this foundation of knowledge, we continued investigating the role of the indirect pathway activity in the expression as well as development of repetitive behaviors using deer mice, capitalizing on the behavioral effects of an enriched environment. The purpose of this dissertation was to identify the mechanisms driving the beneficial effects of EE on repetitive behavior development. The series of studies presented in the following chapters are the first attempts at identifying how repetitive motor behaviors are attenuated by EE and how they develop within EE alongside their neurobiological correlates. Specific Aim #1 tested the hypothesis that alterations in indirect basal ganglia pathway mediate the attenuation of repetitive behavior by EE; an outcome mediated by dendritic morphology and protein expression differences. Specific Aim #2 characterized the developmental trajectory of repetitive behavior of deer mice reared with EE, and identified associated alterations in the indirect basal ganglia pathway across adolescence. Finally, Specific Aim #3 tested the hypothesis that transgenerational effects of EE would benefit repetitive behavior development in non-enriched offspring.

CHAPTER 2
HOW DOES ENVIRONMENTAL ENRICHMENT REDUCE REPETITIVE MOTOR BEHAVIORS? NEURONAL ACTIVATION AND DENDRITIC MORPHOLOGY IN THE INDIRECT BASAL GANGLIA PATHWAY OF A MOUSE MODEL

Repetitive motor behaviors are rigid patterns of behavior that serve no apparent function (Lewis & Bodfish, 1998). These problem behaviors manifest in many clinical populations, notably neurodevelopmental disorders such as autism spectrum disorders and intellectual and developmental disability (Bodfish et al., 2000). Several neurological and psychiatric disorders including fronto-temporal dementia, obsessive compulsive disorder (OCD), schizophrenia, Tourette's syndrome, Parkinson's and Huntington's diseases have repetitive motor behaviors as part of their clinical presentation as well (Ridley, 1994; Langen et al., 2011). Repetitive motor behaviors also develop as a consequence of early environmental deprivation, including congenital blindness and impoverished environments (Fazzi et al., 1999, Rutter et al., 1999). In spite of the large number of affected people, the neurobiological or pathophysiological basis of these behaviors is not well understood. In neurodevelopmental disorders, evidence is limited to a small number of MRI studies that have demonstrated volumetric differences in basal ganglia, mostly caudate/putamen, related to repetitive behavior (Sears et al., 1999; Hollander et al., 2005; Rojas et al., 2006; Langen et al., 2014). As a consequence of very limited information on the underlying neurobiology, effective pharmacological treatments are largely lacking, particularly in neurodevelopmental

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disorders. Identifying the underlying mechanisms for the development of repetitive motor behaviors will promote identification of new therapeutic targets and treatment development.

Animal models of repetitive behavior provide a valuable approach to identify underlying mechanisms of repetitive behavior in response to varying environments. Repetitive motor behaviors can be induced in animals in a variety of ways including by pharmacological agents (e.g., amphetamine), CNS insult (e.g. deletion of genes coding for Shank3, MECP-2, SAP-AP3) and environmental restriction (e.g., standard laboratory caging) (Lewis, 2004; Lewis et al., 2007; Mason et al., 2007; Bechard & Lewis, 2012). Deer mice (*Peromyscus maniculatus*) have proven to be a useful model of repetitive behavior induced by environmental restriction (see review by Lewis et al., 2007). As a consequence of standard laboratory caging, deer mice exhibit high levels of repetitive hindlimb jumping and backward somersaulting, apparent by the time of weaning and persisting across adulthood (Muehlmann et al., 2015; Powell et al., 1999). Furthermore, deer mice reared in enriched environments show significant attenuation of the development of repetitive motor behaviors (Powell et al., 2000; Turner et al., 2002; 2003; Hadley et al., 2006).

Environmental enrichment (EE) in rodents includes increased social and spatial density as well as exposure to novelty and complexity, usually in the form of toys, tunnels, and nesting material. Increased opportunity for exercise (e.g. a running wheel) is also a mainstay of EE. Well-studied in the context of learning and memory, EE increases neurogenesis and synaptogenesis (van Praag et al., 2000) as well as cortical thickness, dendritic spine density and length, synaptic plasticity, and resistance to

disease (Kolb & Wishaw, 1998; Nithianantharajah & Hannan, 2006). A range of behavioral domains are also affected by EE including cognitive, social, and emotional functioning as well as aberrant behaviors (Morley-Fletcher et al., 2003; Branchi, 2006). Attenuation of repetitive behavior is a robust behavioral effect of EE, seen not only in deer mice, but across most captive species (Lewis, 2004; Mason et al., 2007). To date, however, the mechanisms underlying this effect are largely unknown. Previous work in deer mice suggested that EE-induced attenuation of repetitive motor behaviors was associated with increased neuronal activation and dendritic spine density in basal ganglia (Turner et al., 2002; 2003). Importantly, EE effects on neurobiological outcomes were found only for those mice exhibiting EE-induced attenuation of repetitive behavior.

Although this earlier work implicated the basal ganglia, it did not address selective alterations of specific basal ganglia pathways. The basal ganglia include the striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra (SN). The striatum is the main input structure of the basal ganglia and receives projections from the sensory-motor and associative cortical areas, and projects to nuclei via the direct or indirect pathways of the basal ganglia. These projections converge in the output nuclei, the SN, relay to the thalamus and then back to the cortex to complete the loop. The monosynaptic direct pathway projects from striatum to SN pars reticulata (SNR), whereas the indirect pathway projects from striatum to GP which in turn projects to STN before converging on SNR (Schmitt et al., 2014). Appropriate selection, activation and suppression of movement are dependent on the coordination of the direct and indirect basal ganglia pathways, which classically were thought to function in an antagonistic fashion (Alexander et al., 1986). More recent evidence reveals a dynamic

interplay between the basal ganglia pathways with concomitant activity during action sequence initiation but differential encoding of action sequences (Jin et al., 2014), and increased complexity of neuronal cell populations within a given region (Wall et al., 2013; Antal et al., 2014; Macpherson et al., 2014). Direct pathway neurons facilitate selection of relevant motor programs whereas the indirect pathway functions to suppress competing motor programs. In addition, a direct connection between the cortex and STN, known as the hyperdirect pathway, although largely understudied, is thought to modulate response inhibition in situations of conflict (Jahfari et al., 2011; Jahanshahi, 2013). An imbalance in the direct and indirect basal ganglia pathways has been implicated in the dysregulation of cortico-striato-thalamo-cortical circuitry associated with both hyperkinetic and hypokinetic movement disorders (Graybiel, 2000).

In the deer mouse model of repetitive behavior, our work has suggested a functional imbalance of the direct and indirect basal ganglia pathways due to a hypoactivation of the indirect pathway. For example, we found a significant reduction in striatal enkephalin, a marker of indirect basal ganglia pathway neurons, in high versus low repetitive behavior mice with no difference in striatal dynorphin, a marker of direct pathway neurons (Presti & Lewis, 2005). In addition, a significant inverse correlation was found between repetitive behavior scores and striatal enkephalin content (Presti & Lewis, 2005). We also showed that neuronal activity in the STN was reduced in mice with high versus low levels of repetitive motor behavior (Tanimura et al., 2010; 2011). Further, a brief period of EE that was effective in reducing repetitive behavior development was associated with increased neuronal activation in the STN (Tanimura et al., 2010). Targeting striatal indirect pathway neurons with pharmacological agents

designed to increase indirect pathway activation substantially reduced repetitive motor behavior in deer mice (Tanimura et al., 2010).

In the present study, we conducted two experiments to assess the function of the indirect pathway in the EE-induced attenuation of the development of repetitive motor behaviors. We hypothesized that such attenuation is associated with increased neuronal activation of the indirect basal ganglia pathway, an outcome mediated by increased dendritic spine density. In Study 1, we compared neuronal metabolic activation of basal ganglia nuclei in adult deer mice reared in EE to mice reared in standard housing. Neuronal activation is tightly coupled with oxidative energy metabolism, and can be indexed using cytochrome oxidase (CO) histochemistry (Gonzalez-Lima & Cada, 1998). We measured CO as an indicator of long-term neuronal metabolic activity in the dorsal lateral striatum (DLS), GP, STN, SNR, SNC, motor cortex and CA1 region of the hippocampus (HPC). In Study 2, we compared dendritic morphology in basal ganglia nuclei of adult deer mice reared in EE or standard laboratory cages. The dendritic surface receives over 95% of the synapses on a neuron, aided by specialized protrusions (i.e. spines) that act as the basic functional unit of integration for neuronal circuits. Dendritic spines mediate fast excitatory transmission, are heterogeneous in morphology, and modifiable by experience and activity (Markham & Greenough, 2004). Experience-dependent dendritic plasticity is a sensitive index for inferring synapse number and strength, and dendritic remodeling can change the functional properties of a neuron (Harris & Kater, 1994; Kolb & Wishaw, 1998). A number of earlier studies investigating morphological plasticity have found increased dendritic spine density following EE exposure in a variety of brain regions important in

the processing of environmental stimuli (e.g. Comery et al., 1995; 1996; Juraska et al., 1985, 1989; reviewed by Markham & Greenough, 2004). More recently, dendritic remodeling as a consequence of EE has been related to tasks of learning and memory (Farrell et al., 2015) and pathology of Huntington's and Parkinson's diseases (Spires et al., 2004; Murmu et al., 2013; Kim et al., 2013).To our knowledge, however, only our own work has examined dendritic morphological differences in the basal ganglia as a function of EE-induced attenuation of repetitive motor behaviors (Turner et al., 2003).

Materials and Methods

Animals

All procedures were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and approved by the University of Florida Institutional Animal Care and Use Committee. Deer mice were bred and housed in our colony room at the University of Florida, maintained at 70-75ºF and 50-70% humidity, under a 16:8 light:dark cycle, with lights off at 10:00 am. All home environments had access to rodent chow (Teklad) and water *ad lib*, and two Nestlet squares for nest construction. Offspring of monogamous breeding pairs were weaned at day 21 and placed into their randomly assigned housing condition, separated by sex. Litters were split so that siblings were assigned to both EE and standard housing conditions. In Study 1, a total of 26 mice (females: n=16; males: n=10) from 11 different litters were used. We used 1 male and 2 female EE cages, and 2 male and 2 female standard cages in Study 1. In Study 2, a total of 18 mice (females: n=7; males: n=11) from 6 different litters were used. We used 1 male and 1 female EE cage, and 1 female and 2 male standard cages in Study 2.

Enriched housing conditions

Same-sex weanlings with the same birth dates (±1 day) were assigned to EE (Study 1: N=13; Study 2: N=10), which consisted of large dog kennels (1.22 x 0.81 x 0.89 m; group size: n=4-6) customized to have two additional levels created by galvanized wire and connected by ramps (see Fig. 2-1). Various objects (plastic toys, e.g. Legos; domes; tunnels), Habitrail tubes, a running wheel and a large hut were always present; however, toys were rotated weekly to maintain an environment with both novelty and complexity. At the time of toy rotation, bird seed (approximately 2 oz.) was scattered throughout the kennel to promote foraging (see EE paradigm in Turner et al., 2002, 2003; Turner & Lewis, 2003; Hadley et al., 2006; Tanimura et al., 2010). The EE cages underwent weekly toy rotation and seed scattering, and refreshment of bedding, Nestlets, food and water every two weeks.

Standard housing conditions

Same-sex weanlings with the same birth dates (±1 day) were assigned to standard housing (Study 1: N=13; Study 2: N=8) which consisted of standard laboratory cages (29 x 18 x 13 cm; group size: n=3-4). To control for any dietary differences without promoting foraging, a small amount of birdseed (approximately 0.25 oz.) was placed into the corner of the standard cage each week. The standard cages were changed every two weeks for refreshment of bedding, Nestlets, food and water.

Assessment of repetitive motor behaviors

At six weeks post-weaning, mice were removed from their housing conditions and placed into individual test cages (28 x 22 x 25 cm) for measurement of repetitive motor behaviors. In deer mice, these behaviors take the form of vertical hindlimb jumping and backward somersaulting. Both topographies involve vertical activity and so

can be automatically quantified using photobeam arrays (Columbus Instruments) positioned such that when the animal jumps the photobeam is interrupted and a count is recorded (Labview software, National Instruments; see Tanimura et al., 2010, 2011; Muehlmann et al., 2015). At 13.5 cm above the floor, the photobeams are positioned high enough to avoid being broken by drinking, rearing, or any other behavior that does not include all four paws leaving the ground. Random sampling of the videos (Geovision software) recorded during each test session insured the accuracy of the automated counts.

Food and water were available during testing, which lasted for the entire 8 h dark cycle. Mice were given at least 30 min to habituate to the test cages. After the test was complete, animals were returned to their respective home environments. Frequencies of repetitive motor behaviors across the entire 8 h dark cycle were summed for each mouse and the totals were used in subsequent analyses.

Study 1: CO histochemistry

CO is an integral transmembrane protein of the inner mitochondrial membrane that catalyzes the transfer of electrons during the process of generating ATP, and is therefore directly related to neuronal functional activity (Wong-Riley et al. 1998). CO activity is a measurement of long-term (days to weeks) neuronal metabolic activity (Sakata et al., 2005) and has been successfully used to detect alterations in basal ganglia in rodent models of dystonia, ataxia and repetitive motor behaviors (Nobrega et al., 1998; Jacquelin et al., 2013; Turner et al, 2002; Tanimura et al., 2010). The CO staining assay was executed according to the protocol of Gonzalez-Lima and Cada (1998) and has been used previously in our lab to find differences in basal ganglia neuronal activity between deer mice with high and low levels of repetitive motor

behaviors (e.g. Turner et al., 2002; Tanimura et al., 2010; Tanimura et al., 2011). Briefly, the morning after behavioral testing, brains were harvested and immediately snap-frozen in cold 2-methylbutane and stored in a -80^{o}C freezer. Brains were sectioned on a cryostat (-20^{o}C) into 20 µm sagittal sections. We collected tissue from both hemispheres beginning at ~1 mm lateral to the midline, positioning them onto microscope slides (Superfrost Plus, FisherBrand), such that adjacent sections were 100 µm apart. Homogenized brain tissue from non-subject deer mice was used to make standards that were snap-frozen and sectioned at increasing thicknesses (10, 20, 30, 40, 50, 60 µm) to ensure linearity of optical density measurements. Slides were stained and cover slipped with Permount.

Study 1: Quantification of CO histochemistry

Optical density measurements were obtained using ImagePro (Media Cybernetics) from the DLS, STN, GP, SNC, SNR, and other regions of interest including the CA1 region of the hippocampus (HPC) and motor cortex. Although we did not expect CO activity levels in the HPC to be associated with repetitive motor behaviors, this region has been shown to be affected by EE (Turner et al., 2002). Neuronal metabolic activity for each region of interest was calculated by averaging multiple optical density measurements across adjacent sections, such that each animal had one optical density measurement per brain region for use in statistical analyses.

Study 2: Golgi-Cox histochemistry

Golgi-Cox histochemistry was used to assess dendritic morphology in key basal ganglia nuclei (dorsal lateral striatum (DLS), STN, GP, and SNR). At 4 pm on the day following behavioral testing, mice were anesthetized with isoflourane and sacrificed by decapitation. Golgi-Cox histochemistry was completed according to FD Rapid

GolgiStain Kit guidelines (FD NeuroTechnologies, Inc.). In brief, brains were removed and placed into the first solution for Golgi-Cox staining. Time from sacrifice to immersion was less than one minute. Brains were impregnated in solution for 2 weeks at room temperature in a dark environment. After this time, the brains were sliced in a 30% sucrose solution into 200 µm coronal sections using a Vibratome, and allowed to dry at room temperature without exposure to light. The slides were then stained and coverslipped with Permount.

Study 2: Quantification of Golgi-Cox histochemistry

Linear dendritic spine density was quantified using ImagePro (Media Cybernetics) under 40x magnification (Zeiss microscope with Leica DFC camera) by an observer blind to housing condition and sex. Within a given brain region, spines along unobstructed dendritic segments >15 µm were marked on graphic overlays on live digital video images during continuous manual focus adjustment. Any protrusion from the main cylindrical column of the dendrite was counted as a spine. After labeling all the spines along a sample, the approximate dendritic cylinder centerline was traced for return of calibrated length by the software. Dendrites were measured starting beyond the first bifurcation point and at least 50 µm from the soma. It has been shown that enrichment effects on dendritic morphology appear at distances of 50 µm or greater from the cell body (Leggio et al., 2005) and beyond the second order (Spires et al., 2004). In addition, we sought to complement the Turner et al. (2003) findings on first order dendrites. The GP has aspiny and spiny neurons (Kita & Kitai, 1994), so for each mouse, both types were selected for measurement. Within each region of interest, 10-12 dendrites were measured ensuring samples represented both hemispheres and multiple tissue sections. Spine densities were calculated as the total number of spines

divided by the total length of segments in a given brain region, such that each animal had one density measurement per brain region then used in subsequent analyses. The SNR region of one enriched mouse and the GP region of one enriched mouse were not analyzable due to processing error.

Statistical analyses

A General Linear Model (SPSS v21, SPSS Inc, Chicago, USA) with housing, sex and the interaction of these factors was used to assess behavioral and neurobiological differences. If no interaction or main effect of sex was seen, we ran a revised model without sex as a factor. Neurobiological correlates of behavior were assessed using a Pearson's correlation (SPSS v21, SPSS Inc, Chicago, USA). Subsequently, to identify neurobiological effects of EE specific to repetitive behavior, for each analysis, we conducted a secondary analysis of the data comparing only those mice that exhibited the typical EE-induced attenuation of repetitive behavior and mice that developed high levels of repetitive behavior as a consequence of standard housing (SH). The rationale for a secondary analysis of the data derived from earlier deer mouse studies (Powell et al., 1999, 2000; Turner et al., 2002, 2003; Turner & Lewis, 2002; Tanimura et al., 2010, 2010a; Muehlmann et al., 2015) which consistently demonstrated a subpopulation of animals that are atypical in their development of repetitive motor behaviors, given their rearing environment (i.e. enriched mice that develop high levels of repetitive motor behaviors, and standard caged mice that do not develop high levels of repetitive motor behaviors). This secondary analysis of subjects based on repetitive behavior scores was necessary to test our hypothesis that EE-induced attenuation of repetitive behavior is associated with increased functioning of the indirect basal ganglia pathway. This allowed us to interpret our results in the context of attenuation of repetitive behavior

rather than potentially non-selective effects of exposure to EE. Individuals that do not show attenuation of repetitive behavior in response to EE would not be predicted to show increased functioning of the mechanisms hypothesized to mediate repetitive behaviors. To differentiate these subpopulations, we used selection criteria previously established in Turner et al., 2002; 2003, and fit our group criteria to best match the previous studies in terms of frequency (EE < 4000 total jumps > SH) and proportion of atypical individuals (~30%). The frequencies of repetitive motor behaviors and results from secondary analyses are depicted using these stratified group delineations (i.e. EE low repetitive behavior (RB), EE high RB, SH high RB and SH low RB). Raw mean values from both the full and selected data sets are presented in summary tables. In all analyses, Levene's test of equality of error variances and the Kolmogorov-Smirnova test of normality were used to ensure model assumptions were met.

Results

Study 1

EE reduced repetitive motor behaviors

EE significantly decreased the development of repetitive motor behaviors in adult deer mice ($F(1,24)=11.5$, $p=0.002$; see Fig. 2-2). There were no differences in behavior due to sex ($F(1,22)=2.6$, $p=0.12$), and no sex by rearing condition interaction ($F(1,22)=1.1$, $p=0.29$). Three of the 13 mice (23%) reared in EE developed high levels of repetitive motor behaviors, and three of the 13 mice (23%) reared in standard caging developed low levels of repetitive motor behaviors (see Fig. 2-2). Neurobiological analyses were performed on the full data set (EE: n=13 and SH: n=13) and following application of data selection criteria (EE: n=10, SH: n=10).

EE-induced attenuation of repetitive motor behaviors increased neuronal metabolic activity in the indirect basal ganglia pathway

EE significantly increased neuronal metabolic activity in the STN in a sex-dependent fashion (housing*sex: $F(1,22)=4.69$, $p=0.041$; see Fig. 2-3). Enriched males, but not enriched females, were found to have increased activity compared to standard caged animals. There was a significant main effect of sex ($F(1,22)=9.55$, $p=0.005$) with males having overall greater STN CO activity, and a statistical trend for a main effect of EE housing to increase CO activity ($F(1,22)=3.52$, $p=0.074$). In the GP, a statistical trend was evident for EE increases in CO activity (housing: $F(1,22)=3.21$, $p=0.087$) and males had significantly greater CO activity than females ($F(1,22)=9.32$, $p=0.006$) with no housing by sex interaction ($F(1,22)=0.45$, $p=0.50$). There were no significant correlations between total jumps and CO activity in the STN ($r(24)=-0.09$; $p=0.65$) or the GP ($r(24)=-0.22$, $p=0.26$). Outside of the indirect pathway nuclei, there were no significant differences in CO activity due to housing (i.e. in the DLS, SNR, SNC and HPC; all $p>0.05$). In the motor cortex, however, there was a non-significant trend for a housing by sex interaction ($F(1,22)=3.87$, $p=0.062$), with enriched males, but not enriched females, having greater CO activation in this region. There was no significant correlation between total jumps and CO activity in the motor cortex ($r(24)=-0.11$, $p=0.58$). A main effect of sex was found in the DLS ($F(1,22)=6.72$, $p=0.017$), the SNC ($F(1,22)=6.14$, $p=0.021$), and the HPC ($F(1,22)=5.75$, $p=0.025$), always in the direction of males having greater CO activity than females.

Secondary analysis comparing the mice that demonstrated EE-induced attenuation of repetitive behavior and the mice that developed high levels of repetitive motor behaviors induced by standard housing altered our results only in indirect

pathway nuclei. In the STN, a main effect of housing was found with EE increasing STN CO activity ($F(1,16)=5.40$, $p=0.034$; see Fig. 2-4), with the main effect of sex ($F(1,16)=11.7$, $p=0.003$) and housing by sex interaction ($F(1,16)=6.94$, $p=0.018$) remaining. In the GP, secondary analysis based on repetitive behavior scores showed a significant main effect of housing, with EE increasing neuronal activation ($F(1,16)=5.46$, $p=0.033$; see Fig. 2-5) and the significant effect of sex being maintained (females < males: $F(1,16)=6.99$, $p=0.018$). Mice that developed high frequencies of repetitive motor behaviors in spite of enriched housing (i.e. subgroup: EE high RB) showed neuronal activation values in the GP that were quantitatively similar to those of standard caged mice with high frequencies of repetitive motor behaviors (i.e. subgroup: SH high RB; see Fig. 2-5). Table 2-1 provides a descriptive summary of raw mean CO optical density values for EE vs standard housed mice.

Study 2

EE reduced repetitive motor behaviors

As seen in Study 1, rearing mice in EE housing significantly attenuated repetitive motor behavior development ($F(1,16)=9.3$, $p = 0.008$; see Fig. 2-6). There were no differences in behavior due to sex ($F(1,14)=1.0$, $p=0.32$). Three of the ten mice (30%) reared in EE housing developed high levels of repetitive motor behavior, and two of the eight mice (25%) reared in standard housing did not develop high levels of repetitive motor behavior (see Fig. 2-6). Neurobiological analyses were performed on the full data set (EE: n=10 and SH: n=8) and following application of data selection criteria (EE: n=7 and SH: n=6).

EE-induced attenuation of stereotypy increased dendritic spine density in the indirect basal ganglia pathway

Enriched and standard housed mice did not differ in dendritic spine densities in the GP, DLS or SNR (all $p>0.05$). There were no differences in spine densities due to sex (all $p>0.05$). In the STN, no significant effect of EE was seen when mice that failed to show EE-induced attenuation of repetitive behavior were included in the analysis ($F(1,16)=1.9$, $p=0.18$). There was no correlation between total jumps and spine densities in the STN ($r(16)=-0.36$, $p=0.132$). Using those mice exhibiting EE-induced attenuation of repetitive behavior, we found increased dendritic spine density in the STN compared to mice demonstrating high repetitive behavior frequencies ($F(1,11)=8.0$, $p=0.016$; see Fig. 2-7). The mean total spine densities of STN dendrites for deer mice that exhibited EE-induced attenuation of repetitive behavior were quantitatively equivalent to standard caged mice that exhibited atypically low levels of repetitive motor behaviors. STN spine densities of mice that failed to show EE-induced attenuation of repetitive behavior were similar to standard caged mice that developed typically high levels of repetitive behaviors (see Fig. 2-7). Secondary analysis did not alter significance in the GP, DLS and SNR. Table 2-2 provides a descriptive summary of raw mean values of dendritic spine densities for EE vs standard housed mice.

Discussion

As reported in numerous other captive species, rearing deer mice with EE significantly reduced adult levels of repetitive motor behavior. In Study 1, EE-induced attenuation of repetitive motor behavior was associated with increased neuronal activity in the indirect basal ganglia pathway nuclei of the STN and GP. In the STN and motor cortex, the effects of EE were influenced by sex, such that EE males showed greater

increases in CO activity in these regions compared with the other groups. It is notable that males had significantly greater levels of CO in all of the nuclei measured except the SNR. Although not all were statistically significant, on average, EE mice had more CO activity than standard housed mice in all seven of the regions measured. In Study 2, a significant increase in dendritic spine density was seen only in the STN, and only when comparing mice that exhibited EE-induced attenuation of repetitive behavior to standard housed mice with high levels of repetitive behavior typical for that restricted environment. We did not see increased dendritic spine densities in the GP. Although connections with the STN bring some glutamate into the GP that promotes spine density, the influx of GABA from the striatum promotes the competitive selection of spines (Hayama et al., 2013; see discussion below). Analyzing only mice that showed EE-induced attenuation of repetitive behavior and those that developed high levels of repetitive behavior typical of standard caging did not affect the GP spine density results. The current findings suggest that attenuation of repetitive motor behavior by EE is linked to increased neuronal activity and dendritic spine density in the indirect pathway, and is specific to those animals that are behaviorally responsive to EE relative to animals that develop high rates of repetitive motor behavior. These data support our overarching hypothesis that functional activation of the indirect basal ganglia pathway mediates repetitive behaviors.

Rearing animals in EE attenuated the development of repetitive motor behaviors in 70% of mice. Rearing animals in standard conditions generated repetitive motor behaviors in 75% of mice. These percentages are similar to previous findings from our lab that a small proportion of deer mice are atypical for their housing condition (Powell

et al., 2000; Turner & Lewis, 2003; Turner et al., 2003). Why these individuals do not show environment typical levels of repetitive behavior is not known and will require further investigation. Perhaps, mice that do not show EE-induced attenuation of repetitive behavior are less exploratory and interact less with enrichment objects. To this end, future work may wish to include observations of mice interacting with enrichment devices and conspecifics in their home environments.

Turner et al. (2002) demonstrated increased neuronal activation following EE in 12 of the 15 brain regions measured including the DLS, motor cortex and HPC in deer mice. When enriched and standard groups were further divided by behavior, this effect was shown to have been driven by the mice that exhibited EE-induced attenuation of repetitive behavior (Turner et al., 2002). Dendritic spine densities were also significantly increased in the DLS and motor cortex of enriched mice with low frequency of repetitive behaviors relative to standard reared mice with high frequency of repetitive behavior (Turner et al. 2003). Our current findings are consistent with these earlier studies (Tuner et al., 2002, 2003) in highlighting altered cortical-basal ganglia circuitry in the development of repetitive behavior and finding neurobiological differences only in those mice exhibiting EE-induced attenuation of repetitive behavior.

The present findings extend this work on EE effects on basal ganglia, to address selective effects on direct and indirect basal ganglia pathways. We add to a small study conducted by Tanimura et al. (2010) who found increased neuronal activity in the STN, as well as SN (reticulata and compacta) of EE vs standard housed mice. The present study extended the developmental period of EE exposure, now beginning at the time of weaning and lasting for 6 weeks, and included the GP as another indirect pathway

nucleus. As in Tanimura et al. (2010), we found increased activity in the STN as a consequence of EE-induced attenuation of repetitive motor behavior. Moreover, we saw increased CO staining in the GP, another indirect pathway nucleus. These findings provide additional evidence for hypofunctioning of the indirect basal ganglia pathway in repetitive motor behavior development.

A similar pattern emerged following our assessment of dendritic spine densities. Significant main effects due to EE were observed only in the STN, and only when comparing mice that exhibited behavior typical of their housing conditions. Thus, neurobiological results for indirect pathway nuclei seem specific to a repetitive motor behavior phenotype and not a global effect of EE. Moreover, differences were found only in brain regions that are implicated in repetitive motor behaviors. We did not see EE-induced differences in neuronal activation or dendritic spine densities in any nuclei outside of the indirect pathway.

We are the first to report an effect of sex on CO activity levels in association with repetitive motor behaviors. This effect of sex was unexpected given that no differences were found in the repetitive behavior of males and females, and no differences in CO activity levels due to sex have been shown previously in the deer mice. Sex differences in striatal CO activity levels following pharmacological intervention have been reported, however (Jones et al., 2008). Consistent with our prior work in deer mice, in Study 2 we found that males and females did not differ in their behavioral response to housing condition or spine densities in any of the brain regions analyzed (Turner et al., 2003). This result may be specific to deer mice as many studies using other species have found sex differences in dendritic morphology in response to environment (rat: Juraska

et al., 1985, 1989; human: Jacobs et al., 1993). Moreover, our sample size was small and it is therefore possible that we failed to detect an effect of sex on dendritic spine density.

The earlier studies that reported differences in morphological plasticity of spine densities in the DLS as a consequence of EE (Comery et al., 1995; Turner et al., 2003) differed from ours in the approach to quantification. We measured more distal portions of the dendrites; ensuring measurement started beyond the first bifurcation point and at least 50 µm from the soma, and followed the dendrite until it was visually interrupted, whereas first order dendrites of specific lengths (e.g. 15 µm) were measured previously (Comery et al., 1995; Turner et al., 2003). As spine density changes with distance from the soma, the three-fold decrease in reported mean values for spine densities in the DLS supports an overall density difference between first order dendrites and those beyond (Comery et al. 1995, x=~1.30; Turner et al. 2003, x=1.57; current study x=0.45). A major limitation to Study 2 is the lack of assessment of spine structure or the dendritic tree. Spine structure can differ substantially in size and shape, both within a single dendrite and across cell types, and contribute to synaptic function (Harris & Kater, 1994; Comery et al., 1996; Lee et al., 2012).

Predictions for the relationship between neuronal activation and dendritic spine densities are challenging, as this relationship has not been fully elucidated. CO activity is known to reflect contributions of both inhibitory and excitatory inputs, although it is most tightly coupled to the energy demands of mitochondria located in postsynaptic dendritic compartments (Wong-Riley & Welt 1980; Wong-Riley, 1989; Kelly et al., 2010), whereas dendritic spine densities may be more reflective of glutamatergic activity at

presynaptic inputs (McKinney, 2010). Further complicating the relationship between CO activity levels and spine densities, a regulatory role for GABA in local dendritic calcium signaling has been implicated in the shrinkage and competitive elimination of dendritic spines, independent of the generation of action potentials (Hayama et al., 2013). It is noteworthy that elimination of dendritic spines is not necessarily associated with loss of synaptic function between neurons, as morphological remodeling can maintain signal activity (Hasbani et al., 2001; Haws et al., 2014).

We are the first to provide evidence for EE-induced dendritic remodeling in the indirect pathway of the basal ganglia as a potential underlying mechanism for the attenuation of repetitive motor behavior development. Dendritic systems are known to adapt to functional demands (Greenough et al., 1985; Kolb & Whishaw, 1998). Experience-induced morphological plasticity has been observed in several brain regions important in processing of environmental stimuli including cerebellar, primary somatosensory, visual and entorhinal cortices, amygdala, hippocampus, and striatum (Markham & Greenough 2004). Exposure to more complex environments induces structural changes in dendritic morphology that influence the integration of neuronal circuits and storage of information. For example, early exposure to EE induced not only neurogenesis, but a high degree of circuit remodeling involving synaptic inputs (Lonetti et al., 2010; Bergami et al., 2015). MeCP2 mutant mice (a mouse model of Rett Syndrome) exposed to EE show behavioral rescue of impaired motor coordination and increased numbers of excitatory synapses in the cortex and cerebellum. Wild-type mice exposed to EE also had increased densities of excitatory synapses, and interestingly,

reduced numbers of inhibitory synapses, compared to standard caged mice (Lonetti et al., 2010).

As mentioned previously, EE involves a number of components (increased spatial and social density, opportunities for exercise, novelty, etc.). Determining which of these are most influential on repetitive motor behaviors has proven to be challenging (Latham & Mason, 2010; Gross et al., 2011). One component often advanced to account for behavioral and neurobiological changes is exercise. Exercise improves cognitive and motor task performance (e.g. Leggio et al., 2005; Sim, 2014), and increases expression of neurotrophic factors (e.g. BDNF) (Turner & Lewis, 2003; Berchtold et al., 2010; Sim, 2014), hippocampal neurogenesis (van Praag, 2009; e.g. Gregoire et al., 2014), and reduces neuropathology in animal models of neurodegenerative diseases (Larson et al., 2006; Pang et al., 2006; Toy et al., 2014). We have shown that exercise alone did not attenuate the development of repetitive motor behavior in deer mice (Pawlowicz et al., 2010), although its effects were not evaluated here. Thus, the individual contribution of EE component on repetitive behavior and indirect pathway function are as yet to be determined.

A study by Woo and Leon (2013) and their recent follow up study (Woo et al., 2015) applied a direct translation from the EE literature on rodents, focusing on components of novelty and complexity, to children with an ASD diagnosis. Results demonstrated that EE in the form of parent-imposed daily exposure to multiple sensorimotor stimuli (e.g. olfactory, tactile, thermal, motor, balance, auditory, and cognitive tasks), which ensured complexity of stimuli (e.g. combinations of oderant and tactile stimulation) and novelty (new enrichment activities introduced at regular intervals)

successfully improved ASD symptoms for many children in terms of overall severity and cognitive performance (Woo & Leon, 2013, Woo et al., 2015). No independent assessment was made, however, on the effects of their intervention on repetitive behaviors. This research has pioneered EE as a clinical approach for treatment of ASD, and illustrates the potential value of parallel clinical and animal studies in the development of new therapeutics. A better understanding of the mechanisms mediating experience-dependent plasticity will promote the use of EE in conjunction with standard pharmacotherapies.

Figure 2-1. A picture of the environmental enrichment (EE) housing. EE housing employed a large dog kennel with multiple tiers, toys and a running wheel.

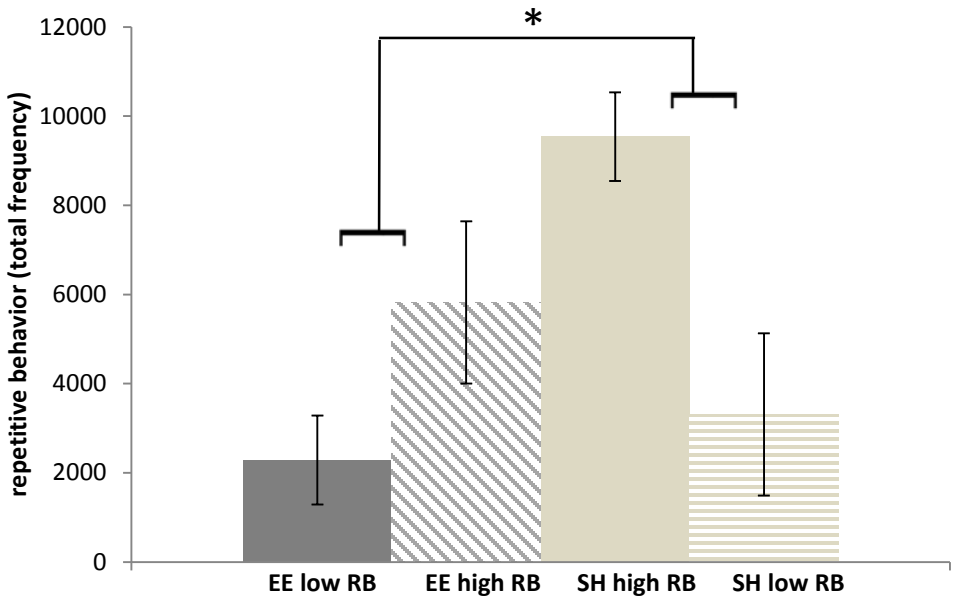

Figure 2-2. Study 1. The effect of housing on the repetitive motor behaviors of adult deer mice. Compared to mice reared in standard housing (SH: n=13), rearing deer mice with environmental enrichment (EE: n=13) significantly reduced the total frequency of adult repetitive motor behaviors occurring over an 8 h dark cycle ($F(1,24)=11.5$, $p=0.002$). Subgroups based on frequency of repetitive motor behaviors (RB): EE low RB (n=10), EE high RB (n=3), SH high RB (n=10), SH low RB (n=3). Data bar shows mean ±SEM.

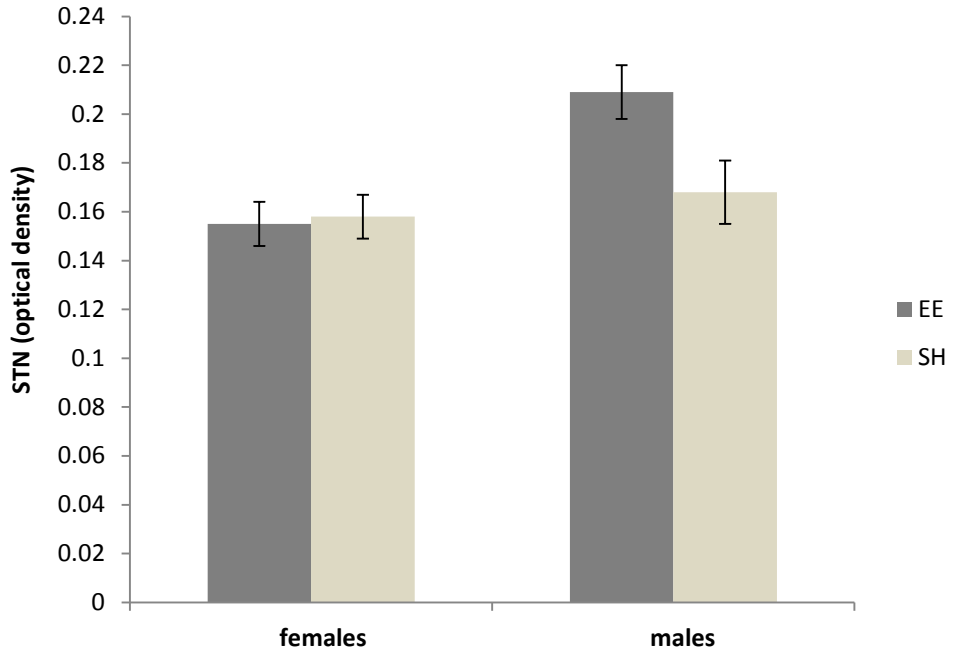

Figure 2-3. Mean optical density measurements in the subthalamic nucleus (STN) following CO histochemistry. Neuronal activation levels in the STN of deer mice reared in EE housing (EE: n=13) were increased compared to mice reared in standard housing (SH: n=13), dependent on sex (housing*sex: $F(1,22)=4.7$, $p=0.041$). Data bar shows mean ±SEM.

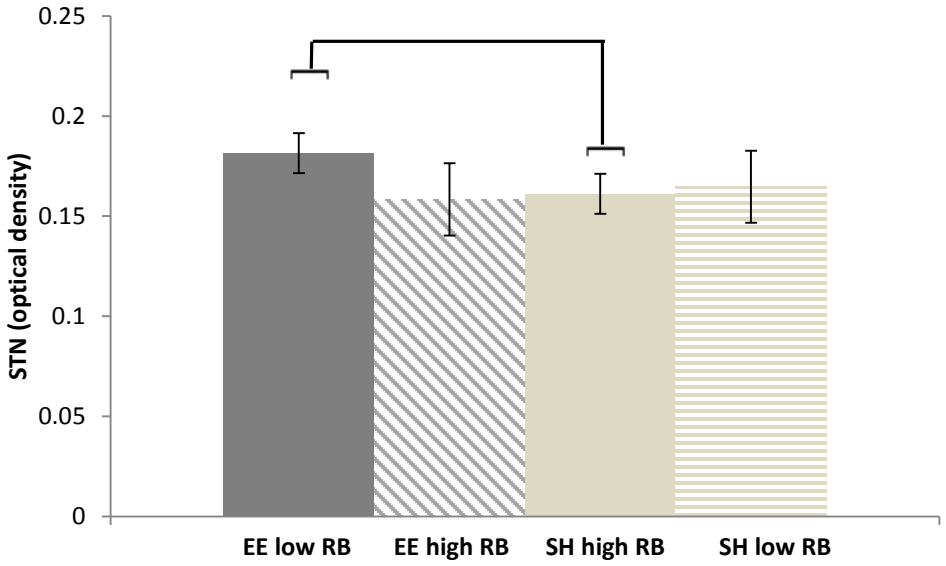

Figure 2-4. Mean optical density measurements in the subthalamic nucleus (STN) following CO histochemistry for deer mice showing EE-induced attenuation of repetitive motor behaviors. Mice showing EE-induced attenuation of repetitive motor behavior development had increased neuronal activation in the STN compared to mice with high levels of repetitive behavior induced by standard housing (SH) ($F(1,16)=5.40$, $p=0.034$). Subgroups based on frequency of repetitive motor behavior (RB): EE low RB (n=10), EE high RB (n=3), SH high RB (n=10), SH low RB (n=3). Data bar shows mean ±SEM.

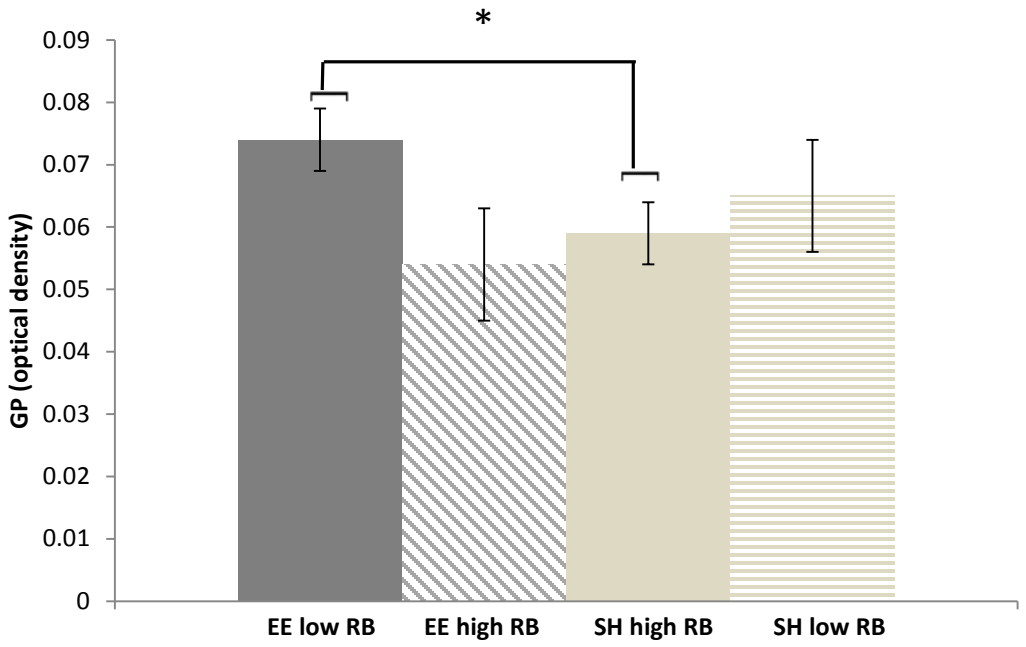

Figure 2-5. Mean optical density measurements in the globus pallidus (GP) following CO histochemistry. Mice showing EE-induced attenuation of repetitive motor behavior development had increased neuronal activation in the GP compared to mice with high levels of repetitive behavior induced by standard housing (SH) ($F(1,16)=5.46$, $p=0.033$). Subgroups based on frequency of repetitive motor behavior (RB): EE low RB (n=10), EE high RB (n=3), SH high RB (n=10), SH low RB (n=3). Data bar shows mean ±SEM.

Table 2-1. Mean (SD) values for CO optical density in a given region.

	EE, n=13	EE, n=10	SH, n=13	SH, n=10
STN	0.176 (0.037)	0.181 (0.041)	0.162 (0.023)	0.161 (0.019)
GP	0.069 (0.016)	0.074 (0.013)	0.060 (0.016)	0.058 (0.015)
DLS	0.130 (0.034)	0.132 (0.039)	0.119 (0.014)	0.118 (0.012)
SNR	0.112 (0.016)	0.114 (0.018)	0.110 (0.014)	0.110 (0.014)
SNC	0.095 (0.016)	0.096 (0.018)	0.089 (0.016)	0.089 (0.011)
M1	0.147 (0.025)	0.149 (0.027)	0.139 (0.010)	0.140 (0.012)
HPC	0.068 (0.016)	0.069 (0.017)	0.064 (0.007)	0.063 (0.008)

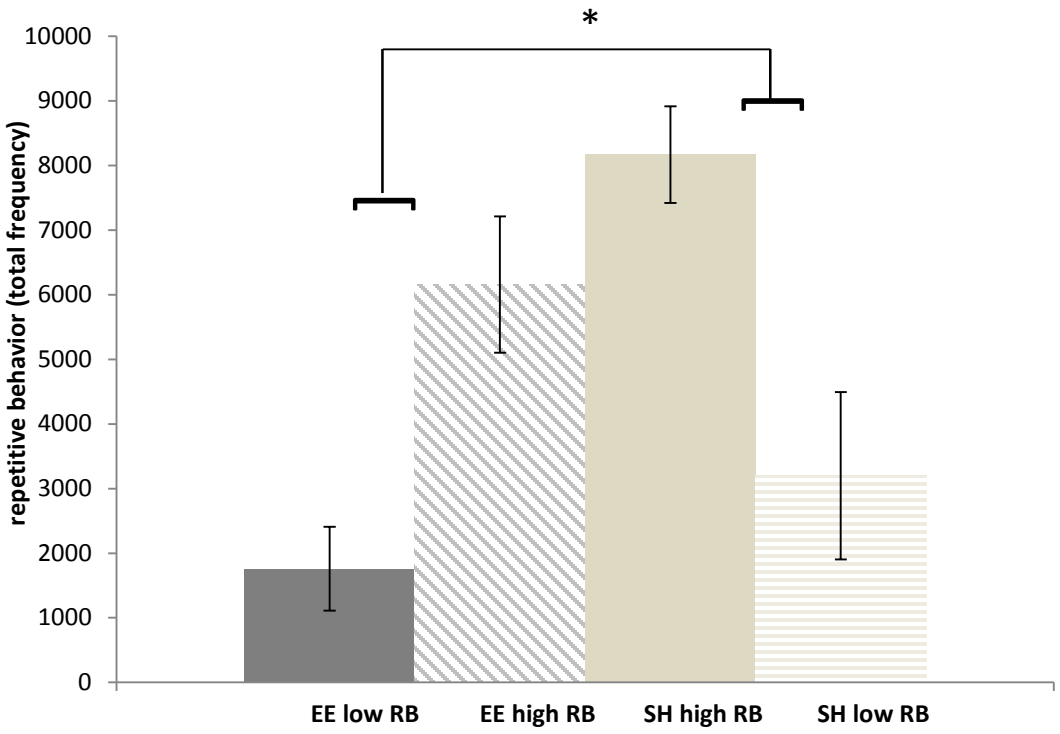

Figure 2-6. Study 2. The effect of housing on the repetitive motor behaviors of adult deer mice. Compared to mice reared in standard housing (SH: n=8), rearing deer mice with environmental enrichment (EE: n=10) significantly reduced the total frequency of adult repetitive motor behaviors occurring over an 8 h dark cycle ($F(1,16)=9.3$, $p=0.008$). Subgroups based on frequency of repetitive motor behavior (RB): EE low RB (n=7), EE high RB (n=3), SH high RB (n=6), SH low RB (n=2). Data bar shows mean ±SEM.

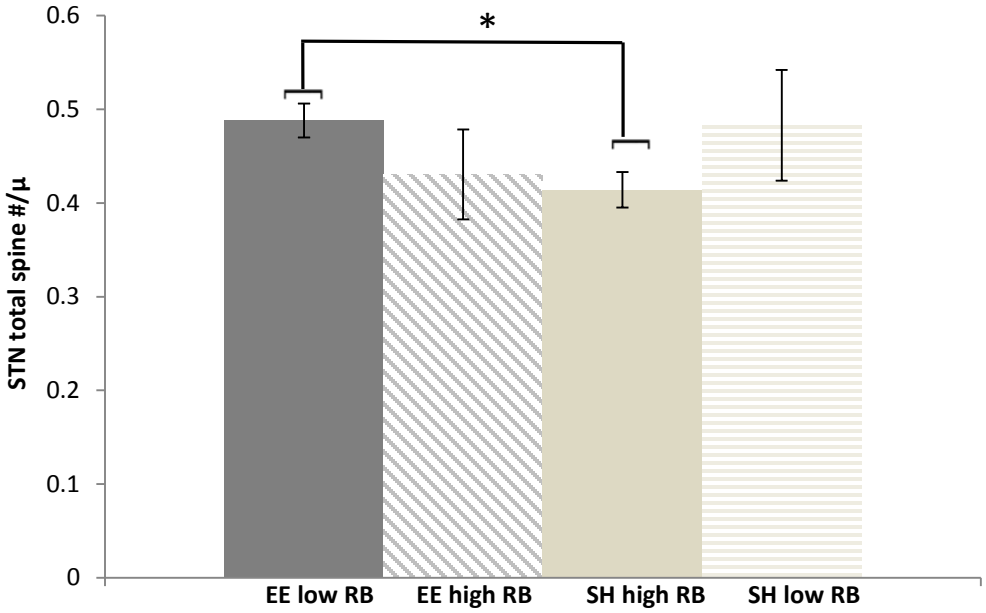

Figure 2-7. Dendritic spine densities in the subthalamic nucleus (STN). EE-induced attenuation of repetitive motor behavior is associated with increased dendritic spine densities in the STN relative to standard housed mice with high levels of repetitive motor behaviors. Subgroups based on frequency of repetitive motor behavior (RB): EE low RB (n=7), EE high RB (n=3), SH high RB (n=6), SH low RB (n=2). Data bar shows mean ±SEM.

Table 2-2. Mean (SD) values for dendritic spine densities in a given region.

	EE, n=10	EE, n=7	SH, n=8	SH, n=6
STN	0.471 (0.067)	0.488 (0.059)	0.431 (0.051)	0.414 (0.025)
GP	0.511 (0.082)	0.506 (0.094)	0.519 (0.058)	0.515 (0.068)
DLS	0.444 (0.042)	0.454 (0.048)	0.479 (0.059)	0.469 (0.065)
SNR	0.434 (0.083)	0.420 (0.079)	0.414 (0.086)	0.412 (0.099)

CHAPTER 3
EFFECTS OF AN ENRICHED ENVIRONMENT ON THE DEVELOPMENT OF REPETITIVE MOTOR BEHAVIORS AND ACTIVATION OF THE INDIRECT BASAL GANGLIA PATHWAY

Repetitive motor behaviors are prevalent across neuropsychiatric, neurological, and neurodevelopmental disorders, including autism spectrum disorders (ASD), for which they are diagnostic (Lewis & Bodfish, 1998). These rigid and rhythmical patterns of movement that seem functionless are also associated with early experiential deprivation, such as congenital blindness and impoverished rearing environments (Fazzi et al. 1999; Rutter et al., 1999). Early in life, repetitive motor behaviors are also performed by typically developing children (Thelen, 1979, 1981; Evans et al.,1997; Kim and Lord, 2010). In spite of their clinical prominence, relatively little is known about the mechanisms underlying repetitive motor behaviors or how they develop across time. Understanding the neurobiological mechanisms mediating normative versus pathological progression of repetitive motor behaviors will promote the development of appropriately timed therapeutic interventions.

Characterization of repetitive behavior development has focused mostly on children with ASD (e.g. Esbensen et al., 2009; Militerni et al., 2002). Repetitive motor behaviors are one of the first behavioral manifestations of ASD, and identifying reliable differences in the developmental trajectories of repetitive behavior in young children is an important tool in early identification and intervention (Barber et al., 2012; Kim and Lord, 2010; Kim and Lord 2012a, 2012b; Sacrey et al., 2015; Watt et al., 2008; Wolff et al., 2014). Kim and Lord (2010) differentiated children (ages 8-56 months) with ASD from those with nonspectrum disorder and typically developing children using the prevalence and severity of early repetitive motor behavior development across time.

Assessments of repetitive behaviors at 2, 3, 5, and 9 years of age revealed increased prevalence and severity of repetitive motor behaviors in children with ASD compared to children with developmental delays.Within the group with ASD, however, trajectory analysis revealed considerable heterogeneity in the development of repetitive motor behaviors over time (Richler et al., 2010). More recently, differences in frequency and intensity, but not topography of repetitive motor behaviors were detected in children with ASD versus typically developing children as early as 9-12 months of age (Wolff et al., 2014).

Although not well studied in terms of function or mechanism, repetitive motor behaviors in normative development typically wane after toddlerhood (Evans et al., 1997; Kim and Lord, 2010). The relationship between normative development and atypical trajectories of repetitive behavior has received little attention, and we know almost nothing about the neurobiological mechanisms mediating normative versus pathological progression. The few neuroimaging studies related to development of repetitive behaviors have typically focused on those behaviors reflecting resistance to change and volumetric differences in basal ganglia, specifically striatum, and reports are often inconsistent. For example, increased striatal growth rate was associated with increased severity of repetitive behavior (insistence on sameness) in young individuals (under 18 years old) with ASD compared to typically developing controls (Langen et al., 2014). In 3-4 year olds with ASD, no systematic association between striatal volume and repetitive behavior was observed (Estes et al., 2011), whereas others found positive correlations between striatal volume and repetitive behavior (rituals and compulsions; Wolff et al., 2013).

Animal models are useful for studying underlying mechanisms of repetitive motor behaviors as they are inducible via CNS insult (e.g. genetic modifications to Shank3, MECP-2), pharmacology (e.g. amphetamines) and restricted environments (Lewis et al., 2007; Bechard and Lewis, 2012). Across species confined to captivity, repetitive motor behaviors are a prominent feature (Mason et al., 2007). Rodent models are an important tool for investigating neurobehavioral developmental trajectories as they have relatively short periods of development. Well-characterized in deer mice (*Peromyscus maniculatus*) reared in standard cages, repetitive hind limb jumping emerges in early development, often before weaning, and increases across adolescence to peak levels by day 56 of age (Muehlmann et al., 2015). Adult repetitive motor behaviors in deer mice manifest as both hind limb jumping and backward somersaulting.

Evidence from both humans and animal models suggest impairments in cortico-basal ganglia circuitry in the development of repetitive behaviors. The basal ganglia include the striatum, globus pallidus (GP), subthalamic nucleus (STN), and substantia nigra (SN). These nuclei coordinate to control movement facilitation, suppression, and refinement via direct and indirect basal ganglia pathways. In the direct pathway, the striatum receives glutamatergic input from the cortex and sends GABAergic monosypnaptic projections directly to the output nucleus, the pars reticulata of the SN (SNR). In the indirect pathway, the striatum sends GABAergic projections to the GP (external GP, in primates), which relays to the STN, before converging on the SNR. A third largely understudied projection, the hyperdirect pathway, directly connects the motor cortex with the STN and modulates inhibition of responses in conflict situations (Jahfari et al., 2011; Jahanshahi, 2013). An imbalance in the dynamic interplay of these

pathways has been implicated in circuitry dysregulation associated with both hyperkinetic and hypokinetic movement disorders (Graybiel, 2000).

The development of high levels of repetitive motor behaviors in deer mice has been associated with dysfunction in cortico-striatal basal ganglia circuitry, and specifically, a hypofunctioning of the indirect basal ganglia pathway (Presti and Lewis, 2005; Lewis et al., 2007; Tanimura et al., 2010, 2011; Bechard et al., 2016). For example, high levels of repetitive behavior in standard housed deer mice were associated with reduced enkephalin (an indirect pathway specific neuropeptide), but not dynorphin (a direct pathway specific neuropeptide; Presti and Lewis, 2005). Pharmacological intervention targeting indirect pathway neuronal activation successfully reduced repetitive motor behaviors in deer mice (Tanimura et al., 2010). Furthermore, developmental trajectories of high versus low repetitive motor behaviors in standard housed deer mice were associated with differences in neuronal activation of the indirect basal ganglia nuclei (e.g., STN), supporting hypoactivation of these nuclei in the development of high levels of repetitive motor behaviors (Tanimura et al., 2011).

Restricted or confined environments give rise to repetitive behavior whereas the attenuation of repetitive behaviors by environmental enrichment (EE) is a robust and well-documented effect (e.g. Lewis, 2004; Mason et al., 2007). Enriching the rearing environments of deer mice attenuated repetitive motor behaviors in adulthood and was associated with increased neuronal activation and dendritic spine densities in nuclei lying within the indirect basal ganglia pathway (Tanimura et al., 2010, Bechard et al., 2016). The development of repetitive motor behaviors within an enriched environment has not been characterized previously, however, and there are no studies on the

neurobiological correlates of repetitive behavior development within an enriched environment. Within a given environment, repetitive motor behavior development in deer mice is quite heterogeneous (e.g. Tanimura et al., 2010a; Muehlmann et al., 2015; Bechard et al., 2016). For example, rearing deer mice with EE attenuates adult repetitive motor behaviors in most, not all, deer mice, but does not completely eliminate them (Turner et al., 2002; 2003; Lewis, 2004, Lewis et al., 2007; Bechard et al., 2016). This heterogeneity both models the clinical presentation and provides an opportunity to investigate neurobiological associations of between and within group behavioral differences (Turner et al., 2002; 2003; Bechard et al., 2016). By including multiple assessments across time, we can address the question of how associated neurobiological mechanisms change with the progression of repetitive motor behaviors.

An important goal of this study was to characterize the developmental trajectory of repetitive motor behaviors in deer mice reared within an enriched environment. Our overall hypothesis was that EE housing would attenuate the development of repetitive motor behaviors, an outcome mediated by increased functioning of the indirect basal ganglia pathway. In Study 1, we sought to characterize repetitive motor behaviors within an enriched environment across development. We hypothesized that rearing mice in EE cages would generate mice with different developmental trajectories of repetitive motor behavior and associated changes in indirect basal ganglia pathway activation. This longitudinal design and statistical approach was used previously in standard reared deer mice to identify three developmental trajectory groups and associated differences in neuronal activation (Muehlmann et al., 2015; Tanimura et al., 2010, 2011). Within the enriched environment, however, this longitudinal approach employing repeated testing

of individual mice was unsuccessful in attenuating repetitive motor behavior development. Therefore, in Study 2, we employed a cohort design to characterize repetitive behavior development in deer mice reared in enriched and standard laboratory cages and associated neurobiological differences in basal ganglia function at key developmental time points. We hypothesized that, compared to standard reared mice, EE reared mice would develop lower frequencies of repetitive motor behaviors with age, and corresponding increased levels of activation in indirect pathway nuclei as indicated by cytochrome oxidase (CO) histochemistry. To our knowledge, we are the first to characterize the development of repetitive motor behavior within an enriched environment and associate neurobiological changes that mediate their progression versus attenuation.

Materials and Methods

Animals

All procedures were approved by the University of Florida's Institutional Animal Care and Use Committee and performed according to the NIH Guide for the Care and Use of Laboratory Animals. Deer mice were bred and housed at the University of Florida in one room maintained at a 16:8 light: dark cycle, 70-75 ^{0}F and 50-70% humidity. Home cages were furnished with SaniChip bedding, Teklad rodent chow, water, and Nestlet squares for nest construction. Offspring of monogamous breeding pairs were weaned at day 21 and litters split to ensure siblings were housed both in EE and standard housing conditions. In Study 1, we tested N=59 subjects; n=40 mice were reared in EE housing and n=19 mice were reared in standard housing. In Study 2, we tested N=216 subjects; n=93 were reared in EE housing and n=123 mice were reared in

standard housing. Cages were comprised of same-sexed, similar-aged mice (± 1 day) grouped together at weaning.

Enriched housing conditions

Our EE paradigm has successfully been used to reduce repetitive motor behaviors in deer mice (Turner et al., 2002, 2003; Tanimura et al., 2010a, Bechard et al., 2016). EE cages were large dog kennels (1.22 x 0.81 x 0.89 m; n=4-6) modified by galvanized wire to have three-tiers, and additionally furnished with multiple objects (e.g. plastic toys, domes, tunnels), Habitrail tubes, a large hut and a running wheel. The objects were rotated weekly to maintain novelty as well as complexity, and simultaneously, bird seed (~2 oz.) was scattered throughout the cage to promote foraging behavior. Food, water, nestlets and bedding were refreshed every two weeks.

Standard housing (SH) conditions

Standard laboratory caging (29 x 18 x 13 cm) reliably induces high levels of repetitive motor behaviors in the majority of deer mice (Powell et al., 1999; Tanimura et al., 2010; Muehlmann et al., 2015). To control for any nutritional differences without promoting foraging, a small amount (~0.25 oz) of bird seed was deliberately placed into the corner of the standard cages each week. Food, water, nestlets and bedding were refreshed every two weeks.

Assessment of repetitive motor behaviors

Mice were removed from their home environments and placed into individual testing chambers (28 x 22 x 25 cm) at least 30 minutes prior to lights off (10:00 am) for assessment of repetitive motor behaviors. Each assessment lasted for the duration of one entire dark cycle: 10:00-18:00 (8 h total), during which food and water were always available. Deer mice display two topographies of repetitive motor behaviors: vertical

jumping, which emerges early in development (by weaning age) and backward somersaulting (emerges later in development). Both topographies involve vertical activity that can be quantified automatically (Labview software; National Instruments) by counting the number of interruptions to photo beam arrays (Columbus Instruments) positioned high enough so that normal activity (e.g. rearing, feeding, drinking, ambulation) does not interfere with them. Simultaneous video recordings (Geovision software) of each test chamber and each test session insured the accuracy of the automated counts. For each mouse, the total number of jumps summed over the entire 8 h test session was calculated and used in subsequent analyses.

The longitudinal design employed in Study 1 involved assessing repetitive motor behaviors repeatedly in the same individuals at 22, 25, 28, 35, 42, 49, 56 and 63 days of age. After each assessment, subjects were returned to their home environments. After completion of the day 63 assessment, brains were harvested for use in neurobiological assays. The cohort design employed in Study 2 tested for repetitive behaviors at corresponding developmental time points: 22, 28, 35, 42, 49, 56, and 63 days of age. In this study, individuals were assessed once at a randomly determined developmental time point and then sacrificed for neurobiological assessment. In both studies, same-aged mice reared in standard cages were tested at the same time as EE-reared mice.

Cytochrome Oxidase (CO) histochemistry

CO activity reflects the oxidative metabolic capacity of neurons due to its functional role in the process of generating ATP (Wong-Riley, Nie, Hevner, Liu, 1998). CO activity is a measurement of long-term (days to weeks) neuronal metabolic activity (Sakata, Crews, Gonzalez-Lima, 2005), and has previously been used to detect

differences in activation of basal ganglia as a function of repetitive motor behaviors (Turner et al., 2002; Tanimura et al., 2010a, 2011; Bechard et al., 2016). The CO staining protocol (Gonzalez-Lima and Cada, 1998) was performed on brains that were snap-frozen in 2-methylbutane and stored in a -80°C freezer. Sagittal sections (20 µm) sliced on a cryostat (-20°C) were collected from both hemispheres at 1-2 mm lateral to the midline and mounted onto microscope slides (Superfrost Plus, FisherBrand). Standards were made from homogenized brain tissue of non-subject deer mice and were included in each assay to ensure linearity of optical density measurements. Slides were stained and cover slipped with Permount.

Quantification of CO histochemistry. Optical density measurements were taken (ImagePro software, Media Cybernetics) from the basal ganglia regions of interest: dorsal lateral striatum (DLS), GP, STN, and SNR, as well as the CA1 region of the hippocampus (HPC) and motor cortex. For each brain region, neuronal metabolic activation values were calculated by averaging the optical density measurements across multiple adjacent sections.

Statistical Analyses

In Study 1, a Repeated Measures General Linear Model (SPSS v23, SPSS Inc. Chicago, IL, USA) with age, housing, and sex as factors was used to assess differences in repetitive behavior development. Subsequent trajectory analysis was completed using a group-based trajectory modeling procedure (Proc Traj; Jones and Nagin, 2007), which clusters individuals within a population based on similar repetitive behavior frequencies across development. For each behavioral assessment (e.g. mouse 1: day 28), the log of the total frequency of repetitive behavior was calculated and entered into Proc Traj (R. 3.0.1) to assign probabilities of group membership to one of three discrete

trajectory groups (High, Medium, Low). Log transformed data were used to ameliorate non-homogeneity of variance in repetitive behaviors scores across development. The three discrete trajectory group assignments matched those used in our previous assessments of developmental trajectories of repetitive behavior for deer mice reared in standard housing (Tanimura et al., 2010; Muehlmann et al., 2015). Trajectory group differences in repetitive behavior frequencies were assessed using a Repeated Measures General Linear Model (SPSS v23) with age, trajectory group, and sex as factors in the model. When our model violated Mauchly's Test of Sphericity ($p<0.05$), which assesses whether the variances of the differences between groups are equal, we report the corrected degrees of freedom using Greenhouse-Geisser estimates of sphericity, which adjusts the F-ratio to reduce the likelihood of committing a Type I error. Post-hoc comparisons were assessed using Bonferroni's test.

In Study 2, a General Linear Model (SPSS v23) with age, housing, and sex as factors was used to assess differences in repetitive behavior development. The effect of age on repetitive behavior development within an environment was assessed for enriched and standard mice separately using a General Linear Model with age as the only factor in the model and Bonferonni's post-hoc test.

A General Linear Model with age, housing, and sex as factors was used to assess neurobiological differences in the STN, GP, SNR, DLS, HPC and motor cortex. We assessed neuronal activation at two key developmental time points: day 42 (EE: n=12, SH: n=17), the first age at which significant differences in repetitive behavior frequencies due to housing were apparent; and day 63 (EE: n=13, SH: n=20), the age at which asymptotic levels of repetitive behavior frequencies were reached. To aid the

interpretation of three-way interactions, we further analyzed the neurobiological data of males (n=35) and females (n=27) in separate analyses, assessing each data set using a General Linear Model with age and housing as factors. For all analyses, Levene's test of equality of error variances and the Kolmogorow-Smirnov test of normality were employed to assure model assumptions were met, and transformations of the data were performed where necessary. The SNR of 2 mice and the GP of 1 mouse were not analyzable due to processing errors.

The effects of repeated testing on repetitive motor behavior development were assessed using a linear mixed model (in R, package lme4). When exploring the data, we considered logarithmic, square root and identity (i.e., no transformation) transformations of the responses. The square root transformation was chosen because it resulted in the lowest (best) AIC scores based on the likelihood for the original-scale responses, and thus provided the best fit. The models were fitted to the data for each environment separately. The full statistical model was: $Y_{ijk} = \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \epsilon_{ijk}$, where Y_{ijk} is the square root transformed response, α_i is the subject-specific random intercept, β_j is the main effect of multiple testing, γ_k is the effect of the day and $(\beta\gamma)_{jk}$ is the interaction term. Model selection was performed via likelihood ratio testing for the significance of the interaction effect as well as the main effect of multiple testing and corresponded to the variance-stabilizing transformation for repetitive behavior scores. Figures of single and multiple testing effects for each environment were produced from the model output and back-transformed to the original scale and thus no error bars are depicted.

Results

Study 1

Developmental trajectories of repetitive motor behaviors within an enriched environment (repeated testing). The frequencies of repetitive motor behaviors increased with age for mice tested repeatedly ($F(3.1,171=45.6)$, $p<0.001$; see Fig. 3-1), but did not differ due to rearing environment or sex. Group-based trajectory analysis for mice reared within EE cages resulted in high (n=22), medium (n=10) and low (n=8) trajectories of repetitive motor behavior development. Although we did not run Proc Traj analysis on the standard housed mice (n=19), for comparison purposes, we included their developmental curve (see Fig. 3-2). Analysis based on trajectory group assignments revealed differences in repetitive behavior frequencies as a function of age (group*age: $F(9.1,154)=2.4$), $p=0.013$; see Fig. 3-2), as well as a main effect of group ($F(3,51)=11.3$, $p<0.001$) and age ($F(3.0,154)=18.2$, $p<0.001$). There were no effects of sex on behavior. Post-hoc tests indicated that the low vs medium trajectory mice ($p=0.25$) and high trajectory vs standard mice ($p=0.17$) did not differ significantly from each other. The low and medium trajectory mice did significantly differ from the high trajectory (vs low: $p<0.001$, vs medium: $p<0.001$) and standard mice (vs low: $p<0.001$, vs medium: $p=0.005$). The finding that medium and low trajectory frequencies were not different suggests a two-trajectory solution for repetitive behavior development within the enriched environment would have been satisfactory.

Study 2

Development of repetitive motor behaviors in EE and standard housing (single test). Figure 3 shows the mean total frequencies of repetitive motor behaviors across development for deer mice reared in standard and enriched environments. To

meet model assumptions, a square root transformation was applied to the behavioral data. Compared to standard housing, EE housing inhibited the development of repetitive motor behaviors (age*housing: ($F(6,188)=4.36$, $p<0.001$; see Fig. 3-3). A main effect of housing ($F(1,188)=40.4$, $p<0.001$) and age ($F(6, 188=15.9)$ $p<0.001$) were also found. Post-hoc analysis for age indicated that days 28 and 35 ($p=0.59$), and 42, 49, and 56 (all $p>0.05$) were not different from each other, whereas all other days showed significant differences in repetitive behavior frequencies (all $p<0.05$).

Within the enriched environment, repetitive behavior significantly increased with age ($F(6,86)=4.04$, $p=0.001$). Repetitive behavior frequencies on day 22 were significantly less than all other days (all $p<0.05$), whereas beyond day 22: days 28, 35, 42, 49, 56, and 63, no further significant increases occurred (all $p>0.05$). Within the standard environment, repetitive behavior again significantly increased with age ($F(6,119)=9.64$, $p<0.001$). Repetitive behavior frequencies on days 22 and 28 were significantly lower than all other days ($p<0.05$), except for Day 35 ($p>0.05$). Day 35 frequencies only differed significantly from day 63 ($p<0.001$). Days 42, 49 and 56 frequencies were not different from one another (all $p>0.05$), but were lower than those on day 63 (all $p<0.01$).

Neuronal activation of the indirect basal ganglia pathway across development. Assessment of neuronal activation in the STN at days 42 and 63 resulted in a significant three-way interaction of sex, age, and housing ($F(1,54)=14.8$, $p<0.001$; see Fig. 3-4). A significant interaction between housing and age ($F(1,54)=8.0$, $p=0.006$) and a main effect of sex, indicating females had overall higher levels of CO ($F(1,54)=5.9$, $p=0.018$), were also found. In the SNR, this same pattern of a significant

sex, age, and housing interaction ($F(1,52)=8.0$, $p=0.007$; see Fig. 3-5) and age by housing interaction ($F(1,52)=5.5$, $p=0.022$) resulted, with no main effect of sex in this brain region. The neuronal activation patterns in the GP, DLS, and HPC also resulted in significant three-way interactions between sex, age, and housing (GP: $F(1,53)=10.5$, $p=0.002$; DLS: $F(1,54)=5.7$, $p=0.02$; HPC: $F(1,54)=4.9$, $p=0.03$), and a significant effect of age in the DLS ($F(1,54)=5.2$, $p=0.026$); no other significant interactions or main effects were found. In the motor cortex, there were no significant differences in activation.

Subsequent neurobiological analyses were conducted for each sex. In males, STN activation differences were significantly affected by age and housing (age*housing: $F(1,23)=30.0$, $p<0.001$). In the SNR, the interaction of age and housing was again significant ($F(1,23)=19.6$, $p<0.001$) as was the main effect of increased activation due to EE housing ($F(1,23)=6.4$, $p =0.019$). In the GP, the interaction of age and housing was significant ($F(1,23)=6.1$, $p=0.021$), with no other main effects being of significance. We found DLS activation differences due to age and housing (age*housing: $F(1,23)=5.7$, $p=0.025$), and overall increased activation with age ($F(1,23)=4.4$, $p=0.047$). In the HPC and motor cortex of male mice, there were no significant differences in neuronal activation. In females, there were no significant differences in neuronal activation in any region (i.e. the STN, SNR, GP, DLS, HPC and motor cortex; all $p>0.05$).

Effects of Repeated Testing on Repetitive Motor Behavior Development

Repeated testing significantly increased repetitive motor behavior development both within the enriched and standard environments. Within the EE housing, the effect of age for single versus multiple tested mice was highly significant ($p=0.002$); see Fig. 3-

6a), Within the SH housing, this effect was again significant (p=0.037); see Fig. 3-6b), although less pronounced.

Discussion

In two studies, we characterized the development of repetitive behavior within an enriched environment and associated developmental changes in indirect basal ganglia pathway functioning. We hypothesized that rearing mice in EE housing would attenuate the progression of repetitive motor behavior development and align with developmental increases in indirect pathway nuclei activation. In Study 1, we characterized the development of repetitive motor behaviors within an enriched environment by repeatedly testing subjects from weaning into adulthood. This longitudinal approach of assessing repetitive motor behaviors was unsuccessful in generating significant differences due to housing. As rearing within an enriched environment typically has a robust attenuating effect on repetitive motor behavior development (Lewis, 2004; Mason et al., 2007), this result was unexpected. Subsequent group-based trajectory analysis revealed a high (55%), medium (25%), and low (20%), trajectory groups of repetitive motor behavior development. The frequencies of repetitive motor behaviors across development of mice belonging to the high trajectory did not differ significantly from those of standard housed mice, but did differ from those of medium and low trajectory mice. Although we found different trajectories of repetitive motor behavior within the enriched environment, we were unsuccessful in generating an asymptotic low trajectory or the expected overall EE-induced attenuated frequencies of repetitive behavior (see Turner et al., 2002, 2003; Bechard et al., 2016).

No previous attempts have been made at the longitudinal characterization of repetitive motor behaviors within an enriched environment. Within a standard laboratory

environment, however, earlier studies identified three distinct developmental trajectories (Tanimura et al., 2010; Muehlmann et al., 2015). The low trajectory group was comprised of mice that showed continuously low levels of repetitive motor behavior across development, whereas the high trajectory group was comprised of mice that showed continuously high levels across development. The middle trajectory was comprised of mice that showed low levels of repetitive motor behavior after weaning and developed high levels of repetitive motor behavior across adolescence and into adulthood. The low trajectory of repetitive motor behavior development was associated with increased neuronal activation of basal ganglia nuclei, including STN, SNR and DLS (Tanimura et al., 2011). Although no sex differences in behavioral trajectories were found, females were overrepresented in the high trajectory group (Muehlmann et al., 2015).

Rearing effects on behavior result from a complex interaction between prior handling, social experience, and test conditions (Holson et al., 1991). Many EE paradigms employ brief repeated handling sessions, as this typically generates mice with less anxiety-like behaviors (e.g. Ábrahám and Kovács, 2000; Río-Álamos et al., 2015). Deer mice, however, are an outbred wild stock, and contrary to *Mus musculus*, appear to resist habituation to handling. In deer mice from both enriched and standard environments, repeated testing exacerbated repetitive motor behavior development, although this effect was more pronounced in the enriched mice. These exploratory analyses further showed that the effect of repeated testing on repetitive motor behavior development does not become apparent until later in development (SH: at 49 days of age; EE: at 42 days of age), suggesting that at least four tests are required to influence

behavior. The relationship between repeated testing, stress, and development of repetitive motor behaviors and the differential effects of housing condition will need further investigation. To this end, conducting observations of repetitive motor behaviors within the home cages of EE mice may be another potential approach, although protracted individual assessment would be very challenging.

Due to the abolition of our typically robust enrichment effect by use of a longitudinal design, in Study 2 we employed a cohort design to assess the development of repetitive motor behaviors within an enriched environment. With this single-test approach to assessing behavior, the repetitive motor behavior development within an enriched environment was significantly attenuated. A one week period of exposure to the enriched environment starting at weaning was sufficient to arrest further progression of repetitive motor behaviors. Differences between EE and standard reared mice were apparent three weeks after mice were introduced to their environments. This aligns with other studies investigating enrichment effects on behavior and structural brain plasticity (Lawlor et al.,1999; Scholz et al., 2015). Within both rearing environments, repetitive motor behaviors significantly increased with age for both enriched and standard reared mice, but significant differences due to housing were apparent by the beginning of adolescence (i.e. day 42). At this age, the slopes of repetitive motor behavior development diverge based on housing condition, with repetitive behavior frequencies of standard mice continuing to increase compared to plateaued frequencies of enriched mice. Follow-up analyses confirm that within EE housing, the progression of repetitive behavior development was complete by day 28, in this study, less than 1 week after being introduced into the enriched environment. In contrast, mean frequencies of

repetitive motor behaviors in standard caged mice rose in a step-wise fashion across adolescence. This novel finding suggests that, rather than reducing already established repetitive motor behaviors, rearing in EE prevents the progression of repetitive motor behavior development. Interestingly, mean frequencies following 1 week of post-weaning EE exposure were no different than those following 6 weeks of post-weaning EE exposure.

In line with our hypothesis, we found increased neuronal activation of indirect pathway nuclei in mice reared with EE that corresponded to lower frequencies of repetitive behavior. Results were dependent on sex, however. In enriched males, CO activity in the STN, GP, SNR and DLS increased across adolescence and levels of repetitive motor behaviors remained low. In standard housed males, CO activity in the STN, GP, and SNR decreased (DLS levels did not change) across adolescence and levels of repetitive behavior increased. However, there was no clear relationship between CO activity and repetitive behavior development in female mice. As a consequence of CO activity in female mice showing minimal changes with age, several significant three-way interactions resulted. Only the motor cortex was unaffected by the interaction of housing, sex, and age. When we separated the analyses by sex, the influence of environment on the activation of nuclei across development was clearer. Males reared in EE housing show similar or slightly lower activation of basal ganglia nuclei in adolescence than standard reared males, yet higher activation in adulthood. As housing differences in repetitive behavior were already apparent at day 42, our data suggest that increased performance of the aberrant behavior preceded changes in neuronal activation as indicated by CO histochemistry. Outside of the basal ganglia

nuclei, there were no differences in activation due to age or housing. No regional, developmental or housing differences in neuronal activation were found in females. Although secondary analyses separated by sex indicated that CO differences between EE and standard males were not significant outside of the basal ganglia, the initial significant three-way interaction in the HPC suggests a potential generalized effect of EE to increase neuronal activation. A generalized effect of EE was not unanticipated, given the wide-ranging therapeutic effects of EE on CNS development (Hannan, 2014). Notwithstanding, the STN and GP lie within the indirect pathway, and therefore these data continue to support an important role for the functioning of the indirect basal ganglia pathway in the development of repetitive motor behaviors.

The current results replicate our recent finding that EE-induced attenuation of repetitive motor behaviors is associated with increased functioning of the indirect basal ganglia pathway in adult male, but not female, deer mice (Bechard et al., 2016). Within the standard environment, they also support Tanimura et al. (2011) findings of reduced activation in the STN and SNR with increasing age (developmental time points: days 28, 46, and 63) and repetitive behavior development, although these were statistical trends. Changes in activation occurred mostly between days 46 and 63, and so neuronal activation changes in the indirect pathway were proposed to lag behind rather than lead repetitive motor behavior development (Tanimura et al., 2011). Our results support this, and also that standard reared male mice show decreased activity in the STN and SNR, and very little change in activity of the DLS and motor cortex, between adolescence and adulthood, a developmental time period in which repetitive motor behavior significantly increased. Unfortunately, no investigation of sex effects was available for comparison.

Neuronal activation differences in basal ganglia nuclei of males and females have been reported previously. For example, differences in CO activity in the striatum of males and females have been reported following caffeine administration (Jones et al., 2008), and in the frontal cortex regions of preweanlings following mother-infant separation and early handling (Spivey et al., 2011). CO histochemistry may be interacting with female hormonal phases. In support of this, differences in the specific activation of CO based on phase of estrus cycle were found in mouse ovarian cells (Chapman et al., 1992) and brown adipose tissue of rats (Puerta et al.,1998). Since we did not evaluate the phase of estrus cycle in females, the variations in CO activity may have masked any effects of age and housing, a hypothesis now needing direct testing.

In summary, to our knowledge we are the first to have characterized the development of repetitive motor behaviors in EE housing. Using a longitudinal approach, we report a novel effect of repeated testing to exacerbate repetitive motor behavior development in deer mice, virtually eliminating the typically robust effect of EE-rearing to generate mice with low levels of repetitive behaviors. Using a cohort, single-test approach, we found that progression of repetitive motor behaviors across early development was prevented by EE, and required at least three weeks of EE exposure to differ from those of standard controls. Moreover, at least in deer mice, 1 week of post-weaning exposure to EE generated mice with repetitive behavior levels similar to those of mice having 6 weeks of exposure. We also found associated differences in the activation of nuclei lying within the indirect basal ganglia pathway across development that continue to support its importance in mediating the development of repetitive motor behaviors.

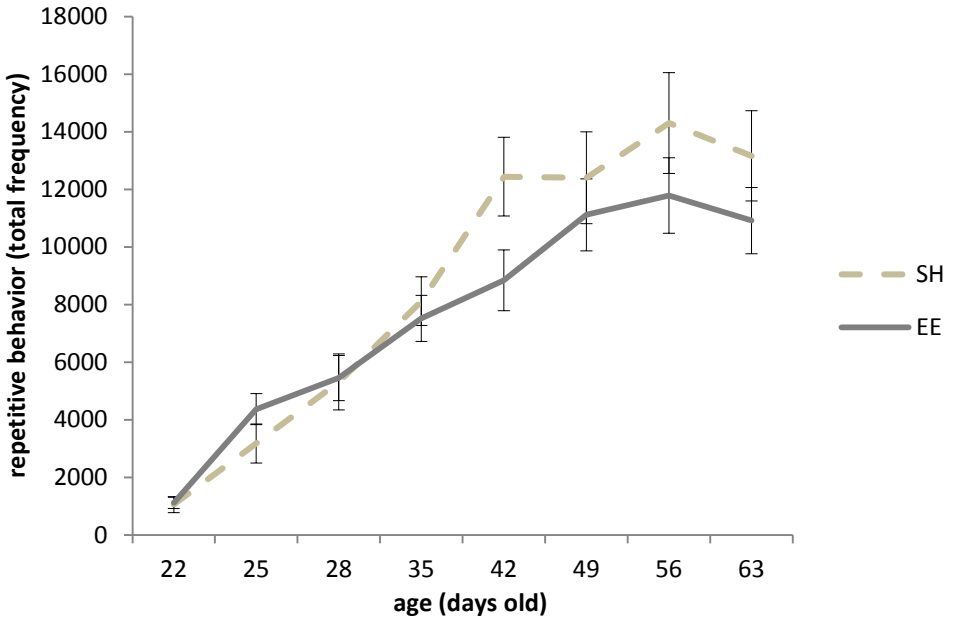

Figure 3-1. The effect of housing on repetitive motor behavior development in repeatedly tested deer mice reared in enriched (EE: n=40) or standard housed (SH: n=19) environments. Error bars are ± SEM.

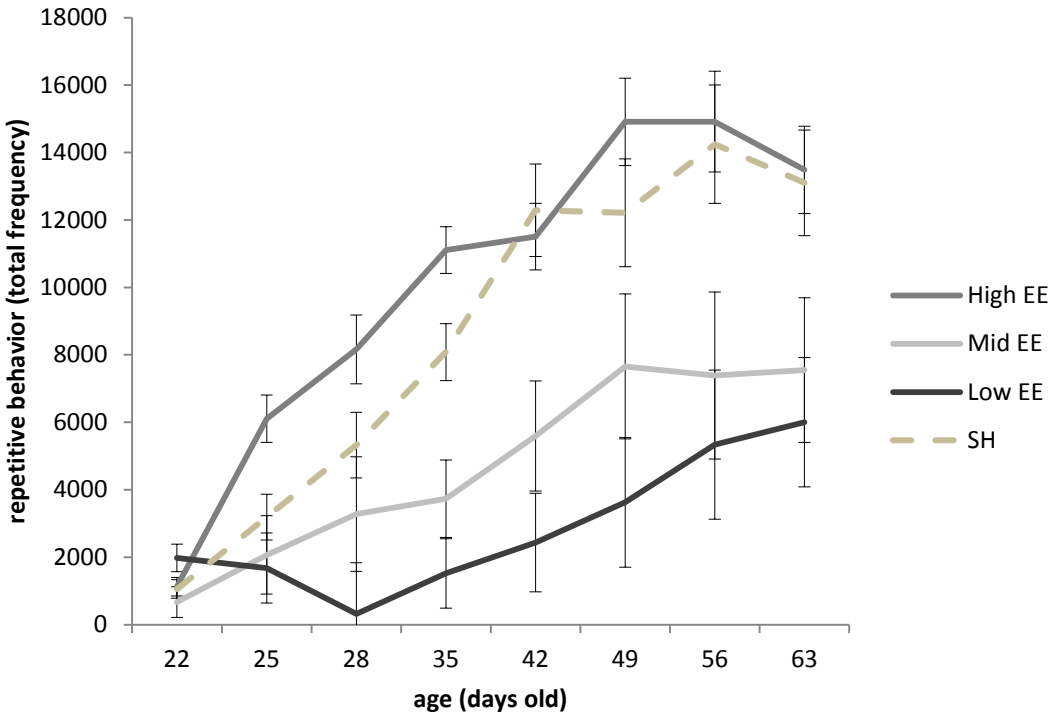

Figure 3-2. The developmental trajectories of repetitive motor behaviors for repeatedly tested deer mice reared in enriched housing (High: n=22, Medium: n=10, and Low: n=8) compared to the developmental curve of repeatedly tested standard housed deer mice (SH: n=19). Error bars are ± SEM.

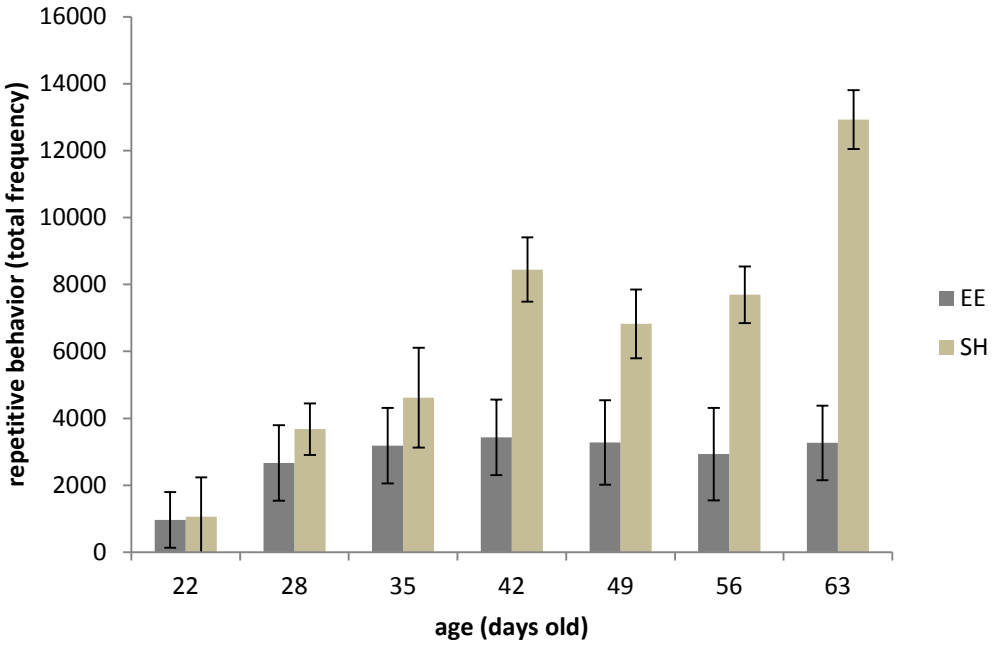

Figure 3-3. Mean total frequencies of repetitive motor behaviors across development for mice reared in enriched (EE: n=93) and standard housed (SH: n=123) environments. Error bars are ± SEM.

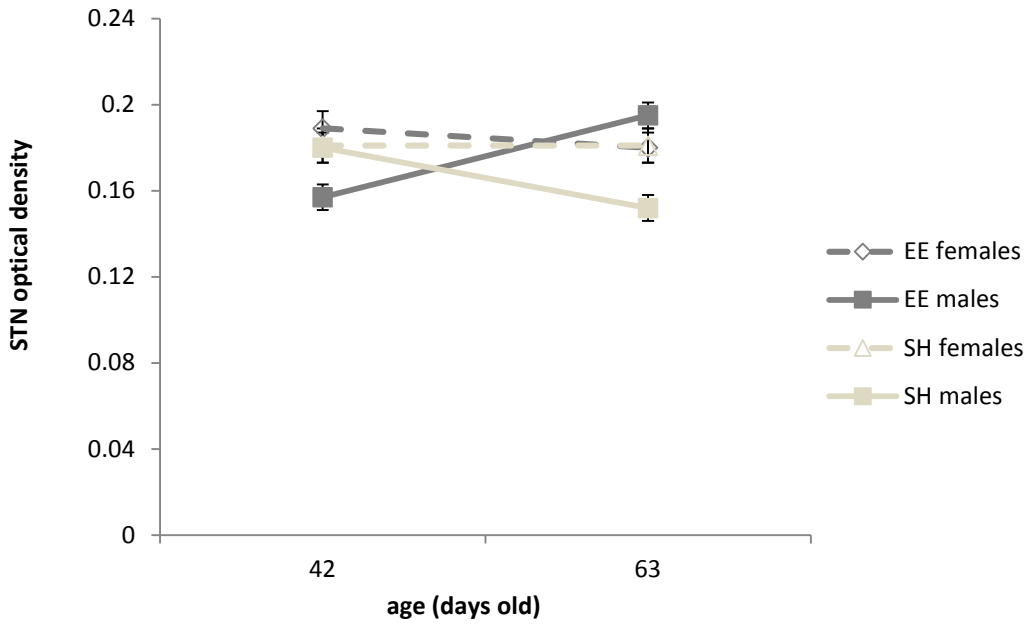

Figure 3-4. The effect of housing on neuronal activation in the subthalamic nucleus (STN) at two developmental time points for deer mice reared in enriched (EE: males n=11, females n=14) and standard housed (SH: males n=16, females n=21) environments. Error bars are ± SEM.

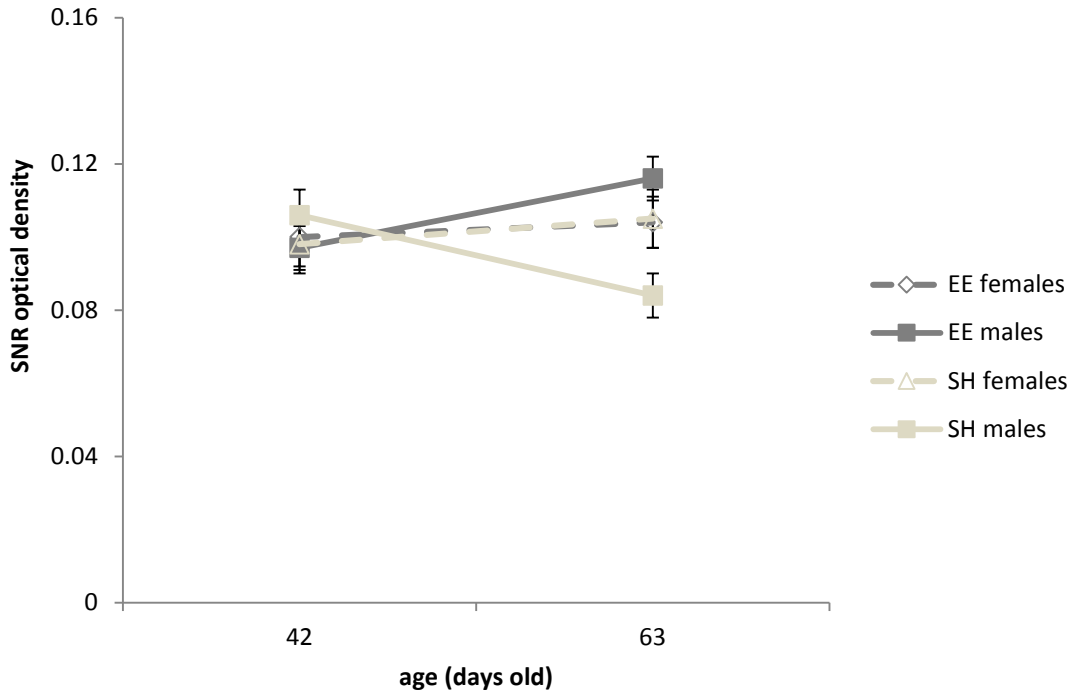

Figure 3-5. The effect of housing on neuronal activation in the substantia nigra pars reticulata (SNR) at two developmental time points for deer mice reared in enriched (EE: males n=11, females n=14) and standard housed (SH: males n=15, females n=20) environments. Error bars are ± SEM.

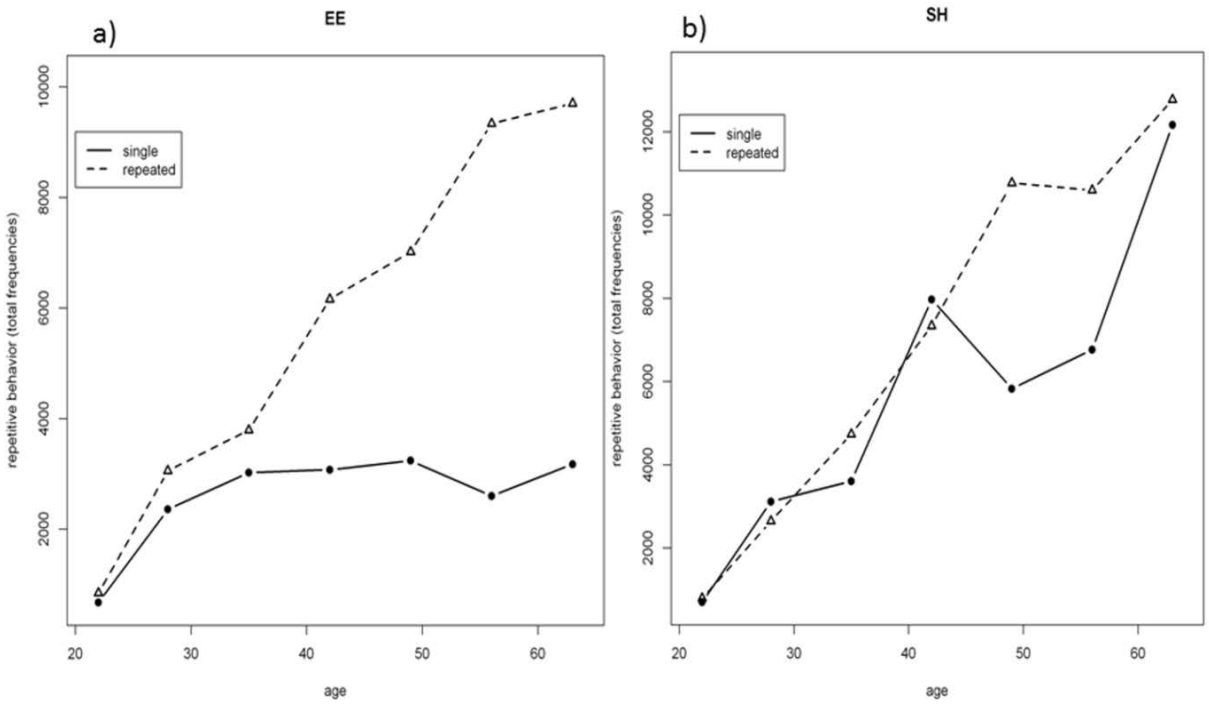

Figure 3-6. The effect of repeated testing on repetitive motor behavior development for deer mice reared in (A) enriched (repeatedly tested EE: n=40, single tested EE: n=93) and (B) standard environments (repeatedly tested SH: n=19, single tested SH: n=123). Data values are back-transformed mean square root repetitive behavior frequencies.

CHAPTER 4
MOLECULAR UNDERPINNINGS OF REPETITIVE MOTOR BEHAVIORS: A PROTEOMIC APPROACH

Repetitive motor behaviors are invariant sequences of behavior that are seemingly functionless. These abnormal behaviors are most strongly associated with autism spectrum disorder (ASD), for which they are diagnostic, but are prevalent across many neurodevelopmental, neurological and neuropsychiatric disorders (e.g. intellectual and developmental disabilities, schizophrenia, fronto-temporal dementia, Alzheimer's and Parkinson's diseases). Repetitive behavior development is also induced by barren rearing environments, in both humans (Fazzi et al., 1999; Rutter et al., 1999) and animals (Mason et al., 2007).

The practice of housing animals in highly impoverished environments consistently results in high levels of aberrant repetitive behaviors that are often ameliorated by implementation of environmental enrichment (EE) (Mason et al., 2007). The EE paradigm exerts a cascade of positive effects on behavior and environmentally mediated changes on brain function, including increased synaptic plasticity, dendritic branching and spine densities, neurogenesis, and delays or even prevention of disease onset (Lewis, 2004; Mason et al., 2007; Nithianantharajah & Hannan, 2006).

In both human and animal research, findings suggest dysfunction of cortico-basal ganglia circuitry is mediating the development of repetitive behaviors. The basal ganglia include the striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra (SN) that function mainly via a direct and indirect pathway. The sensory-motor and associative cortical areas project to the striatum, which sends projections either directly to the output nuclei, the SN pars reticulata (SNR), or indirectly, via the GP and STN. Direct pathway neurons promote appropriate action selection, whereas indirect pathway

neurons suppress competing actions. A third largely understudied hyperdirect pathway connects the cortex directly to the STN, and is thought to modulate response inhibition in conflict situations (Jahfari et al., 2011). Perturbations in the coordinated actions of these pathways have been associated with the development of repetitive behaviors (Graybiel, 2000; Lewis, 2007).

In deer mice (*Peromyscus maniculatus*), rearing in standard laboratory cages induces high levels of repetitive motor behavior (Muehlmann et al., 2015), whereas rearing in EE cages arrests the development of repetitive behaviors (Bechard, Bliznyuk, Lewis, subm.). Moreover, low levels of repetitive behaviors induced by EE housing have been associated with increased neuronal activation and dendritic spine density in the STN (Bechard et al., 2016), a nucleus on the indirect basal ganglia pathway.

The molecular mechanisms underlying the morphological and functional differences in the indirect basal ganglia pathway mediating the expression of repetitive behaviors are not known. Thus, we sought to identify novel protein candidates and accompanying molecular pathways that mediate early EE effects on repetitive motor behavior development using two mass spectrometry based methods of proteomic profiling. A proteomics approach to identifying differences in protein expression was employed as this powerful tool can assess very large numbers of proteins simultaneously. This is a particular strength in the present case as *a priori* hypotheses about specific genes or their protein products that mediate repetitive behaviors are lacking. Proteomic approaches have been used previously to identify biomarkers of ASD, implicating differences in apolipoproteins and complement proteins in serum of children with ASD compared to controls (Corbett et al., 2007), and myelin-related

proteins, both in post-mortem cortical tissue of children with ASD (Broek et al., 2014) and a mouse model (Wei et al., 2016). Biological markers specific to repetitive behavior have, to date, not been identified.

Targeted searches for the molecular underpinnings of repetitive motor behaviors in animal models have mostly employed genetic mutations of candidate genes. For example, mutations in the methylCpG binding protein 2 (MECP2) are implicated in Rett syndrome, and mice with this genetic mutation perform repetitive forelimb behaviors similar to those seen in human patients (Moretti et al., 2005). A hyper-grooming phenotype is inducible in mice via changes affecting the homeobox protein Hox-B8 (Greer and Capecchi, 2002). Mutations in regulatory molecules of excitatory synaptic structure and function, such as the scaffolding protein SAPAP3, have emerged as potential mediators of repetitive motor behaviors (Welch et al., 2007). In support, mutations in ProSAPs/SHANK genes encoding another postsynaptic scaffolding protein family enriched at excitatory synapses have recently been associated with some human cases of ASD and intellectual and developmental disabilities (Berkel et al., 2010; Qin et al., 2009; Sato et al., 2012), and mouse models with these genetic mutations display repetitive behaviors (Schmeisser et al., 2012; Wang et al., 2011; Won et al., 2012; Yang et al., 2012). Neuroligins, a family of postsynaptic cell-adhesion molecules that associate with presynaptic neurexins to regulate synaptic maturation, have been implicated in ASD, as neuroligin 1 deficient mice show an over-grooming phenotype that can be rescued with a NMDA receptor agent (Blundell et al., 2010). The Grin1 (glutamate receptor, ionotropic, NMDA1) gene mutation has also been linked to repetitive behaviors in mice (Moy et al., 2008, 2014). Repetitive behaviors also

accompany progressed stages of Huntington's disease (e.g., chorea); a disorder caused by an expansion of repeat CAG chains within the HTT gene, which then generates the abnormal Htt protein responsible for damaging the brain. Transgenic mouse models of Huntington's develop an early (~2mo) motor phenotype characterized by increased rearing at night, which, by 4-6 months, progresses into decreased locomotion and associated decreases in striatal enkephalin; all of which precede the presence of mutated Htt protein microaggregates in striatal neurons (Menalled et al., 2002). Although Htt is necessary for development, known to interact with several other proteins, including upregulation of brain-derived neutrophic factor (BDNF), and associated with vesicles and microtubules (Hoffner et al., 2002), its exact function is still unclear.

We aimed to identify changes in the proteome mediating the attenuation of repetitive behavior development by EE. To this end, we generated animals with low levels of repetitive behaviors by rearing in EE housing and compared their STN proteome to those of standard reared animals with high levels of repetitive motor behaviors. Two methods of approach for mass-spectrometry proteomic analysis were employed: a modified stable isotope labeling by amino acids in cell culture (SILAC) (Study 1) and a label-free (Study 2) approach. We hypothesized that differences in repetitive behavior would be associated with protein expression differences in the STN. Moreover, we sought to elucidate novel proteins that mediate the development of repetitive behaviors and associated molecular pathways. Identifying novel protein candidates and pathways important in mediating repetitive behaviors will provide new targets for pharmacotherapies which are currently lacking.

Materials and Methods

Animals

All animals and procedures were approved for use by the University of Florida's IACUC and followed the guidelines of the NIH use for animal care. Deer mice (*Peromyscus maniculatus*) were housed in a colony room maintained at 16:8 light:dark cycle, 20-25°C, and 50-70% humidity. Subjects were born to monogamous breeding pairs housed in standard cages (48 x 27 x 15 cm). At 21 days of age, litters were weaned such that siblings were split into both standard and EE housing. All mice had ad libitum access to food, water and nest building materials, and their cage bedding (SaniChip) refreshed every two weeks. In addition to the standard provisions, EE mice belonged to larger social groups (EE: n=6 versus SH: n=3) with greater territory (122 x 81 x 89 cm), as EE cages were large dog kennels modified by galvanized wire to have three tiers interconnected by ramps. A variety of objects (e.g. toys, tunnels, mouse houses) were rotated weekly to promote novelty as well as complexity, and a running wheel provided an additional opportunity for exercise. To encourage foraging, we scattered birdseed (~2oz) throughout the kennel weekly. All subjects remained in their assigned housing condition for 6 weeks post-weaning.

Repetitive Behavior Assessment

Repetitive motor behaviors in deer mice manifest as vertical activities (hind limb jumping and backwards somersaulting) that can be automatically quantified using software that records each time there is a break in a photo beam array. The beams are positioned high enough to selectively capture vertical jumps, and video recordings of each session ensured the accuracy of the automated counts. At 6 weeks post-weaning, mice were assessed for the total frequency of repetitive behavior occurring across one

entire 8 h dark period. Mice were placed into individual testing chambers (22 x 28 x 25 cm) furnished with bedding, food and water, at least 30 min before the start of the test.

The total frequency of repetitive behaviors for each mouse was calculated by summing the number of jumps that occurred across the 8 h test. The mean frequency of repetitive behaviors for each STN sample was then calculated by averaging the total frequencies of the six mice that contributed to the pooled sample. Differences in mean repetitive behavior frequencies between high standard housed and EE-induced low repetitive behavior mice were assessed using a GLM (SPSS v23) with group as a factor in the model.

Proteomic Profiling

Study 1: a super-SILAC approach

Mice were anaesthetized with isofluorane and their brains rapidly removed, snap-frozen in isopentane, and stored at -80°C. Basal ganglia nuclei were subsequently sectioned using a bilateral microdissection-by-punch technique from 300µ coronal slices sliced on a cryostat set at -10°C. The STN proteomic profiles of standard (n=30) and enriched (n=30) mice were compared for differences. Due to the extremely small size of the nucleus, we pooled the STN from six mice of like sex, housing condition and repetitive behavior levels into one biological sample. In the end, there were n=5 (2 male, 3 female) STN samples from standard housed mice with high levels of repetitive behavior and n=5 (2 male, 3 female) STN samples from enriched mice with low levels of repetitive behavior subjected to mass spec analysis.

Samples were analyzed using LC-MS/MS at the Florida Center of Excellence for Drug Discovery and Innovation, USF. A C57BL6 male MouseExpress (*Mus musculus*) SILAC-labeled (^{13}C6 Lysine) brain, incorporation 98%, was acquired (Cambridge

Isotope). The brain was lysed in 4% SDS, 100 mM Tris-HCl, pH 7.6, 100 mM dithiothreitol at 95 °C for 5 min prior to probe sonication and clearance of lysate in a microcentrifuge at 15,000 x g for 5 min. The SILAC-labeled brain lysate (i.e., spike-in internal standard) was aliquoted and stored at -80 °C. Tissue samples were put in a cold room and placed into low-retention 1.5 ml centrifuge tubes prior to storage at -80 °C. Samples were thawed on ice and spun at 500 x g for 5 s to bring all tissue punches to the bottom of the tube. Twenty-six µl of lysis buffer containing 4% SDS, 100 mM Tris-HCl, pH 7.6, and 100 mM dithiothreitol were added to the samples prior to incubation for 5 min at 95 °C. No mechanical homogenization or probe sonication was employed. Samples were then spun at 15,000 x g for 5 min to clear lysates. Protein concentration for both the internal standard and STN samples were measured using the 660 nM Protein Assay with Ionic Detergent Compatibility Reagent as described above. SILAC-labeled internal standard was added to samples in a ratio of 2:1, ensuring total volume did not exceed 30 µl and total protein mass did not exceed 300 µg, which is the maximum allowed for FASP digestion. FASP filters were pre-wetted with 200 µl of 8 M Urea and spun for 10 min. Proteins were buffer exchanged in microcentrifuge spin columns from the detergent laden lysis buffer first into chaotropic 8 M urea for alkylation of reduced cysteine residues before additional buffer exchange into 50 mM ammonium bicarbonate for sequence-grade trypsin digestion at a ratio of 1:100 enzyme to protein overnight (Wisniéwski, 2009). Peptides were desalted using solid phase C18 columns and speedvaced prior to fractionation on an offline strong cation exchange column. Samples were fractionated by charge for additional protein depth into 6 fractions weighted by unique peptide load. Fractions were centrifuged under vacuum until

dryness and then resuspended in 0.1% formic acid in water for inline reversed-phase liquid chromatography separation and high-resolution mass spectrometric analysis on a hybrid linear ion trap-Orbitrap mass spectrometer (Orbitrap XL, Thermo Fisher Scientific).

Spectra were analyzed using the MaxQuant analysis suite employing constant modification of carbamidomethyl cysteine and variable methionine oxidation. A reverse concatenated reference database for *Mus musculus* from Uniprot was searched in order to establish a false discovery rate of 1% for peptides and proteins. The median peptide level across all peptides for a protein group across all biological replicates was reported as the protein level to improve proteome depth. Due to possible noise introduced by the ratio-of-ratios, proteins had to have a minimum of two ratio counts and two independent peptides, to be considered. Perseus, a statistical processing suite included with MaxQuant, was used to establish significance at a $p<0.05$ using the outlier test, SigA. Significant protein ratios were then input into Ingenuity Pathway Analysis to investigate pathway enrichment.

Study 2: a label-free approach

Brain tissue was processed using Study 1's methods. Briefly, mice were sacrificed after completion of the repetitive behavior assessment, brains harvested and stored at -80°C, and subsequently, regions were isolated using a microdissection-by-punch technique. The STN proteomic profiles of standard housed (n=16) and enriched (n=16) mice were compared for differences. Due to the extremely small size of the nucleus, we pooled the STN from four mice of like sex, housing condition, and repetitive behavior levels into one biological sample. In the end, there were n=4 (2 male, 2 female) STN samples from standard mice and n=4 (2 male, 2 female) STN samples

from EE-induced low repetitive behavior mice subjected to mass spec analysis at the Florida Center of Excellence for Drug Discovery and Innovation at USF. Samples were processed using the SDS lysis procedure described above. Given the smaller amount of protein obtained by using less STN tissue (<50 µg) and possible sample loss that could occur on the FASP filter, 5µg of total STN lysate from each replicate and group were separated by 1D SDS-PAGE. After staining with Coomassie to visualize protein bands, the gels were cut into three molecular weight fractions that span the entire gel lane. The gel lane fractions were destained and proteins were then reduced and alkylated in-gel using DTT and iodoacetamide, respectively. After reduction and alkylation, proteins in each fraction were digested in-gel with trypsin overnight at 37°C. Peptides were then extracted, dried down and resuspended in 0.1% formic acid in water and analyzed by LC-MS/MS using a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific). Mass spectrometric data was searched similar to as described above, however, this time we implemented label-free quantitation (LFQ) in MaxQuant. LFQ intensity values (with a minimum ratio count of two) were used for relative quantitation of protein expression in the STN samples from standard and EE housed mice. Relative quantitation was performed by calculating the median LFQ intensity for the reference group and then calculating a ratio for each individual replicate in the comparison group. Ratios were calculated only for proteins in which non-zero values were observed in at least two out of four samples within each experimental group. The SigA outlier test in Perseus was then used to determine statistically significant proteins ($p<0.05$) within each experimental group replicate. Differentially

expressed proteins that were significant in at least two out of the four replicates were input into Ingenuity Pathway Analysis for bioinformatic analysis.

Study 2's label-free approach to proteomic profiling differs from the spike-in SILAC approach employed in Study 1 in that protein abundance profiles are generated using all of the detectable MS features (i.e. the extracted ion currents (XICs) of peptides). The initial processing of the sample did not use the spike-in SILAC internal standard in order to calculate ratios for relative quantitation. In Study 1, however, the mouse brain standard used as a spike-in standard was labeled with stable isotope-labeled lysine rather than double labeled with lysine and arginine, which resulted in only lysine-terminated tryptic peptides that could be used for relative quantitation. The reduction in quantifiable peptides resulted in a significant decrease in quantitation confidence because fewer peptides for a particular protein are used to generate the protein ratio. Another limitation of Study 1 is the difference in proteome composition of the STN compared to the whole brain protein extract used as the internal standard. For STN proteins that are at much higher or lower abundance compared to the whole brain profile, the peptide pair intensity differences could be outside a range for accurate quantitation even after the ratio-of-ratio calculations. Additionally, it is possible that a small number of peptide pairs would not be detected given amino acid differences that are present between *Mus musculus* and *Peromyscus maniculatus*. This sequence difference could result in skewing of protein ratios values that are derived from the individual ratio values of the peptides if detectable features are present in the expected m/z range that would result in erroneous ratio values. This effect could be exacerbated for low abundance proteins where the number of peptides used for quantitation is

smaller. In comparison, the label-free approach allowed for relative quantitation of both arginine and lysine-terminated peptides and only considered peptides for quantitation that are identical to sequences in the *Mus musculus* database. Moreover, Study 2 used instrumentation with improved analytical performance which included ultra-performance liquid chromatography (UPLC) and a hybrid quadrupole-Orbitrap (Q Exactive Plus) compared to conventional HPLC coupled to a hybrid linear ion trap-Orbitrap (Orbitrap XL) instrument that was used in Study 1. The improved analytical performance resulted in much greater depth of proteome coverage without the need to carry out extensive fractionation. Improvements in label free quantitation features in MaxQuant, including normalization to obtain LFQ values, have allowed quantification with fairly high accuracy (Cox et al., 2014).

Ingenuity Pathway Analysis (IPA)

Pathway analysis helps to interpret the large amount of molecular data in the context of biological processes and pathways, and promotes a more global perspective of the data by identifying molecular relationships. These downstream effects analyses further generate an activation z-score based on the predicted direction of effects. IPA uses z-scores to calculate statistical significance of activated and inhibited genes, and intersects these with sets of genes associated with a particular biological function or pathway (Kramer et al., 2014). The p-values associated with functional and pathway analyses are a measure of the significance of overlap in observed genes and those associated with a particular disorder, and are generated using the right-tailed Fisher Exact Test. IPA also identifies upstream regulators based on prior knowledge of expected effects between transcriptional regulators and their target genes. This causal analysis similarly generates an overlap p-value and an activation z-score. Here, the

overlap p-value compares dataset genes with known targets of the transcriptional regulator for overlap, with the purpose of identifying transcriptional regulators that explain observed gene expression changes. As a note, it is unlikely that any of the chemical reagents identified as upstream regulators are actually present in the mice (with the exception of genistein, due to soybean being a main ingredient in our rodent chow); however, their identification does implicate a potential impairment in the related molecular pathways.

Results and Discussion

Study 1

Repetitive behavior of standard and enriched mice

The STN samples (n=5 high, n=5 low) subjected to super-SILAC proteomic profiling represented two groups of mice differing in levels of repetitive behavior as a consequence of rearing environment, with significantly less repetitive behavior displayed by EE reared mice ($F(1,8)=26.1$, $p=0.001$; see Fig. 4-1). This result aligns with several previous studies of EE effects on repetitive motor behaviors in deer mice (Turner et al., 2002; Bechard et al., 2016; Bechard and Lewis, 2016).

Differentially expressed STN proteins in standard versus enriched mice

Mass spec analysis identified 3439 proteins in the STN, of which 250 were significant, and 85 of these were differentially expressed at a 2-fold difference or greater. Of these 85 proteins, there were 28 upregulated, and 57 downregulated proteins in the standard versus enriched group comparison (see Table 4-1). The top proteins upregulated included ryanodine receptor 2 (cardiac) (RYR2, 17.5 fold increase), which is involved in calcium ion binding; synaptotagmin (SYT12, 10.1 fold increase), that functions in calcium-dependent exocytosis and binding of syntaxin, and

is important for long-term synaptic potentiation, synaptic vesicle endocytosis and regulation of neurotransmitter secretion; and, succinate dehydrogenase complex (SDHC, 10.1 fold change), which plays a role in the mitochondrial electron transport chain. Apolipoprotein D (ApoD) was also highly upregulated in standard mice with high frequencies of repetitive behaviors (4.6). ApoD is a protein associated with neurological disorder and myelin related nerve injury, and increased levels have been found in patients with schizophrenia, bipolar disorder and Alzheimer's disease (AD) (Muffat and Walker 2010). Expression of the regulatory subunits (PPP2R2A and PPP2R5A) of protein phosphatase 2 (PP2) was increased in standard mice with high levels of repetitive behavior. PPP2 has been suggested as a potential drug target for Parkinson's and Alzheimer's diseases, although which isoform and direction of expression for the best therapeutic effects are unknown (Braithwaite et al., 2012; Sontag and Sontag, 2014).

The regulatory subunits 1A (PPP1R1A) (-2.1 fold change) and 1B (PPP1R1B) (-2.2) of protein phosphatase 1 (PP1) showed decreased expression in the STN of standard mice with high levels of repetitive behavior. PP1 is important in many basic biological functions, including muscle contraction, protein synthesis, cell division, apoptosis, and regulation of membrane receptors, and reduced activity in both grey and white matter has been found in AD patients (Gong et al., 1993). Apolipoprotein E (apoE) is known for lipoprotein metabolism and association with cardiovascular disease. In mice, apoE deficiency leads to profound susceptibility to atherosclerosis (Getz and Reardon, 2016). More recently, it has been implicated in immune regulation, oxidation, and AD (Sando et al., 2008). We found decreased expression of apoE (-2.6), and

apolipoprotein A-1 (apoA1) (-1.6), important in beta-amyloid, lipoprotein, phospholipid and cholesterol binding, in standard reared mice with high repetitive behaviors compared to low repetitive behavior mice reared in EE housing. Moreover, apolipoprotein 0 (apoO) (-1.6) was down-regulated in high repetitive behavior mice. ApoO localizes with mitochondria and enhances mitochondrial uncoupling and respiration to the extent that it has been suggested to promote lipotoxicity of the heart (Turkieh et al., 2014). Mitochondrial intermembrane chaperone proteins, TIMM10 (-6.5) and TIMM44 (-2.6), were also downregulated in the STN of mice with high levels of repetitive behavior. Reduced expression of cytochrome c oxidase subunits, COX-7A2 (-1.7) and 6B1 (-1.7), a mitochondrial membrane protein indicative of neuronal metabolic capacity, corroborate a series of histochemistry studies showing decreased activation of cytochrome oxidase in the STN of standard-reared mice with high levels of repetitive behavior compared to the STN of EE mice with low levels of repetitive behaviors (Tanimura et al., 2010a; Bechard et al., 2016). The greatest downregulated STN protein in high versus low repetitive behavior mice was Ces1b/Ces1c (-10.2). Carboxylesterase 1c has been linked to the binding and stabilization of evorolimus, an orally active inhibitor of mTOR used in cancer therapy and as an immunosuppressant (Tang et al., 2014).

Upstream Regulators

IPA upstream regulator analyses identify the cascade of upstream transcriptional regulators that may explain the observed protein expression changes in the molecular data set. IPA predicts which transcriptional regulators are involved with the proteins in the data set and whether they are activated or inhibited. Two upstream regulators were predicted as activated (i.e. activation z-score ≥ [2]), and six upstream regulators were

predicted as inhibited (see Table 4-2). PML (z=2.4, p=6.1E-09, 9 proteins) was identified as a significant and activated upstream regulator based on 9 proteins in the data set. Due to its regulatory role in calcium homeostasis of the endoplasmic reticulum, of PPAR signaling, and sequestering of MTOR, promyelocytic leukemia protein (PML) services numerous important biological functions, such as tumor suppression, transcriptional regulation, apoptosis, and viral defense. The identification of the chemical drug, methapyrilene (z=2.2, p=3.97E-08, 12 proteins), was based on 12 proteins in the data set. The administration of this drug has been linked to hepatoxicity, due to the associated expression of genes related to oxidative stress in the liver of rats (Leone et al., 2014). The top upstream regulators with predicted inhibition included two transcription regulators: x-box-binding protein 1 (XBP1) (z=-2.2, p=6.6E-02, 4 proteins) and heat shock factor protein 1 (HSF1) (z=-2.2, p=4.5E-02, 5 proteins). Many important functions and processes employ XBP1, such as response to oxidative stress, negative regulation of apoptosis, and positive regulation of histone modification and cell growth, and HSF1 is a promising drug target in cancer treatment. The predicted inhibition of beta-estradiol (z=-1.7, p=7.8E-07, 22 proteins), which also plays a role in apoptosis, neuroprotection against cell death, cancer progression, cell cycle and hormone binding, continued to implicate the underactivation of these biological processes in standard versus enriched mice.

The top upstream regulators identified based on highly significant p-values, but not activation scores, included the TP53 (p=2.9E-10, 43 proteins), a tumor suppressor and apoptosis inhibitor; HTT (p=2.9E-09, 28 proteins), important for microtubule-mediated transport, the apoptotic process and brain development, and highly

associated with Huntington's disease; RICTOR (p=2.6E-11, 17 proteins), a subunit of mTORC2 and important in cell growth and survival in response to hormone signals; and, PPARGC1A (p=5.6E-07, 13 proteins), that regulates key mitochondrial genes also involved in energy metabolism, oxidative stress, and cell death. Other identified upstream regulators with established and noteworthy roles in disease manifestation included FMR1 (p=1.4E-03, 4 proteins), linked to fragile-X mental retardation, ASD and Parkinson's disease; and amyloid precursor protein (APP) (p=5.8E-07, 27 proteins) and microtubule-associated protein tau (MAPT) (p=4.1E-06, 13 proteins), which are both strongly connected to AD pathology.

Pathways implicated in repetitive motor behaviors

The top canonical pathways implicated in the development of repetitive behavior were oxidative phosphorylation (p=5.4E-10, n=13/109 overlap i.e. 13 proteins in our data set/209 known proteins in pathway), mitochondrial dysfunction (p=1.6E-08, n=14/171 overlap), production of nitric oxide and reactive oxygen species in macrophages (p=1.0E-05, n=11/180 overlap), cardiac-adrenergic signaling (p=2.9E-05, n=9/133 overlap), and synaptic long term depression (p=4.9E-05, n=9/142 overlap; see Fig. 4-2). Evidence linking mitochondrial dysfunction, oxidative stress and inflammation in the brains of individuals with ASD has recently emerged (reviewed by Rossignol and Frye, 2014).

The top diseases and disorders implicated were neurological disease (n=95 proteins), psychological disorders (n=66 proteins), skeletal and muscular disorders (n=71 proteins), hereditary disorder (n=61 proteins), and metabolic disease (n=59 proteins). The top three diseases with predicted inhibition were in the category of neurological disease, and were related to degeneration of neurons (e.g. degeneration of

the nervous system, z=-2.9, p=2.6E-03; see Fig. 4-3). The diseases most implicated in repetitive behavior expression based on significance and predicted increased activation were mostly related to cell death and fell within the categories of cancer, cell death and survival, organismal injury and abnormalities, and tumor morphology (e.g. cell death of cancer cells: z=3.2, p=3.7E-03). Based just on significance, the top five diseases were in the category of neurological disease and included: movement disorders (p=4.4E-10, 46 proteins, see Fig. 4-4), neurological signs (p=5.1E-10, 34 proteins), disorder of the basal ganglia (p=3.7E-09, 36 proteins), chorea (p=5.0E-09, 30 proteins), and neuromuscular disease (p=5.6E-09, 39 proteins). In the category of cell death and survival, the pathways for apoptosis (p=1.5E-07, 77 proteins) and cell death (p=7.2E-07, 89 proteins) had high significance and overlap with the proteins in our data set. Within the category of behavior, pathways that had highly significant p-values but lower activation z-scores included behavior (p=5.3E-04, 30 proteins), learning (p=9.5E-03, 13 proteins), locomotion (p=6.6E-09, 22 proteins), vertical rearing (p=1.08E-03, 6 proteins), dyskinesia (p=7.0E-09, 31 proteins), Huntington's disease (p=1.8E-08, 29 proteins) and AD (p=2.5E-07, 26 proteins).

Study 2

Repetitive behavior of standard and enriched deer mice

The STN samples (n=4 high, n=4 low) subjected to label-free proteomic profiling represented two groups of mice differing in levels of repetitive behavior as a consequence of rearing environment, with significantly less displayed by EE reared mice ($F(1,6)=22.3$, $p=0.003$; see Fig. 4-5). This result again replicates a number of earlier studies showing EE reduces repetitive motor behaviors in deer mice (e.g. Bechard et al., 2016).

Differentially expressed STN proteins in standard versus enriched mice

Mass spec analysis identified 3200 proteins in the STN, of which 120 were significant, and 14 differentially expressed at a 2-fold difference or greater. Of these 14 proteins, there were 11 upregulated, and 3 downregulated proteins in the standard /EE mice group comparison (see Table 4-3). The top upregulated proteins included six that were entirely absent from the profile of the EE-induced low repetitive behavior group (and thus designated at a 10.0 fold change). For example, WDR11 (10.0) is involved in a number of cellular processes, such as cell cycle progression, signal transduction, apoptosis, and gene regulation, and has been implicated in gliomas and tumors. A protease that removes conjugated ubiquitin from target proteins and thereby inhibits protein degradation, USP11 (10.0), was also identified only in the STN profile of high repetitive behavior mice. Relatedly, the highly upregulated E3 ubiquitin ligase, HECTD3 (4.1), has also been implicated in a variety of cancers. Also linked to tumor invasion and metastasis was CTSB (2.0), which functions in intracellular degradation and turnover of proteins. A wide variety of diseases have been associated with elevated levels of CTSB, as it causes numerous pathological processes including cell death, inflammation, and production of toxic peptides.

The protein identified as most highly downregulated in the STN of standard housed versus enriched mice was NUMB (-8.2). NUMB plays a role in the determination of cell fates during development, and a loss of its expression has been demonstrated in several cancers, such as breast cancer. Downregulation of MYO9B, an actin based motor molecule that may service intracellular movements or remodeling of the cytoskeleton, was also noted. Finally, a subunit of the mitochondrial ATP synthase

(ATP5D) (-2.6) that functions in energy production was also downregulated in the STN of standard housed mice with high levels of repetitive behavior.

Upstream regulators

In Study 2, there were three activated, and one inhibited, upstream regulators identified by IPA (see Table 4-4). Genistein (z=2.5, p=6.7E-03, 7 proteins) was activated in the standard/EE STN group comparison. Genistein is an angiogenesis inhibitor and phytoestrogen belonging to the category of isoflavones, and common sources include soybean. It is a tyrosine kinase inhibitor and activator of all PPR isoforms (Wang et al., 2014). Due to its interaction with estrogen receptors, this chemical drug can induce effects resembling those of estrogen. It has been found to accelerate estrogen-dependent breast cancer (Ju et al., 2006) and induce apoptosis in testicular cells (Kumi-Diaka et al., 1998). In high doses, genistein is toxic to normal cells (Jin et al., 2007), and its use has been suggested to both treat adult leukemia (Raynal et al., 2008) and increase risk of infant leukemia when ingested during pregnancy (Spector et al., 2005). The activated cytokine, OSM (z=2.1, p=9.2E-03, 8 proteins), is an inflammatory mediator belonging to the group of interleukin 6 cytokines. The role of OSM is not well defined, yet it may be involved in liver, blood, CNS and bone development (Walker et al., 2010). A protein highly expressed during muscle atrophy and deficient in mice resistant to muscle atrophy (Gomes et al., 2001; Bodine et al., 2001), FBXO32 (z=2.0, p=1.3E-03, 4 proteins), was activated in the STN of mice with high levels of repetitive behavior. FBXO32 (also known as atrogin-1) has recently been suggested to regulate cell survival, and its silencing due to epigenetic mechanisms (i.e. methylation) has been implicated in certain carcinomas (Guo et al., 2014; Sukari et al., 2016). Lastly, the chemical reagent: 1,2-dithiol-3-thione (z=2.0, p=2.9E-02, 4 proteins) was identified as

activated in the STN of high compared to low repetitive behavior mice. Dithiolethiones indirectly inhibit the toxicity and carcinogenicity of many chemical carcinogens via induction of genes controlling antioxidant enzymes, and thus are of interest as cancer chemoprevention agents (Kensler et al., 1987, 1992). The only upstream activator predicted to be inhibited was a chemical drug, prednisolone (z=-2.0, p=3.9E-02, 4 proteins), a synthetic glucocorticoid and derivative of cortisol that is primarily used to treat asthma, but also inflammatory and autoimmune disorders.

The top upstream regulators based on the significance of the p-value of overlap but not activation scores included: HTT (p=1.9E-06, 16 proteins), APP (z=-1.7, p=9.0E-05, 15 proteins), BDNF (z=1.0, p=2.7E-04, 8 proteins), MAPT (p=5.5E-04, 7 proteins), and one chemical drug, topotecan (z=-1.0, p=7.8E-05, 7 proteins). Topotecan is a topisomerase inhibitor used as a chemotherapeutic agent in the treatment of certain cancers, such as ovarian and lung cancer. Experimentally, topotecan has been used to unsilence the paternal UBE3A gene in the treatment of Angelman's syndrome, a disorder caused by dysfunction of the expressed UBE3A maternal allele in neurons (Huang et al., 2012). Other notable upstream regulators identified included the tumor suppressor, TP53 (z=-0.9, p=1.9E-03, 17 proteins), and beta-estradiol (z=1.4, p=3.5E-02, 16 proteins).

Pathways implicated in repetitive motor behaviors

The top canonical pathways resulting in Study 2 were: RhoA signaling (p=1.0E-03, n=5/122 overlap), protein ubiquitination pathway (p=1.1E-03, n=7/255 overlap), axonal guidance signaling (p=1.6E-03, n=9/434 overlap), D-myo-inositol-5-phosphate metabolism (p=2.8E-03, n=5/145 overlap), and cardiac hypertrophy signaling (p=2.8E-03, n=6/223) (see Fig. 4-6).

The most highly implicated disease pathways in repetitive behavior development were neurological disease (50 proteins), developmental disorder (28 proteins), cancer (118 proteins), organismal injury and abnormalities (121 proteins), and reproductive system disease (63 proteins). Identified disease pathways implicated in repetitive behavior development based on predicted activation and significance of the p-value of overlap were largely related to infectious diseases, whereas those based on predicted inhibition and significance were mostly in the category of cell death and survival. For example, a predicted state of activation was found for the pathways of replication of Influenza A virus (z=3.1, p=6.0E-05, 10 proteins), viral infection (p=5.7E-04, z=3.1, 27 proteins), and replication of RNA virus (z=2.6, p=2.5E-03, 11 proteins). Other activated pathways included the development of the CNS (z=2.4, p=2.2E-05, 17 proteins), formation of the brain (z=2.0, p=2.1E-04, 13 proteins), and developmental process of the synapse (z=2.0, p=1.9E-04, 8 proteins). The disease pathway with the greatest predicted inhibition was organismal death (z=-4.1, p=3.9E-06, 42 proteins, see Fig. 4-7), followed by apoptosis (z=-2.1, p=3.7E-03, 36 proteins). The most significant p-values of overlap were associated with the categories of cell development and morphology, and nervous system development and function, and included pathways for the development of neurons (p=1.4E-10, 28 proteins), neuritogenesis (p=5.2E-10, 23 proteins), and formation of cellular protrusions (p=1.7E-07, 25 proteins). The pathways of synaptic depression (p=3.5E-07, 9 proteins), cognitive impairment (p=3.8E-07, 14 proteins), movement disorders (p=1.0E-05, 24 proteins) and transport of molecules (z=1.8, p=9.3E-07, 34 proteins) were also highly significant. Within the category of behavior were significant pathways for behavior (p=8.5E-07, 25 proteins), learning (p=1.1E-06,

15 proteins), locomotion (p=5.5E-03, 8 proteins), and vertical rearing (p=4.0E-04, 5 proteins).

General Discussion

Using a deer mouse model of repetitive behavior, we present the first study to complete a comprehensive proteomic analysis of the STN from standard housed mice with high levels of repetitive behavior and EE housed mice with low levels of repetitive behavior, which included quantitation of proteins both with and without isotopic labels. In Study 1, using a super-SILAC proteomic approach, we identified a number of significantly upregulated and downregulated proteins implicated in neurological disorders, cell growth, survival, and death, and metabolism. The top upstream regulators were also related to cell growth and cell death. Novel potential targets from this list for future pharmacological study include RICTOR and beta-estradiol. These functional categories are reflected in the implicated canonical pathways, such as those for mitochondrial dysfunction, production of ROS, and oxidative phosphorylation. Pathways involved in diseases mostly associated with CNS degeneration were found to overlap with our dataset and to be comprised of molecular relationships consistent with the predicted direction. Many genes implicated in disorders known to include a repetitive behavior phenotype, such as disorder of the basal ganglia, Huntington's, Alzheimer's and Parkinson's diseases, significantly overlapped with our dataset, although there were fewer consistencies with predicted relationships.

In Study 2, using a label-free proteomic approach, identified proteins were mostly involved in the cell cycle, such as cell development, division, and fate, as well as metabolism. These differentially expressed proteins are reflected in the identified canonical pathways for RhoA and axonal guidance signaling, protein ubiquitination, and

metabolism. The most highly implicated upstream regulators were also involved with cell growth, proliferation and death, and often related to cancer. However, neurological disease and developmental disorder preceded cancer when ranked by significance despite the cancer pathway having more proteins that overlapped with our data set. Interestingly, an inbred mouse model of repetitive motor behavior, the C58/J mouse, which has a similar repetitive behavioral trajectory as the deer mouse (Muelhmann et al., 2012, 2015), also develop leukemia at one year of age, are susceptible to diet-induced atherosclerotic aortic lesions, and lack interleukin-3 receptor (jax.org/strain/000669).

In comparing the results from Study 1 and 2, there were far fewer proteins quantified as differentially expressed at the level of a 2-fold change using Study 2's label-free approach, although the coverage was about the same for both studies. Moreover, only 11 proteins were identified in both studies as differentially expressed, and of these, 4 were inconsistent in the direction of fold change. The change in methods between Study 1 and 2 accounts for much of this non-convergence (see methods section for approach comparison), and notably, Study 2's label-free approach is the more conservative in its rate of false discoveries and quantitation estimates. This promotes the emphasis of a global comparison (i.e. upstream regulators and pathways) of the two studies, with significance (not activation) scores given priority. In this light, the low number of overlapping protein hits is outweighed by many of the implicated upstream regulators and disease pathways that were consistent across the two studies. Of the 15 upstream regulators identified as significant in Study 2, 11 were also identified in Study 1 (see Fig. 4-8). For example, HTT, TP53, APP and MAPT were all identified

as highly significant in Study 1 and 2. Identified as highly significant and inhibited in both studies was APP, which plays a role in synaptic formation and repair, but is known for its relationship as a precursor to beta-amyloid (the primary component of plaques) that is characteristically increased in AD pathology. Familial AD has been linked to gene mutations in APP that cause mismetabolism and beta-amyloid deposition, leading to tau phosphorylation and tangle formation, and eventually, cell death (Hardy and Allsop 1991; Citron et al., 1992). Although increased levels of APP occurs in normal aging, it is a decline in production and loss of a neuron's APP in proximity to mature plaques that services the pathology of dementia (Barger et al., 2008). More recently, a protective mutation in the APP gene against sporadic AD was identified (Jonsson et al., 2012), suggesting that, certain fragments of APP may be differentially influencing beta-amyloid production or the ratio of beta-amyloid isoforms (Saito et al., 2014). Several studies implicate cholesterol metabolism as a modulator of AD risk and pathogenesis (Wellington et al., 2004; Shobab et al., 2005), although inconsistencies between human data and most animal and cell studies, as well as other potential interacting factors such as genotype and age, have left this hypothesis in debate (Wood et al., 2014). The cholesterol-transport protein, apoE, was downregulated in Study 1. Certain allelic variants of human apoE are an established risk factor for AD (Stratman et al., 2005); however, cholesterol-rich apoE containing lipoproteins have also been suggested to bind to beta-amyloid, and promote its clearance and degradation (Trommer et al., 2005), so the story is not straightforward. Moreover, in Study 1 we identified activation of a potent liver X receptor (LXR) agonist, TO901317, which also has implications for AD pathology. Initially used in the treatment of diabetes, patients treated with such

cholesterol biosynthesis inhibitors were noted to have reduced prevalence of AD, an effect likely related to the role this chemical reagent plays in suppression of liver gluconeogenesis and increased insulin sensitivity, potentially, via the inhibition of reactive oxygen species (ROS) production and increased anti-oxidant gene expression (Dong et al. 2015). Also of interest for its role in characteristic AD pathology was the identification of MAPT, for which misfolding and aggregation is highly linked to the formation of neurofibrillary tangles, and PSEN1, which generates amyloid beta from APP.

Beta-estradiol was identified in both studies as a highly significant upstream regulator, although the direction of predicted activation was inconsistent. Beta-estradiol is a natural antioxidant of membrane phospholipid peroxidation suggested to have protective effects from oxidative-stress-induced cell death, and therefore has implications in aging-related dementia, such as AD (Behl et al., 1995). As such, we would hypothesize that beta-estradiol would be increased in EE-induced low repetitive behavior mice, which aligns with the results of Study 1, but not Study 2. Several upstream regulators that function in processes notoriously aberrant in cancer pathologies were identified in both Study 1 and 2, for example, TP53, MTOR, IGF1R, and the chemical reagent: 1,2-dithiol-3-thione.

The category of neurological disease was the most highly implicated in repetitive behavior expression in both Study 1 and 2 (see Fig. 4-9a). Within this category, dyskinesia, movement disorders, seizure disorder, disorder of the basal ganglia, and Huntington’s disease, all which are highly associated with repetitive behaviors, were implicated in both Study 1 and 2 (see Fig. 4-9b). Other highly significant and relevant

disease categories found in both Study 1 and 2 included cellular assembly and organization, cell morphology, cellular function and maintenance, and cell death and survival. Differences between Study 1 and 2 were found in lipid metabolism, molecular transport and small molecule biochemistry, which seemed to be highly implicated in Study 1, but much less so in Study 2. Study 1 also had several pathways identified related to free radical scavenging, whereas this category was absent in Study 2's results. Moreover, Study 2 had high activation scores for several pathways in the category of infectious disease (e.g. viral infection, z=3.1), whereas, in Study 1, only viral infection was identified, and at a lower activation score (z=1.3). There was a high convergence of disease pathways across the two studies, especially when ranked by significance. Aberrations in the generation, development, and morphology of neurons (including formation of cellular protrusions and dendritic growth/branching) were highly implicated in both studies, as were disorders known to involve the basal ganglia (e.g. movement disorders, disorder of basal ganglia, Huntington's disease, dyskinesia). These results support earlier studies of neuronal morphology in basal ganglia nuclei of standard and enriched mice, which showed increased dendritic branching, and spine densities in the striatum (Turner et al., 2003) and STN, of enriched mice with low levels of repetitive behavior (Bechard et al., 2016). Upon close examination, there are some diseases and functions that may be even more similar than at first glance. For example, degeneration of the nervous system was downregulated in Study 1, and although this was not a pathway specifically implicated in Study 2, neuronal cell death (z=-1.6, p=5.3E-04), and cell death of the brain (z=-1.0, p=3.6E-04) were, and clearly have mechanistic overlap. The relationship between apoptotic processes (mostly

downregulated in the STN of high versus low repetitive behavior mice) and cancer (mostly upregulated) also deserves further investigation for potential molecular overlap with those implicated in repetitive behavior.

The current findings also had an environmental factor at play, as our low repetitive behavior mice were generated in EE-housing. EE has far-reaching effects on the CNS, and so many of the proteins increased in our low repetitive behavior mice are likely due to exposure to a more complex early environment. A within housing study design comparing the STN of high versus low repetitive behavior deer mice may be useful for converging on candidate proteins involved specifically in spontaneous repetitive motor behavior development. We did not see expression differences in the proteins previously implicated in repetitive behavior by targeted mutation, however, the heterogeneous nature and numerous differentially expressed proteins suggests there are many ways to alter functioning of the circuitry underlying repetitive behaviors. Moreover, several proteins and pathways identified were involved in processes regulating synaptic maturation and function, which are the same functions targeted by ASD studies using specific mutations. One major limitation to the presented work is the lack of validation studies. Due to the extremely small size of the STN nucleus, there was not enough sample lysate following mass spec analysis for validation purposes. However, the change in methods implemented in Study 2 suggests something more than just replication, although undoubtedly, future studies will need to replicate the current findings and validate candidate proteins using an alternate method.

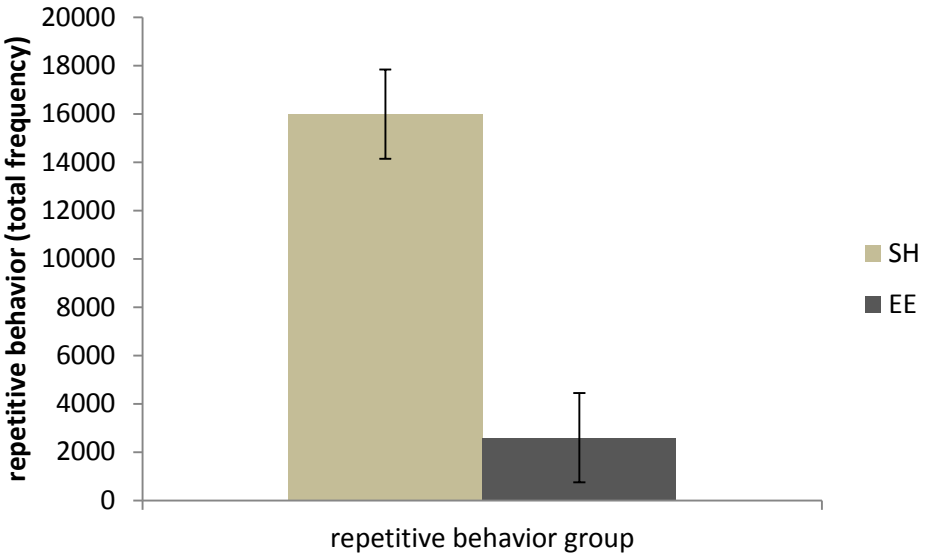

Figure 4-1. Shows mean repetitive behavior frequencies for biological replicates of standard-reared deer mice with high levels of repetitive behavior (n=5) and EE-reared mice with low levels (n=5) that were subjected to super-SILAC proteomic profiling.

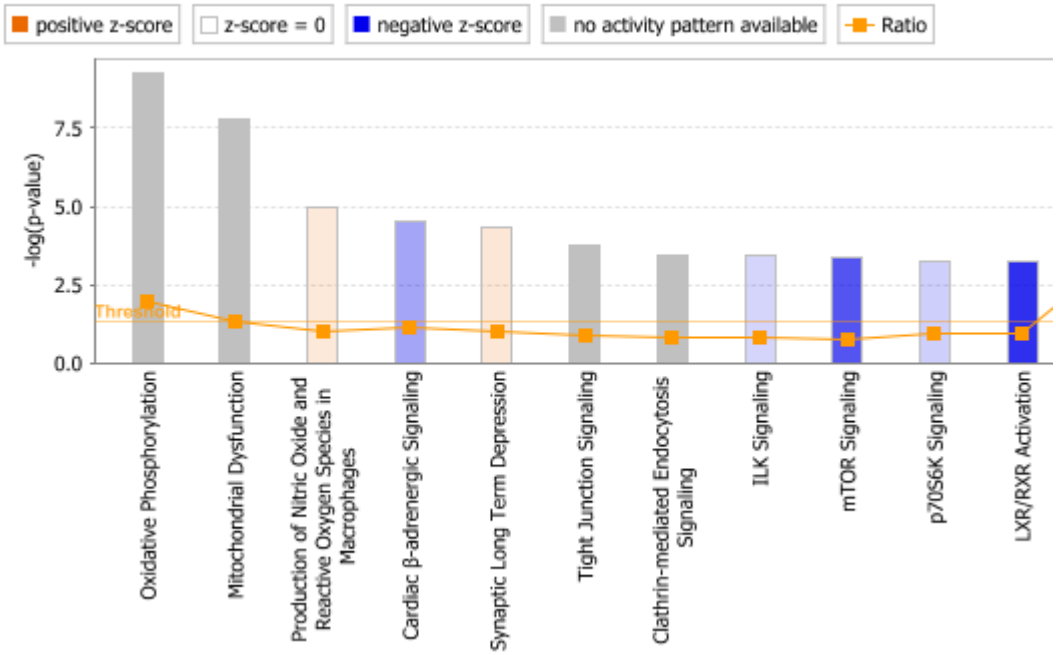

Figure 4-2. Shows a bar chart of the canonical pathways implicated in the development of repetitive behavior using a super-SILAC approach.

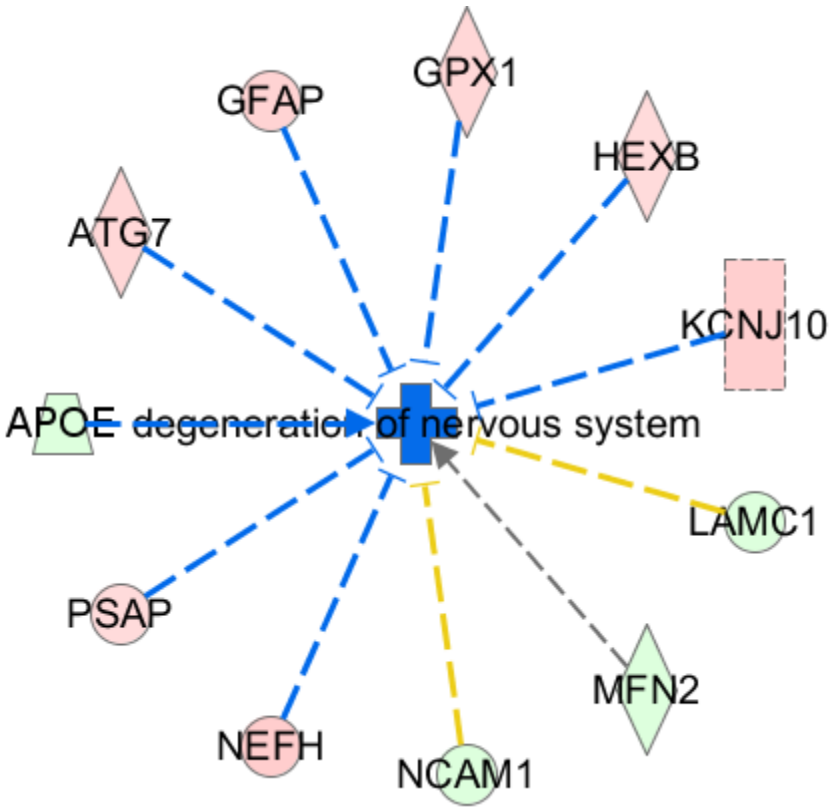

Figure 4-3. Shows the IPA generated pathway for the degeneration of the nervous system in the category of neurological disorders (z=-2.9, p=2.6E-03). Molecules in red indicate upregulated genes, whereas green indicate downregulated genes. The blue lines indicate predicted inhibition and grey lines indicate no relationship prediction. Dotted lines indicate indirect interactions and a solid line indicates direct action on the disease. The arrowheads indicate that A causes B to be activated and the flat ends indicate A causes B to be inhibited.

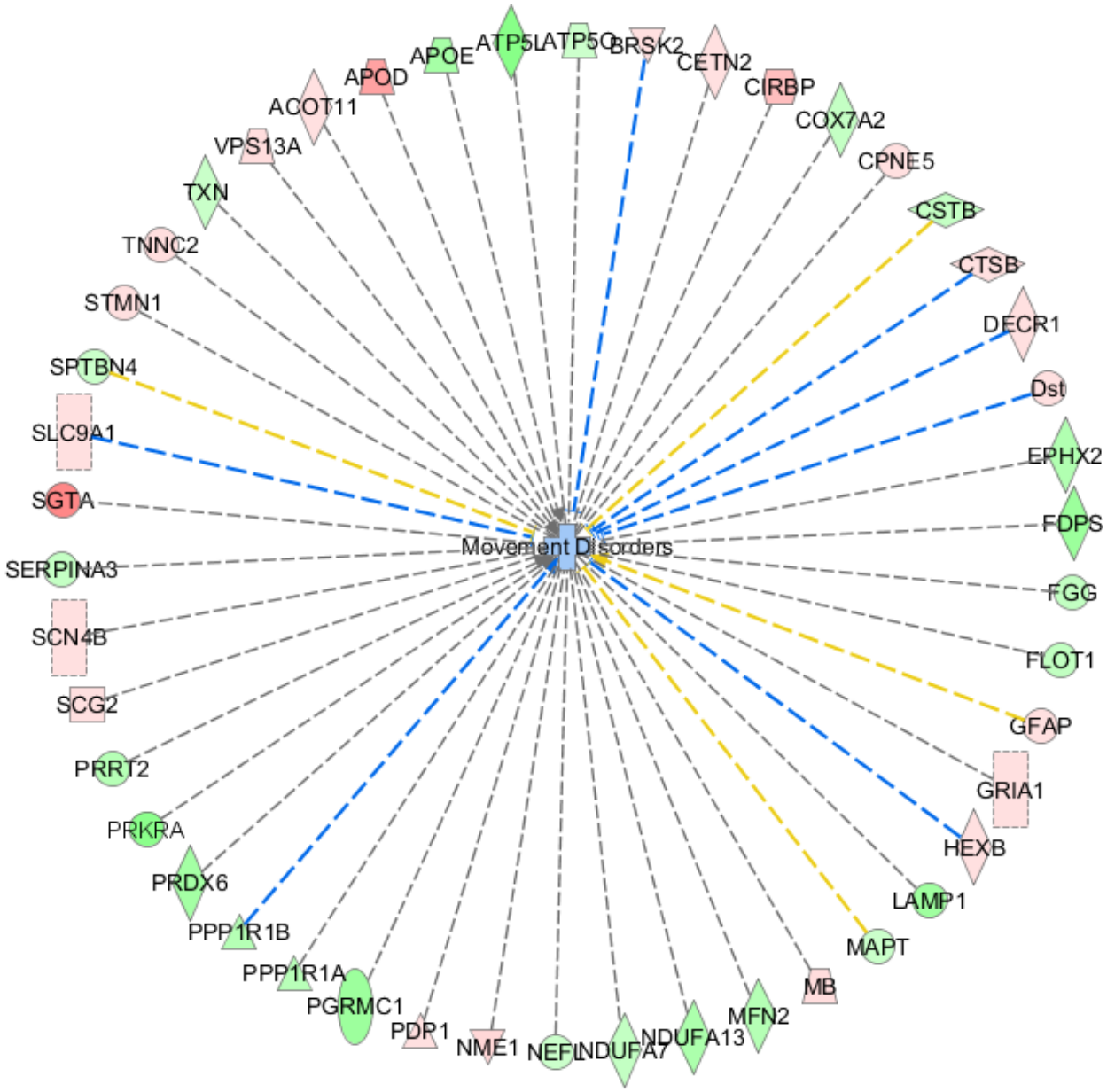

Figure 4-4. The pathway for movement disorders, in the category of neurological disease, was highly implicated in the STN of standard versus enriched mice group comparison (p=4.4E-10, 46 proteins). The red color indicates upregulated proteins, whereas the green color indicates downregulated proteins. The blue lines indicate predicted inhibition, yellow lines indicate a relationship inconsistent with that predicted, and grey lines indicate no relationship prediction. Dotted lines indicate indirect interactions and a solid line indicates direct action on the disease. The arrowheads indicate that A causes B to be activated and the flat ends indicate A causes B to be inhibited.

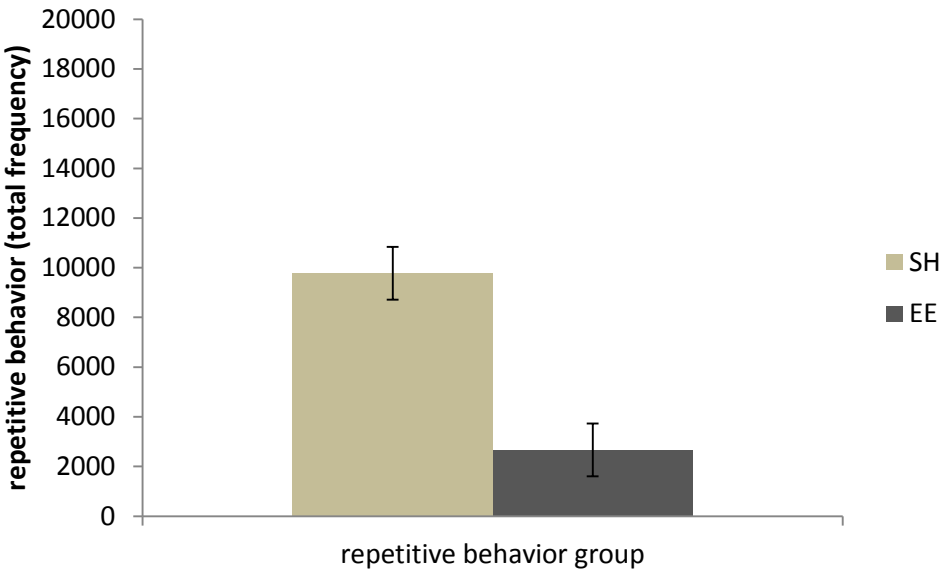

Figure 4-5. Shows mean repetitive behavior frequencies for biological replicates of standard-reared deer mice with high levels of repetitive behavior (n=5) and EE-reared mice with low levels (n=5) that were subjected to label-free proteomic profiling.

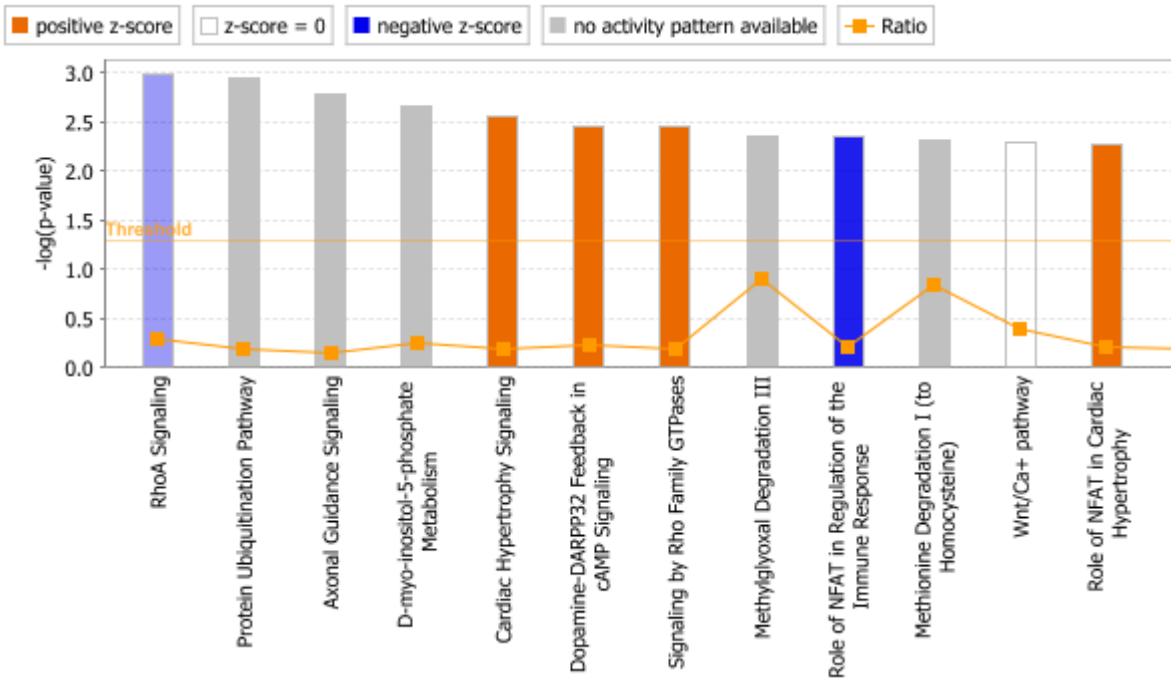

Figure 4-6. Shows a bar chart of the canonical pathways implicated in the development of repetitive behaviors using a label-free approach.

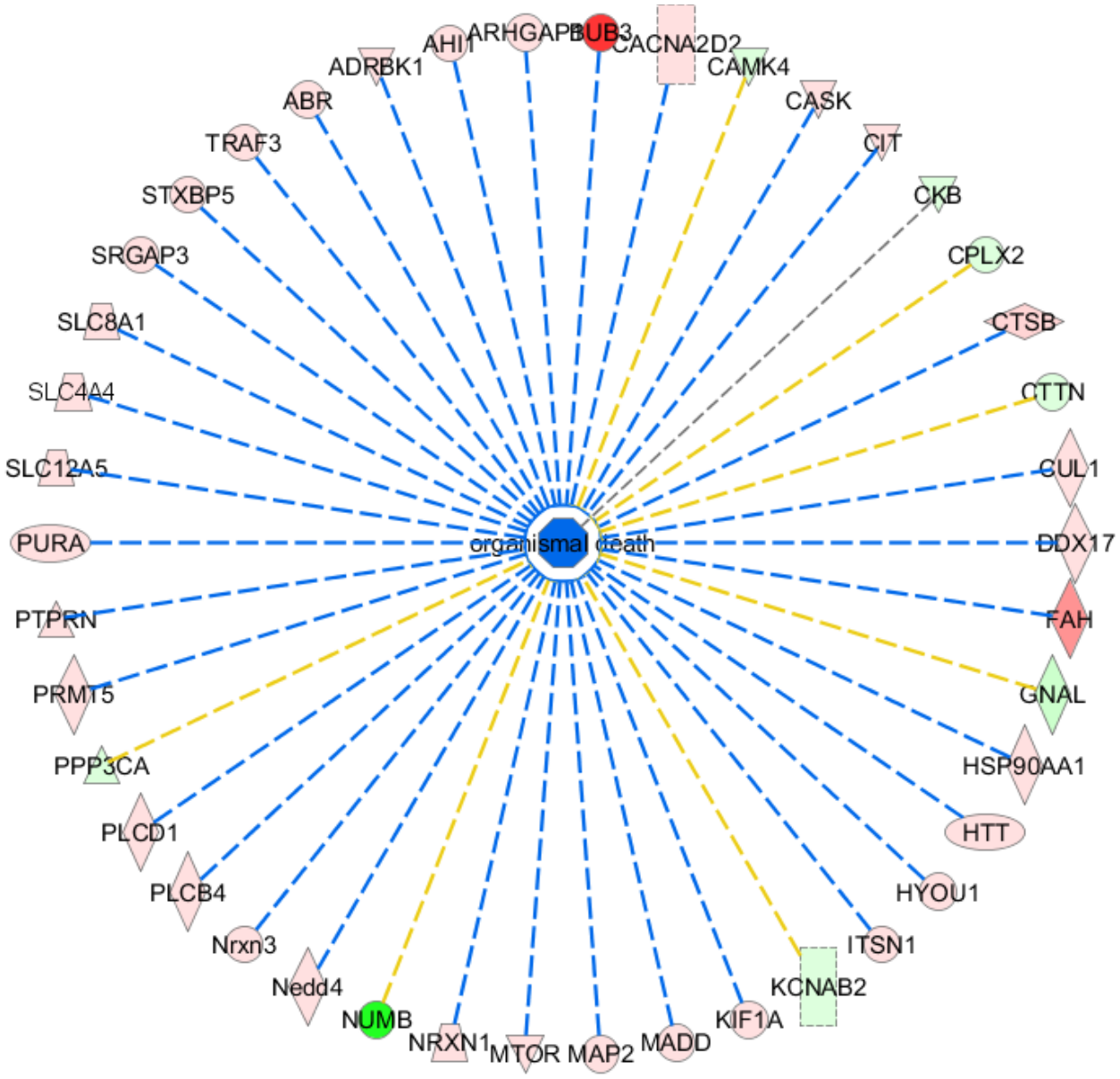

Figure 4-7. Shows the inhibited pathway for organismal death (z=-4.1, p=3.9E-06, 42 proteins) within the category of organismal survival identified in the standard/EE group comparison using a label-free proteomic approach.

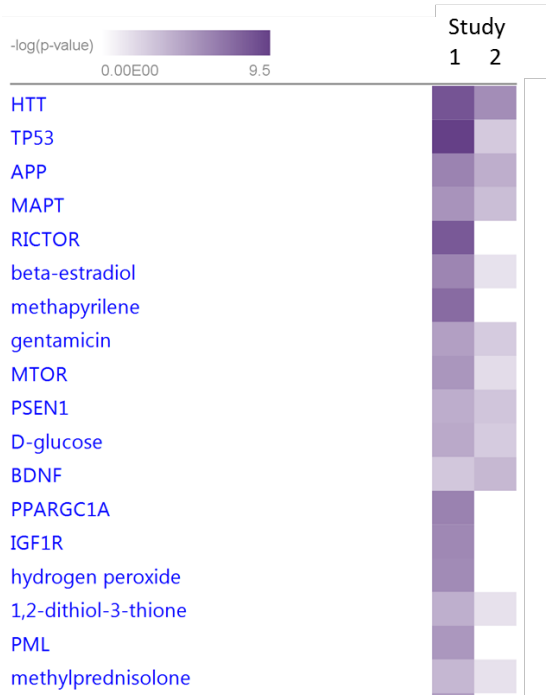

Figure 4-8. Shows the results of the comparison analysis for significance of upstream regulators implicated in repetitive behavior from Study 1 (super-SILAC) and Study 2 (label-free).

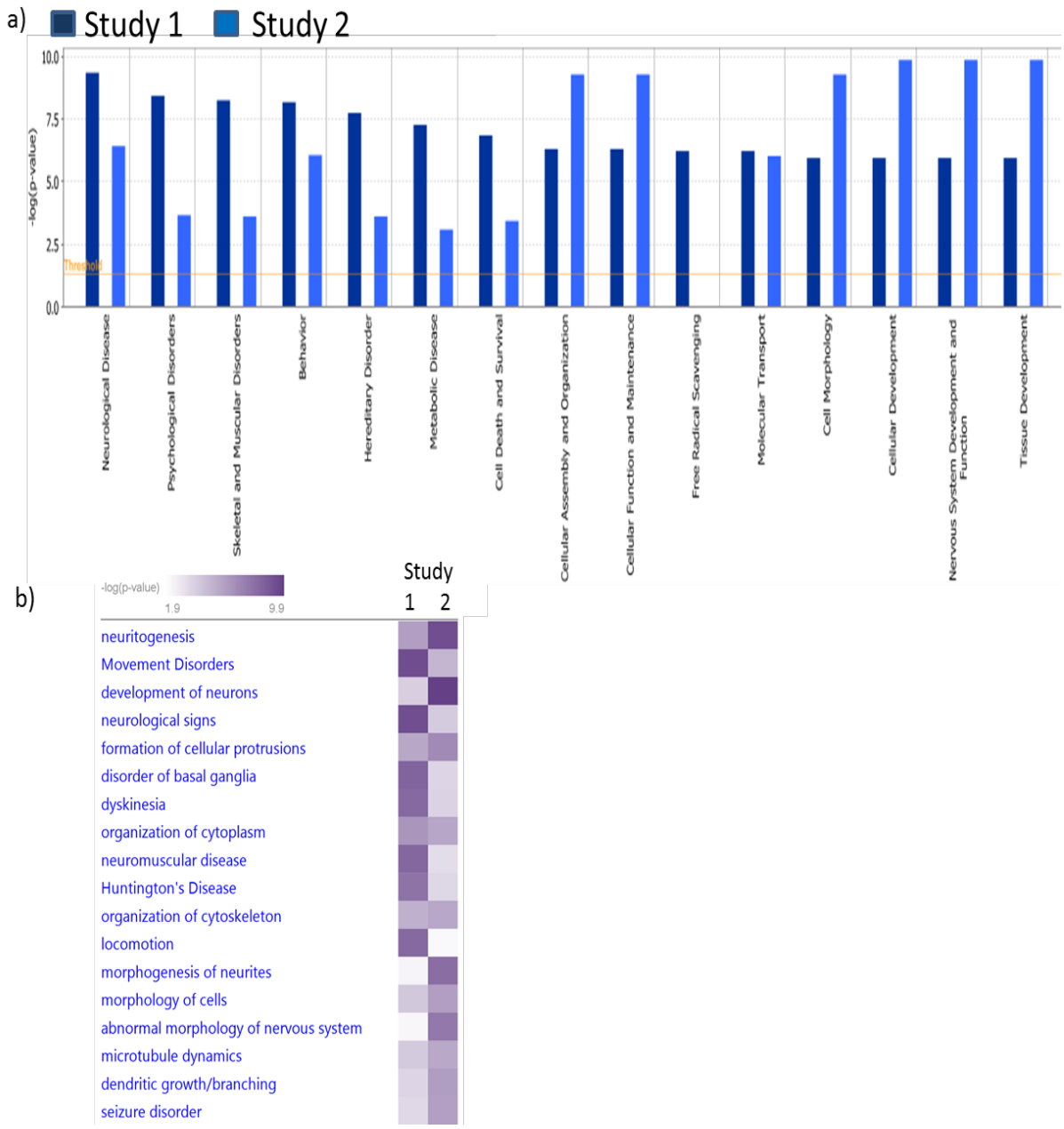

Figure 4-9. Shows the results of the comparison analysis for significance of a) disease categories and b) diseases and functions implicated in repetitive behavior from Study 1 (super-SILAC) and Study 2 (label-free).

Table 4-1. Lists the STN proteins differentially expressed at a 2-fold change or greater from the standard/enriched deer mice group comparison using a super-SILAC approach (Study 1).

Fold Change	ID	Symbol	Entrez Gene Name	Location	Type(s)
-10.2	P23953	Ces1b/Ces1c	carboxylesterase 1C	Cytoplasm	enzyme
-6.6	P62073	TIMM10	translocase of inner mitochondrial membrane 10 homolog (yeast)	Cytoplasm	transporter
-4.4	E9QN99	ABHD14B	abhydrolase domain containing 14B	Cytoplasm	enzyme
-4.3	Q9R1P3	PSMB2	proteasome subunit beta 2	Cytoplasm	peptidase
-4.1	P51174	ACADL	acyl-CoA dehydrogenase, long chain	Cytoplasm	enzyme
-4.0	Q9DCT8	Crip2	cysteine rich protein 2	Plasma Membrane	other
-3.5	Q9CPQ8	ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G	Cytoplasm	enzyme
-3.4	Q9WTX2	PRKRA	protein kinase, interferon-inducible double stranded RNA dependent activator	Cytoplasm	other
-3.4	Q0VBD0	ITGB8	integrin, beta 8	Plasma Membrane	other
-3.3	Q78IK2	USMG5	up-regulated during skeletal muscle growth 5 homolog (mouse)	Cytoplasm	other
-3.2	Q8R164	BPHL	biphenyl hydrolase-like (serine hydrolase)	Cytoplasm	enzyme
-3.2	P85094	ISOC2	isochorismatase domain containing 2	Cytoplasm	enzyme
-3.2	P23116	EIF3A	eukaryotic translation initiation factor 3, subunit A	Cytoplasm	other
-3.0	Q8K1Z0	COQ9	coenzyme Q9	Cytoplasm	other
-2.9	Q920E5	FDPS	farnesyl diphosphate synthase	Cytoplasm	enzyme
-2.9	O55022	PGRMC1	progesterone receptor membrane component 1	Plasma Membrane	transmembrane receptor
-2.8	P11438	LAMP1	lysosomal-associated membrane protein 1	Plasma Membrane	other
-2.8	Q8BFQ8	PDDC1	Parkinson disease 7 domain containing 1	Cytoplasm	other
-2.7	Q9D9V3	ECHDC1	ethylmalonyl-CoA decarboxylase 1	Cytoplasm	enzyme
-2.7	Q6GT24	PRDX6	peroxiredoxin 6	Cytoplasm	enzyme
-2.6	Q9D1I5	MCEE	methylmalonyl CoA epimerase	Cytoplasm	enzyme
-2.6	P08226	APOE	apolipoprotein E	Extracellular Space	transporter
-2.6	Q8QZS1	HIBCH	3-hydroxyisobutyryl-CoA hydrolase	Cytoplasm	enzyme
-2.6	O35857	TIMM44	translocase of inner mitochondrial membrane 44 homolog (yeast)	Cytoplasm	transporter
-2.6	P56376	ACYP1	acylphosphatase 1, erythrocyte (common) type	Cytoplasm	enzyme
-2.6	P29699	AHSG	alpha-2-HS-glycoprotein	Extracellular Space	other

Table 4-1. Continued

Fold Change	ID	Symbol	Entrez Gene Name	Location	Type(s)
-2.5	Q5U3K5	RABL6	RAB, member RAS oncogene family-like 6	Cytoplasm	other
-2.4	Q9D7X3	DUSP3	dual specificity phosphatase 3	Cytoplasm	phosphatase
-2.4	Q9ERS2	NDUFA13	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	Cytoplasm	enzyme
-2.4	Q91YJ2	SNX4	sorting nexin 4	Cytoplasm	transporter
-2.4	O35295	PURB	purine-rich element binding protein B	Nucleus	transcription regulator
-2.3	P38060	HMGCL	3-hydroxymethyl-3-methylglutaryl-CoA lyase	Cytoplasm	enzyme
-2.3	Q3TCD4	ECI2	enoyl-CoA delta isomerase 2	Cytoplasm	enzyme
-2.3	P34914	EPHX2	epoxide hydrolase 2, cytoplasmic	Cytoplasm	enzyme
-2.3	Q9QZ23	NFU1	NFU1 iron-sulfur cluster scaffold	Cytoplasm	other
-2.2	Q9D114	HDDC3	HD domain containing 3	Other	other
-2.2	O88737	BSN	bassoon presynaptic cytomatrix protein	Plasma Membrane	other
-2.2	Q3B7Z2	OSBP	oxysterol binding protein	Cytoplasm	transporter
-2.2	Q8WTY4	CIAPIN1	cytokine induced apoptosis inhibitor 1	Cytoplasm	other
-2.2	Q60829	PPP1R1B	protein phosphatase 1, regulatory (inhibitor) subunit 1B	Cytoplasm	phosphatase
-2.2	P32037	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	Plasma Membrane	transporter
-2.2	Q80U63	MFN2	mitofusin 2	Cytoplasm	enzyme
-2.2	E9PUL5	PRRT2	proline-rich transmembrane protein 2	Other	other
-2.1	Q9Z1Q9	VARS	valyl-tRNA synthetase	Cytoplasm	enzyme
-2.1	Q9Z2W0	DNPEP	aspartyl aminopeptidase	Cytoplasm	peptidase
-2.1	Q9ERT9	PPP1R1A	protein phosphatase 1, regulatory (inhibitor) subunit 1A	Cytoplasm	phosphatase
-2.1	P24270	CAT	catalase	Cytoplasm	enzyme
-2.1	Q8K2C9	HACD3	3-hydroxyacyl-CoA dehydratase 3	Cytoplasm	enzyme
-2.0	Q8BGD9	EIF4B	eukaryotic translation initiation factor 4B	Cytoplasm	translation regulator
-2.0	Q9CY64	BLVRA	biliverdin reductase A	Cytoplasm	enzyme
-2.0	P83940	TCEB1	transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)	Nucleus	transcription regulator
-2.0	Q61838	Pzp	pregnancy zone protein	Extracellular Space	other
-2.0	Q99KF1	TMED9	transmembrane p24 trafficking protein 9	Cytoplasm	transporter
-2.0	O35678	MGLL	monoglyceride lipase	Plasma Membrane	enzyme
-2.0	Q8BJI1	SLC6A17	solute carrier family 6 (neutral amino acid transporter), member 17	Cytoplasm	transporter
-2.0	Q99L04	DHRS1	dehydrogenase/reductase (SDR family) member 1	Cytoplasm	enzyme
2.0	Q5EBJ4	ERMN	ermin, ERM-like protein	Extracellular Space	other

Table 4-1. Continued

Fold Change	ID	Symbol	Entrez Gene Name	Location	Type(s)
2.0	Q62351	TFRC	transferrin receptor	Plasma Membrane	transporter
2.1	Q9CQW1	YKT6	YKT6 v-SNARE homolog (S. cerevisiae)	Cytoplasm	enzyme
2.1	Q91V77	S100A1	S100 calcium binding protein A1	Cytoplasm	other
2.2	Q6PD03	PPP2R5A	protein phosphatase 2, regulatory subunit B', alpha	Cytoplasm	phosphatase
2.3	A2AEC2	TCEAL6	transcription elongation factor A (SII)-like 6	Other	other
2.3	Q6ZQ58	LARP1	La ribonucleoprotein domain family, member 1	Cytoplasm	translation regulator
2.3	Q9WU78	PDCD6IP	programmed cell death 6 interacting protein	Cytoplasm	other
2.3	F6X5P5	ABHD10	abhydrolase domain containing 10	Cytoplasm	enzyme
2.5	Q4VA93	PRKCA	protein kinase C, alpha	Cytoplasm	kinase
2.5	Q8CGK3	LONP1	lon peptidase 1, mitochondrial	Cytoplasm	peptidase
2.6	Q9CPW4	ARPC5	actin related protein 2/3 complex, subunit 5, 16kDa	Cytoplasm	other
2.8	M0QWQ1	RFTN2	raftlin family member 2	Other	other
2.8	E9Q5C9	Nolc1	nucleolar and coiled-body phosphoprotein 1	Nucleus	other
2.9	Q8K1C0	ANGEL2	angel homolog 2 (Drosophila)	Nucleus	other
3.0	Q9DD20	METTL7B	methyltransferase like 7B	Other	enzyme
3.1	Q8R1B4	EIF3C	eukaryotic translation initiation factor 3, subunit C	Other	translation regulator
3.2	P60824	CIRBP	cold inducible RNA binding protein	Nucleus	translation regulator
3.3	Q6P1F6	PPP2R2A	protein phosphatase 2, regulatory subunit B, alpha	Cytoplasm	phosphatase
3.6	O09111	NDUFB11	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11, 17.3kDa	Cytoplasm	enzyme
4.5	E0CXA9	MOB4	MOB family member 4, phocein	Cytoplasm	other
4.6	P51910	APOD	apolipoprotein D	Extracellular Space	transporter
5.5	O35841	API5	apoptosis inhibitor 5	Cytoplasm	other
5.9	Q8BJU0	SGTA	small glutamine-rich tetratricopeptide repeat (TPR)-containing, alpha	Cytoplasm	other
9.8	Q8BRR9	PDE1A	phosphodiesterase 1A, calmodulin-dependent	Cytoplasm	enzyme
10.0	Q9CZB0	SDHC	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa	Cytoplasm	enzyme
10.1	Q920N7	SYT12	synaptotagmin XII	Plasma Membrane	transporter
17.5	F6U7V1	RYR2	ryanodine receptor 2 (cardiac)	Plasma Membrane	ion channel

Table 4-2. Top STN upstream regulators and their target molecules identified using a super-SILAC proteomic approach.

Upstream Regulator	Activation z-score	p-value of overlap	Target molecules in dataset	Molecule Type
APP	-0.5	5.79E-07	ALDOA,APOE,CAMK2D,CAT,CD59,COX7A2,CTSB,GALK1,GFAP,GRIA1,MADD,MAPT,MT-CO1,NDUFB11,NEFL,NME1,NPTX1,PAK3,PPP2CA,PRDX6,PRKCA,PTGDS,SIRPA,STMN1,SUCLG1,TTR,TXN	other
BDNF	0.9	1.53E-03	CALB2,GFAP,GRIA1,MAPT,MYO6,PDE1A,PPP1R1B,RNH1,RYR2,TFRC	growth factor
beta-estradiol	-1.7	7.77E-07	ALDOA,APOA1,APOD,APOE,CKM,CSTB,CTSB,DECR1,DUSP3,EIF3A,FASN,FLOT1,FNBP1,GCSH,GFAP,GPX1,GRIA1,ILF3,MAPT,MB,MGLL,MT-CO1,NME1,NPTX1,PGRMC1,PPP2CA,PPP2CB,PSMB2,PTGDS,SCG2,SDC3,SERPINA3,SLC2A3,SLC3A2,SLC9A1,TARS,TCEB1,TF,TMED9,TNNC2,TTR,TXN	chemical - endogenous mammalian
cerivastatin	-2.0	9.82E-03	APOA1,FDPS,GPX1,PTGDS	chemical drug
ESR1	0.4	6.0E-03	ABI2,APOA1,APOE,ARHGAP1,ARHGEF2,CD59,CTSB,DECR1,FASN,GCSH,GFAP,GPR158,MADD,MAPT,METTL7A,MYO6,NME1,POR,RAB5C,SLC3A2,TFRC,TMOD1,TTR,YKT6	Ligand-dependent nuclear receptor
gentamicin	0.9	1.55E-05	ACADSB,ASL,DECR1,EPHX2,ETFDH,NME1,PPP1R1A,Pzp,S100A1,TARS,TFRC,TTR	Chemical drug
HSF1	-2.1	4.48E-02	ATG7,FASN,MAPT,RAB5C,TTR	transcription regulator
HTT		2.89E-09	ALDOA,APOA1,ATP5O,CKM,COX4I1,CPNE5,FASN,FDPS,GFAP,GRIA1,MOBP,NDUFA11,NDUFA7,NEFL,NPTX1,PDXK,POR,PPP1R1A,PPP1R1B,PPP2R2A,PURB,SCN4B,SERPINA3,SGTA,SIRPA,STMN1,TFRC,TRAP1	transcription regulator
hydrogen peroxide	-0.9	1.51E-06	ATG7,CAT,CD59,CTSB,DDAH2,FASN,FDPS,GPX1,ISCU,MB,MFN2,MIF,MT-CO1,PDCD10,SLC30A1,STMN1,TFRC,TXN	chemical - endogenous mammalian
IGF1R	0.1	1.05E-06	ALDOA,ATP5L,ATP5O,CETN2,COX4I1,Cox5b,FASN,MT-CO1,NME1,PDCD10,SCG2,TXN,UQCRC2	transmembrane receptor
IL3	-2.0	1.02E-02	CAT,Ces1b/Ces1c,CIAPIN1,FASN,GPX1,SERPINA3,SLC2A3,SLC3A2,TARS	cytokine
lovastatin	-2.0	3.97E-02	APOE,CAT,FASN,FDPS	chemical drug
MAPT		4.08E-06	ALDOA,BSN,CAMK2D,COX7A2,GFAP,MAPT,NME1,PAK3,PCLO,PRDX6,STMN1,SUCLG1,TXN	other

Table 4-2. Continued

Upstream Regulator	Activation z-score	p-value of overlap	Target molecules in dataset	Molecule Type
methapyrilene	2.2	3.97E-08	ACADL,ALDOA,ALDOB,APOE,CAT,CTSB,DECR1,PDP1,PPP2R2A,SLC3A2,STMN1,TTR	chemical drug
MTOR	0.6	5.49E-06	ACADL,ATG7,COX4I1,DDAH2,EIF3A,FASN,FDPS,MADD,MAPT,MT-CO1,PGRMC1,PPP2CA,UQCRC2	kinase
PML	2.4	6.12E-06	ACADL,APOA1,APOE,CAT,CIAPIN1,FASN,PRKCA,STMN1,TXN	transcription regulator
PPARGC1A	0.3	5.63E-07	ACADL,ATP5O,CAT,COX4I1,Cox5b,FASN,GPX1,HAPLN1,HMGCL,MB,MFN2,MT-CO1,NCEH1	transcription regulator
prednisolone	-0.8	3.84E-02	API5,APOE,ATG7,CAT,CTSB,MB	Chemical drug
RICTOR	0.2	4.86E-09	ATP5L,ATP5O,ATP6V0C,ATP6V1G2,COX4I1,Cox5b,COX6B1,COX7A2,NDUFA11,NDUFA7,NDUFB7,Ndufs5,PRKCA,PSMA3,PSMB2,SDHC,UQCRC2	other
TO901317	-2.1	1.82E-04	APOD,APOE,EPHX1,FASN,FDPS,MGLL,POR,PSMB2,PTGDS,SERPINA3	chemical reagent
TP53	0.3	2.91E-10	ACOT11,API5,APOA1,APOE,ARHGAP1,ARHGEF2,ASL,ATG7,CAMK2D,CARHSP1,CAT,CD47,CD59,Ces1b/Ces1c,CKM,Cox5b,COX7A2,Crip2,CSTB,CTSB,EPHX1,FASN,FDPS,GPX1,INPP4A,IPO9,MB,MYO6,NME1,NPTX1,PADI2,PAK3,PDCD6IP,PDP1,PPP2CA,PRDX6,PRKCA,PTGDS,SERPINA3,STMN1,SUCLG1,TIMM44,TRAP1	transcription regulator
XBP1	-2.2	6.60E-02	APOA1,CAT,FASN,TTR,TXN	transcription regulator
1,2-dithiol-3-thione	-0.837	9.59E-05	ALDOA,EIF3C,EPHX1,HACD3,MGLL,PSMB2,SERPINA3,TFRC,TTR,TXN	chemical reagent

Table 4-3. Lists the STN proteins differentially expressed at a 2-fold change or greater from the standard/enriched mice group comparison using a label-free approach.

Fold Change	ID	Symbol	Entrez Gene Name	Location	Type(s)
-8.2	Q05BE7	NUMB	numb homolog (Drosophila)	Plasma Membrane	other
-7.0	Q9QY06	MYO9B	myosin IXB	Cytoplasm	enzyme
-2.6	Q9D3D9	ATP5D	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	Cytoplasm	transporter
2.0	P10605	CTSB	cathepsin B	Cytoplasm	peptidase
3.9	F6VF36	SRPR	signal recognition particle receptor (docking protein)	Cytoplasm	other
4.1	Q3U487	HECTD3	HECT domain containing E3 ubiquitin protein ligase 3	Cytoplasm	enzyme
4.4	A6H5Z3	EXOC6B	exocyst complex component 6B	Other	other
5.3	P35505	FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	Cytoplasm	enzyme
10.0	Q9WVA3	BUB3	BUB3 mitotic checkpoint protein	Nucleus	other
10.0	Q8BVP5	CSNK1G2	casein kinase 1, gamma 2	Cytoplasm	kinase
10.0	B9EJ54	NUP205	nucleoporin 205kDa	Nucleus	other
10.0	G8JL76	PPA2	pyrophosphatase (inorganic) 2	Cytoplasm	enzyme
10.0	Q99K46	USP11	ubiquitin specific peptidase 11	Nucleus	peptidase
10.0	Q8K1X1	WDR11	WD repeat domain 11	Cytoplasm	other

Table 4-4. Top STN upstream regulators and their target proteins identified using a label-free approach.

Upstream Regulator	Activation z-score	p-value of overlap	Target molecules in dataset	Molecule Type
APP	-1.7	9.06E-05	AP3B2,ATP2B2,ATP5D,CKB,CLTC,CPLX2,CTSB,CTTN,HSP90AA1,MADD,MAP2,NEFL,PAK3,PURA,TRAF3	other
BDNF	1.0	2.74E-04	AP3D1,CPLX2,HSP90AA1,KIF1A,MYO5A,NRXN1,SLC12A5,TRAF3	growth factor
beta-estradiol	1.4	3.48E-02	AP3D1,ATP2B2,BUB3,CAMK4,CKB,CTSB,CTTN,DDX17,FARSA,FARSB,PCBD1,PPP3CA,PRMT5,PTPRN,SLC8A1,TRAF3	chemical - endogenous mammalian
ESR1	1.3	1.23E-02	ARHGAP1,ATP6V0D1,BUB3,CIT,CKB,CTSB,DDX17,HSP90AA1,MADD,MAP2,MYO9B,NUP205,PCBD1,VPS35	ligand-dependent nuclear receptor
FBXO32	2.0	1.28E-03	HYOU1,ISYNA1,PPP3CA,RPN1	enzyme
genistein	2.5	6.71E-03	ADRBK1,AP3B2,ATP6V0A1,ATP6V0D1,CTSB,PLCXD3,SLC4A4	chemical drug
gentamicin	0.8	2.60E-03	ACSL3,CAND1,DDC,HSP90AA1,HYOU1,Nedd4	chemical drug
HTT		1.90E-06	ADRBK1,ATL2,ATP2B2,BAIAP2,CAMK4,CIT,CPLX2,CTTN,GNAL,HTT,MAP2,MOBP,MTOR,NEFL,PPP3CA,TRAP1	transcription regulator
MAPT		5.53E-04	ATP5D,CKB,CLTC,CPLX2,HSP90AA1,MAP2,PAK3	other
MTOR		1.61E-02	AKR1B1,CLTC,MADD,MAP2,MTOR	kinase
OSM	2.1	9.19E-03	ACSL3,BAIAP2,CASK,MAP2,PLCB4,PLCD1,SEPT9,USP9X	cytokine
prednisolone	-2.0	3.94E-02	CTSB,HSP90AA1,HTT,RAB24	chemical drug
topotecan	-1.0	7.83E-05	CSNK1G2,EXOC6B,MAT2A,NRXN1,Nrxn3,PPP3CA,TRAP1	chemical drug
TP53	-0.9	1.95E-03	ACSL3,AKR1B1,ARHGAP1,CAND1,CKB,CTSB,DDX3X,EZR,HSP90AA1,HTT,PAK3,PPP3CA,PTPRA,PURA,RPN1,TRAP1,USP9X	transcription regulator
1,2-dithiol-3-thione	2.0	2.96E-02	CUL1,EIF3C,HACD3,HSP90AA1	chemical reagent

CHAPTER 5
TRANSGENERATIONAL EFFECTS OF ENVIRONMENTAL ENRICHMENT ON REPETITIVE MOTOR BEHAVIOR DEVELOPMENT

The beneficial effects of environmental enrichment (EE) on behavior and brain development have long been recognized (Greenough, 1975; Hebb, 1949). In humans, EE operationalized as daily exposure to multiple sensorimotor stimuli and motor and cognitive tasks, in various novel combinations, was found to benefit children with autism spectrum disorder (ASD) (Woo and Leon, 2013; Woo et al., 2015). In rodents, larger, more complex rearing environments increased brain weight, dendritic branching and spine densities, synaptic plasticity, neurogenesis, neurotrophic factors, and gene expression (Nithianantharajah and Hannan, 2006). Functionally, such changes are reflected in improved cognitive, affective, and motor performance including attenuation of repetitive behavior and amelioration of deficits associated with modeling neurodegenerative diseases (Hannan, 2014; Lewis, 2004; Nithianantharajah and Hannan, 2006).

A growing body of evidence suggests that EE may benefit the offspring of enriched animals, despite their lack of exposure to EE. Denenberg and Rosenberg (1967) first documented transgenerational effects of early experience in rats, showing weight and activity differences in offspring two generations removed. More recently, Arai et al. (2009) observed increased long-term potentiation (LTP) not only in mice exposed to EE, but also in their F1 offspring that had never directly experienced EE. Some

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benefit of EE on LTP also accrued to F2 mice. Transgenerational benefits of EE were associated with a signaling cascade in the CA1 region of the hippocampus and passed on by the mother (Arai et al., 2009). Wei et al. (2015) found the EE-induced maternal weight loss resulted in reduced fat accumulation and improved glucose tolerance and insulin sensitivity, effects associated with altered methylation patterns of metabolic genes in the liver of offspring (Wei et al., 2015). In rats, transgenerational effects of prenatal maternal EE included improvements in exploration and balance, and reductions in hippocampal DNA methlylation at weaning age (Mychasiuk et al., 2012). Moreover, pre-reproductive maternal EE was effective in improving offspring's motor and cognitive performance as well as increased brain-derived neurotrophic factor (BDNF) (Caporali et al., 2014; Cutuli et al., 2015). To our knowledge, there have been no attempts to investigate the transgenerational benefits of EE on the development of repetitive motor behaviors.

Repetitive motor behaviors are most strongly associated with ASD, for which they are diagnostic. However, these repetitive invariant patterns of behavior that seemingly lack function are associated with many neurological, neuropsychiatric and neurodevelopmental disorders (e.g., intellectual and developmental disabilities, obsessive compulsive disorder, tic disorder, fronto-temporal dementia, and schizophrenia) (Lewis and Kim, 2009). Early environmental deprivation (e.g., congenital blindness; orphanages) can also induce repetitive motor behaviors (Fazzi et al., 1999; Rutter et al., 1999). Typically developing children sometimes perform repetitive motor behaviors early in development that wane with age (Evans et al., 1997; Kim and Lord,

2010; Thelen, 1979). Repetitive motor behaviors are a prominent feature of animal species maintained in confined conditions (Mason et al., 2007).

Deer mice (*Peromyscus maniculatus*) develop high levels of repetitive hind limb jumping and/or backward somersaulting in response to standard laboratory caging (Muehlmann et al., 2015). EE significantly attenuates the development of repetitive behaviors in deer mice (Bechard et al., 2016).

The present, exploratory study assessed transgenerational effects of EE on the development of repetitive motor behaviors in deer mice. We hypothesized that EE would reduce the development of repetitive behavior in the parent generation, and that offspring of enriched animals would also develop less repetitive behavior, despite never having directly experienced EE. We also assessed maternal care as a potential mediator of transgenerational effects. Opportunistically, we additionally investigated the effect of a single reproductive experience (in this study, mating, pregnancy, and pup rearing) on the expression of repetitive behavior in the dam. To our knowledge, we are the first to assess the effects of reproductive experience on repetitive behavior expression.

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida and followed the NIH Guidelines for the Care and Use of Laboratory Animals. Our laboratory maintains a colony of deer mice at 70-75°F and 50-70% humidity, under a 16:8 light:dark cycle, from which our parent (F0) generation of deer mice were derived. Figure 5-1 shows a timeline for the breeding and behavioral assessments. F0 females were weaned at 21 days of age, and siblings split between standard (SH; n=5) and EE (n=10) housing. EE cages were large dog kennels

(1.22 x 0.81 x 0.89 m) modified to have three levels interconnected via ramps, with same-sex group sizes of n=4-6. Furnishings inside EE housing consisted of a variety of toys, tunnels and mouse houses, that were systematically rotated each week, and permanent structures that remained undisturbed, such as a large hut and a running wheel. To promote more naturalistic foraging behavior, birdseed was scattered throughout the kennel (~2oz/wk). Nestlets were available for nest construction and refreshed every two weeks, along with food, water, and Sani Chip bedding. Standard environments grouped n=2-3 same-sex individuals within standard rat cages (29 x 18 x 13 cm) provisioned with nestlets, food, water, and bedding, and were refreshed in two week intervals. To control for diet without promoting foraging behaviors, a small amount of birdseed (~0.25 oz/wk) was placed in the corner of standard cages at the same time as EE cages.

An age-matched cage of males for standard and EE environments was also generated for the purpose of breeding the subsequent generation. F0 littermates were split between EE and standard housing. At 65 days of age, one male mouse from the enriched male cage was selected at random and placed into the enriched female cage, and similarly, one standard housed male was placed into the standard female cage. After three weeks, the males were removed and females were moved to individual standard cages to give birth to and rear their F1 offspring.

F1 subjects (EE: n=39, SH n=21) were born in standard cages, and weaned at 21 days of age into standard cages, grouped by similar ages (± 1 day), sex, and housing environment of parents. Following behavioral assessment at day 63 of age, 2 F1 females and 2 F1 males from the standard reared parents, and 3 F1 females and 3

F1 males from EE reared parents were selected for breeding the F2 generation. Pairs remained together for three weeks before males were removed. Unfortunately, 1 EE female lost her pups at birth, and a second EE female lost her pups due to a leaky water bottle that resulted in a flooded cage. In the end, 2 litters of F2 standard pups (n=6) and one litter of F2 EE pups (n=2) were generated. Subjects were left undisturbed in their assigned housing except for routine husbandry procedures. During the rearing of the F1 generation, however, cages were not changes so as to prevent disturbances to maternal behavior.

Across PND 1-8, instantaneous scan sampling was used to identify dam location and behavior (see Table 5-1) once every ten minutes, for 1 hour (12:00-1:00pm), resulting in 6 observations per day. We also scored the nest quality of each dam daily (see Table 5-1). As an additional proxy of maternal investment, dam (F0) and pup (F1) weights were recorded following the observation session on PND 3, 7, and 21, for a subset of litters.

The frequency of repetitive behavior was assessed for each mouse using automated software (Labview software, National Instruments) that records each time there is a break in a photo beam array (Columbus Instruments). The photo beams are positioned high enough so that all four paws of the mouse must leave the floor in order to break them. For each assessment, video surveillance (Geovision software) accompanied the automated data and ensured the accuracy of the automated counts. On the day of testing, mice were placed into individual testing chambers (28 x 22 x 25 cm) at least 30 min prior to lights off (at 10am) until lights on (at 6pm). Food, water, and Sani Chip bedding were provided for the duration of the 8 h assessment period. F0

parent mice were tested for levels of repetitive behavior at 63 days of age and dams were retested 1 week after their offspring were weaned (~2 months after the day 63 assessment). F1 and F2 mice were tested for levels of repetitive behavior at 28 and 63 days of age.

In order to smooth the data for maternal behavior analyses, we calculated the average of behavior over 2 consecutive days (i.e. PND 1 and 2). These binned data values were then used to assess housing conditions on maternal care, which included the proportion of observations spent: in contact with the pups; active; inactive; mothering; and performing repetitive behaviors, as well as nest quality. A Repeated Measures General Linear Model (GLM; SPSS v23) was used to assess differences in maternal care, with rearing environment and age as factors in the model. Differences in litter sizes due to rearing environment were assessed using a GLM with parental (F0) rearing environment as the only factor in the model. This same model was used to assess weight data, which were analyzed separately for each time point (i.e. PND 3) since dam and litter weights were not collected from all litters at every time point.

For each assessment, the total frequency of repetitive behavior across the 8 h test was calculated and used in subsequent analyses. A GLM with housing as a factor in the model was used to assess differences in mean frequencies of repetitive behavior of females exposed to EE. A Repeated Measures GLM was used to assess differences in mean frequencies of repetitive behavior for F1 offspring, using parental (F0) environment, sex and age of the offspring as factors in the model. This same model was used to assess F2 differences in repetitive behavior development. To assess the effects of reproductive experience on repetitive behavior of the dam, a Repeated

Measures GLM was used with time (before mothering i.e. d63, and after mothering) and housing as factors in the model.

Rearing in EE reduced repetitive behavior development ($F(1,13)=6.3$, $p=0.026$; Fig. 5-2a). Rearing environment had no significant effects on maternal care, as indicated by maternal contact, behaviors, and nest provisioning (all $p>0.05$). Across PND 1-8, we found non-significant trends for dams from both rearing conditions to spend less time in the nest with their pups ($F(3,39)=2.38$, $p=0.084$) and more time active ($F(3,39)=2.85$, $p=0.05$). There were no differences in mean litter size (EE: 3.3 pups vs SH: 3.6 pups), nor were there differences in dam or offspring weights at any time point (all $p>0.05$).

The F1 offspring of EE parents developed significantly less repetitive behavior compared to F1 offspring of SH parents ($F(1,56)=5.03$, $p=0.029$; see Fig. 5-2b). For all F1 offspring, repetitive motor behaviors increased with age ($F(1,56)=39.2$, $p<0.001$). Repetitive behavior of the dam was a significant predictor of adult offspring repetitive behavior at day 63 ($F(1,65)=4.37$, $p=0.04$) but not at day 28. Although the F2 offspring of EE parents displayed lower mean frequencies of repetitive behavior than F2 offspring of SH parents, differences failed to meet the level of significance ($F(1,6)=2.26$, $p=0.18$; see Fig. 5-2c).

The effects of reproductive experience on repetitive behavior development of the dams was dependent on rearing condition ($F(1,12)=7.17$, $p=0.02$; see Fig. 5-3). Rearing in SH housing generated adults with increased levels of repetitive behavior that were reduced following reproductive experience. Rearing in EE housing generated adults

with reduced levels of repetitive behavior that did not change following reproductive experience.

Using a mouse model, we found a beneficial transgenerational effect of EE on the development of repetitive behavior in offspring never having experienced EE. F1 offspring of EE reared parents developed fewer repetitive motor behaviors than F1 offspring of standard reared parents. Although not statistically significant, the development of repetitive behavior in F2 offspring was similar to that of F1 mice: EE F2 mice displayed less repetitive behavior both in early adolescence and adulthood than standard housed F2 mice. The small number of F2 EE pups limited the strength of this comparison, however. Both direct and indirect measurements of maternal care suggested that this effect was not maternally mediated, as EE and standard reared dams demonstrated no differences in maternal investment across PND 1-8. We believe this assessment for differences in maternal behaviors of the F0 generation strengthens the findings, having directly tested one potential mechanism for mediating beneficial effects of parental environment on repetitive behavior development.

Evidence from both human and animal studies in support of transgenerational inheritance has been growing. Environmental factors such as stress, diet and toxins have been shown to influence transgenerational inheritance relevant to neurobiological disease, including depression, anxiety, addiction, and ASD (Roth et al., 2009; Saab and Mansuy, 2014). Fewer studies have investigated the transgenerational effects of enriching environments on behavior. Of those studies that have, positive effects for offspring, such as enhanced plasticity and memory (Arai et al., 2009; Cutuli et al.,

2015), motor coordination and balance (Caporali et al., 2014; Mychasiuk et al., 2012), and metabolic health (Wei et al., 2015) were found.

The novel transgenerational effect of EE housing to reduce repetitive behavior development in non-enriched offspring was, we believe, not maternally-mediated. Although the F2 results need to be replicated these pilot data are promising, suggesting that transmission of the phenotype will persist across several generations and derive from an epigenetic mechanism. Future studies are needed to establish if there are associated changes in the epigenome by which they are mediated.

In addition, we demonstrated that a single reproductive experience affected repetitive behavior levels, although this was dependent on rearing environment. Reproductive experience for standard reared females had an enrichment effect, and reduced levels of repetitive motor behaviors. We thus suggest that reproductive experience may be a special case of environmental enrichment. Other studies support this with beneficial findings of reproductive experience on dam anxiety, cognition, affect, stress response, and neural function (Macbeth and Luine, 2010). For EE reared females, however, reproductive experience did not alter levels of repetitive motor behavior. Potentially, its ameliorating effect in EE females was masked by degradation of environmental complexity associated with being moved into standard cages. Notwithstanding, these data are some of the first to empirically support the enriching effects of reproductive experience on repetitive behavior.

In summary, novel findings from this exploratory study support a beneficial influence of an enriched parental environment on offspring development of repetitive behavior. Moreover, maternal behavior did not seem to mediate the transgenerational

effect, although repetitive behavior was affected by reproductive experience. The transgenerational effect of EE on repetitive behavior development now requires replication and the identification of epigenetic mechanisms mediating this effect.

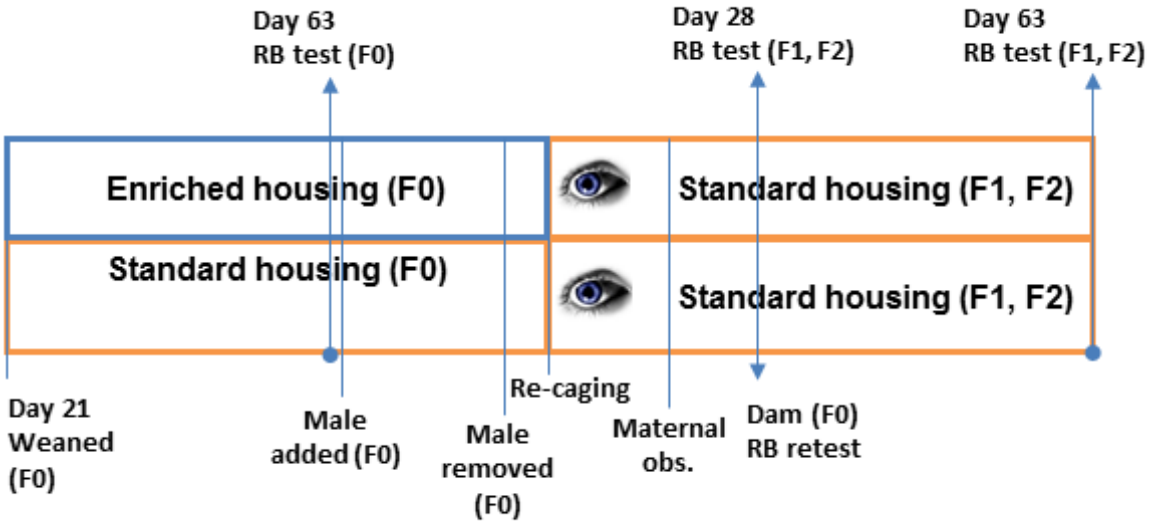

Figure 5-1. The timeline for the breeding and testing schedules.

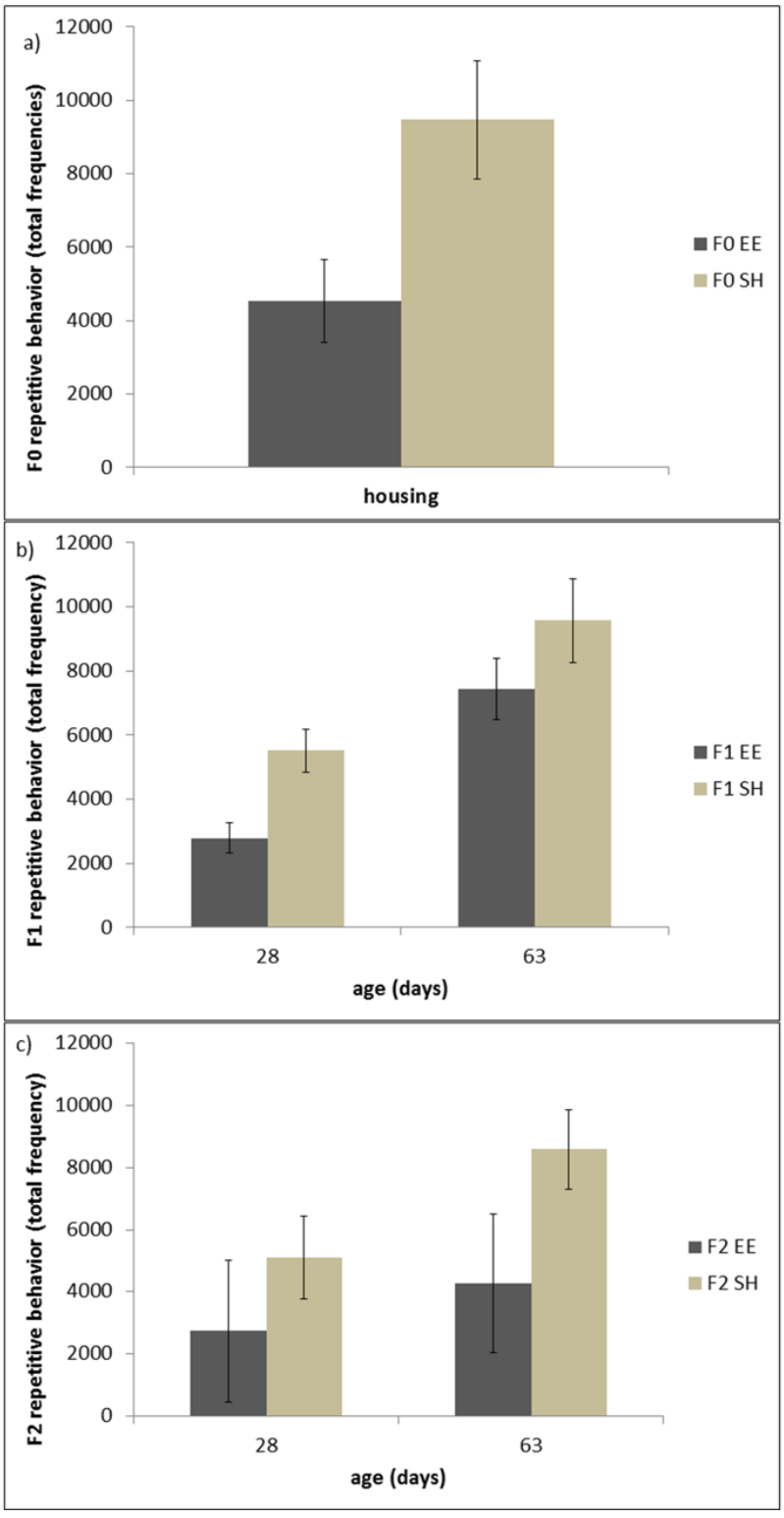

Figure 5-2. The mean total frequencies of repetitive motor behaviors for a) F0 females reared in environmental enrichment (EE) and standard housing (SH), b) non-enriched F1 offspring, and c) non-enriched F2 offspring. Data bar shows mean± SEM.

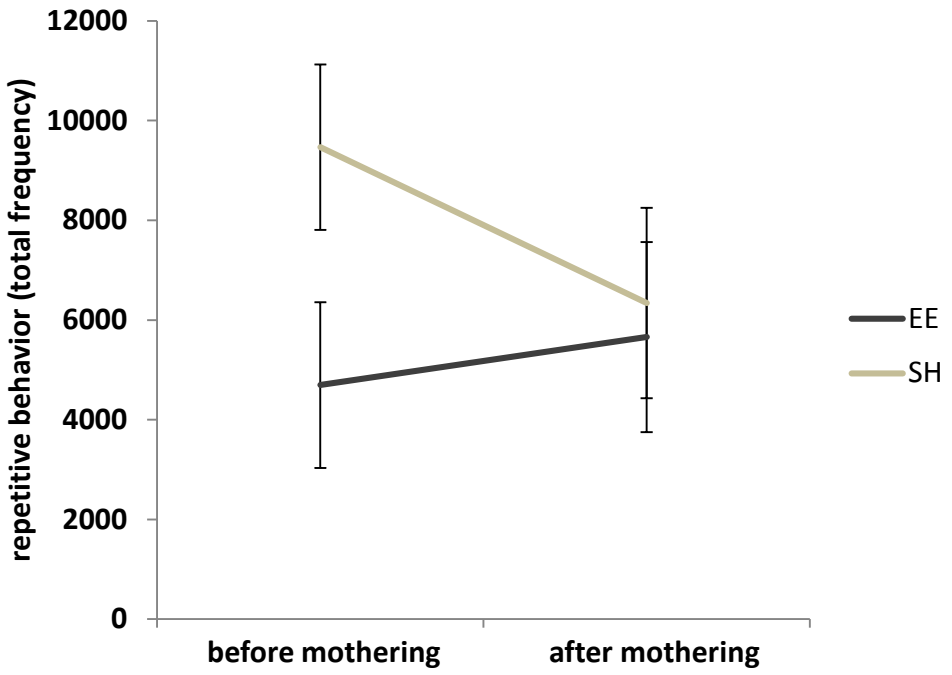

Figure 5-3. Shows the effect of reproductive experience on the expression of repetitive behaviors of F0 females reared in environmental enrichment (EE: n=10) and standard housing (SH: n=5).

Table 5-1. Description of dam location and behavior.

Location	
Dam in nest	Majority of dam positioned inside nest
Dam out of nest	Majority of dam positioned outside of nest
Behavior	
Active	Walking, drinking, eating, grooming
Inactive	Sleeping, resting
Mothering	Nursing, licking, nosing, grooming, nest building
Repetitive behaviors	Pattern of topographically similar behavior performed in bouts (minimum of 3 consecutive events (e.g. hind limb jumps) in less than 3 seconds)
Not visible	Cannot see what the dam is doing. No observation made.
Nest Score (0-3)	
0	no nest
1	0 < 0.5 cm walls
2	0.5-2.0 cm walls
3	> 2 cm walls/enclosed

CHAPTER 6
GENERAL DISCUSSION

In spite of many advances, the specific mechanisms mediating normative versus pathological progression of repetitive behavior development are not well understood, and no selective pharmocotherapies currently exist. Repetitive behaviors are extremely heterogeneous in nature and expression is dependent on both genetic and environmental factors. Along with their prevalence in neurodevelopmental and neurological disorders, repetitive behaviors are apparent in both humans and animals kept in barren environments. There is a long history of attenuating repetitive behaviors in captive animals via environmental enrichment (EE), yet relatively little investigation has been made into the specific neurobiological mechanisms underlying this effect. We aimed to identify how EE attenuates repetitive motor behaviors in order to identify controlling neural circuitry, specific neurobiological mechanisms, and to promote new targets for early therapeutic intervention.

The application of such findings to early interventions for neurodevelopmental disorders, such as ASD, has great therapeutic potential. For example, by targeting specific neural circuitry, molecules and pathways of repetitive behavior, the selectivity of pharmacological effects should increase, and side-effects should decrease. Also, an understanding of how EE is altering the brain to promote beneficial neural plasticity will promote the development of enviromimetics (drugs which mimic or enhance the beneficial effects of environmental stimulation) which can be used synergistically with the EE paradigm to maximize treatment outcomes.

To this end, we conducted a series of studies investigating the development of repetitive motor behaviors in deer mice in response to varying early environments. Our

overall purpose was to identify mechanisms by which EE attenuates the development of repetitive behaviors. The deer mouse model of repetitive behavior was selected for these studies, as prior work from our lab showed that high levels of repetitive motor behaviors (hindlimb jumping, backward somersaulting) are induced as a consequence of standard laboratory caging, and suppressed as a consequence of being reared in EE. Moreover, high levels of repetitive behavior in deer mice were associated with decreased activation of indirect basal ganglia pathway nuclei. To build on this foundation of knowledge, we set out to test the overarching hypothesis that EE-induced attenuation of repetitive motor behaviors is mediated by increased functioning of the indirect basal ganglia pathway.

Summary of Results

In Chapter 2, we provided a novel test of the hypothesis that increased neuronal activation of indirect pathway nuclei mediated EE-induced attenuation of repetitive motor behaviors in adult deer mice. Using cytochrome oxidase (CO) techniques, we found increased neuronal activation in indirect pathway nuclei of adult mice with low levels of repetitive behavior induced by EE housing compared to standard-reared mice with high levels of repetitive behavior. EE reared mice also exhibited greater dendritic spine densities in these brain nuclei, the likely anatomical basis for the increased neuronal activation. Unexpectedly, we found significant sex by housing effects in our CO data, indicating results were driven by enriched versus standard males. Thus, Chapter 2 results provide functional and morphological evidence for alterations in the indirect pathway mediating the effects of EE on repetitive behavior.

In Chapter 3, we continued to investigate environmentally mediated mechanisms underlying repetitive behavior by assessing the neurobiological correlates of attenuated

repetitive behavior across development. We first set out to characterize repetitive behavior development within an enriched environment using a longitudinal design. We found the unexpected effect that repeated testing exacerbated repetitive behavior development in both standard and EE mice, although this effect was stronger in EE mice. Characterization of the effects of EE on repetitive behavior development was re-assessed using a cohort design, and the expected attenuation of repetitive behaviors by EE was restored. Novel findings on the temporal progression of repetitive behaviors in EE included that one week of EE exposure was needed to arrest repetitive behavior development, although it took three weeks until significant differences were observed between EE and standard-reared mice. We then assessed activation of the hypothesized mediating circuitry in adolescence when differences due to housing first emerged, and compared levels of activation to those of adult animals. We found increasing levels of activation in indirect pathway nuclei corresponding to the maintenance of low levels of repetitive behavior in enriched male compared to standard male mice. As previously seen in adult deer mice, the CO values for females did not vary by housing or repetitive behavior levels. Chapter 3 findings substantiate and extend results from Chapter 2 by providing evidence for the importance of the indirect pathway in mediating the development of repetitive behavior. These results also provide the first characterization of the trajectory of repetitive behavior development in EE.

In Chapter 4, we investigated the underlying molecular mechanisms of repetitive behavior development in varying environments using a super-SILAC and label-free proteomics approach. Although there was low convergence of top protein hits across the two methods, there were many global similarities in significant upstream regulators

and disease pathways. Highly implicated in repetitive behavior development were aberrant pathways in the category of neurological disease, such as those for generation, development and death of the neuron, and disorders with a repetitive behavior phenotype (e.g. Huntington's and Alzheimer's diseases, movement disorders, disorder of basal ganglia). Findings from this Chapter begin to determine specific molecular alterations mediating the circuitry changes established in Chapters 2 and 3.

Finally, in Chapter 5, we conducted an exploratory study on the transgenerational effects of an enriched environment on repetitive behavior development of non-enriched offspring. We found novel significant effects for the transmission of benefits on repetitive behavior development from parental EE and excluded the mother as a mediator of this effect. Although in need of replication with a larger sample size, these preliminary data suggest this effect may carry over into the F2 generation. We also opportunistically investigated the effects of mothering on repetitive behavior development, and present novel data for an enrichment effect of a single reproductive experience, although only for standard reared dams. Chapter 5 findings suggest that epigenetic mechanisms may also mediate EE effects on repetitive behavior and highlight the need to determine if such epigenetic changes are selective for indirect pathway brain regions.

Conclusions

When taken collectively, the results briefly reviewed in this chapter provide strong evidence that the attenuation of repetitive motor behavior development by environmental enrichment is mediated by increased functioning of the indirect basal ganglia pathway. Cellular mechanisms suggested to be driving the increased functioning of the indirect pathway nuclei (e.g. the STN) include morphological properties of the cell, such as increased dendritic spine densities, and potentially,

regulatory mechanisms for cell growth and survival. The characterization of repetitive motor behavior development within an enriched environment continues to support the EE paradigm as a quick and powerful tool to reduce repetitive behaviors, requiring only one week of exposure during early development to maximize behavioral effects. Moreover, the positive changes induced by enriching environments that attenuate repetitive behaviors are also transmitted to non-enriched offspring.

Future Directions

More work is required to elucidate the environmentally mediated neurobiological changes that attenuate repetitive behavior development. Findings from the proteomic analysis require validation, and following this, pursuit of a number of novel targets for assessment of their effects on repetitive behavior development. For example, future studies may try altering functioning of HTT or APP and assessing the downstream effects for corresponding changes in repetitive behavior of standard housed mice. Moreover, it would be interesting to see if tumor suppressing drugs have an effect on repetitive behavior development. Altering levels of beta-estradiol would also be interesting, due to its role both in cancer and AD pathologies.

Although no differences between males and females were found in their development of repetitive behaviors, the neuronal activation studies using CO histochemistry did show significant sex effects. The speculative explanations for this, such as estrous cycle and CO interactive effects, beg for direct testing. The unexpected effect of repeated testing on repetitive behavior development also warrants future investigation, potentially, as a third environmental treatment group (e.g. enriched mice with high levels of repetitive behavior) for comparison of differential mediating mechanisms.

The preliminary data on transmission of environmentally mediated effects that attenuate repetitive behavior in non-enriched offspring point to the tip of an iceberg for research on the potential epigenetic transmission of repetitive behavior. Results need first to be replicated, and carried out across as many generations as is required to observe a loss of transmission of effects. Subsequent to this, identification of an epigenetic change and mediating mechanism are required. Findings from the proteomic study suggest potentially focusing on changes in genes involved in cell growth and cell death. The potential transmission of EE effects on repetitive behavior across generations suggests considering the influence of the parents' environment on offspring repetitive behavioral trajectories.

Findings from the current studies identify critical neuronal projections mediating repetitive behavior and provide novel targets for development of pharmacotherapeutics and enviromimetics that prevent the development of repetitive motor behaviors. Moreover, they promote the direct translation of EE techniques into clinical populations at risk for neurodevelopmental and neurological disorders associated with repetitive behaviors, such as ASD. EE paradigms are relatively inexpensive and accessible, and their employment in high-risk clinical populations is a promising avenue of research.

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BIOGRAPHICAL SKETCH

Allison Rollande Bechard was raised in Pittsford, New York, and graduated from Pittsford Sutherland High School. She moved to Toronto, Canada, to attend the University of Toronto, where she received a Bachelor of Science in zoology. After graduating, Allison remained in Toronto to work in the laboratory of Dr. John Roder, at the Samuel Lunenfeld Research Institute, Mount Sinai Hospital. Leaving her position in pursuit of a degree in Animal Behavior and Animal Welfare, Allison next joined the Department of Animal and Poultry Sciences at the University of Guelph under the supervision of Dr. Georgia Mason. Here, she investigated early environments and development of abnormal behaviors in laboratory mice. After many travels and teaching English abroad, Allison moved to Gainesville, Florida, to work at the University of Florida in the ecology and evolution laboratory of Dr. Christine Miller. She then joined the Behavioral and Cognitive Neurosciences program in the Department of Psychology, where she studied the mechanisms underlying repetitive behavior development and their response to varying early environments, in the laboratory of Dr. Mark Lewis. Allison received her Ph.D. from the University of Florida in the summer of 2016 and continues in the Department of Psychology as a postdoctoral researcher in the laboratory of Dr. Lori Knackstedt investigating the neurobiology of cocaine addiction and post-traumatic stress disorder in a rat model.